Clinical and molecular monitoring of *Plasmodium falciparum* resistance to antimalarial drug (artesunate+sulphadoxine-pyrimethamine) in two highly malarious district of Madhya Pradesh, Central India from 2012–2014

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ABSTRACT

The spread of *P. falciparum* resistant strain has led to a significant resurgence of malaria morbidity and mortality. The current cornerstone in malaria treatment in India is Artemisinin based Combination (Artesunate + Sulphadoxine-Pyrimethamine) Therapy (ACT) for treatment of uncomplicated *P. falciparum* malaria since 2010. In the present study we assessed the therapeutic efficacy of ACT and molecular monitoring of antimalarial resistance. Therapeutic efficacy was determined by in vivo method using 28 days follow-up. Molecular genotyping of *dihydrofolate reductase* (*dhfr*), *dihydropteroate synthase* (*dhps*) and *kelch13* genes were analyzed. *msp-1* and *msp-2* genotyping were used to differentiate recrudescence. Therapeutic efficacy of ACT was determined in 237 patients over the three year period. Most of the patients showed adequate clinical and parasitological response (99.6%). Molecular study revealed that 72% parasites were of mutant genotype (27.2% single mutants, 43.5% double mutants and 1.3% triple mutants) for *pf dhfr* while *pf dhps* showed 78.2% wild type alleles and 21.8% mutants (18.1% single mutants and 3.7% double mutants). Analysis of total 135 samples revealed mutation in *k13* gene along with non-synonymous single mutation at codon M579T (1.5%) and double mutations at codon M579T & N657H in 37%. ACT remains effective for the treatment of uncomplicated *P. falciparum* malaria in Madhya Pradesh, Central India. However, increasing mutation in *pf dhfr* (particularly triple mutations) and *pf dhps* may reduce susceptibility to partner drug SP and mutation in *k13* propeller gene, highlighting the need for continuous monitoring of the efficacy of ACT.

KEYWORDS

Malaria; *Plasmodium falciparum*; drug resistance; therapeutic efficacy; ACT (Artesunate + Sulphadoxine-Pyrimethamine); Central India; molecular monitoring; *k13* gene

Introduction

Malaria is a global public health problem, and India alone contributes to 70% of all malaria cases in South-East Asian Region [1]. Madhya Pradesh is highly endemic state in the country, contributing 7% of total malaria cases with more than 50% *Plasmodium falciparum* infection [2,3]. Treatment of *P. falciparum* malaria is complicated by the emergence and spread of parasite resistance to many of the first line antimalarial drugs. Resistance to

the Chloroquine (CQ) and Sulphadoxine-Pyrimethamine (SP) has spread across most parts of the India [4–8]. Combination therapies rather than monotherapies have shown to be better alternative for the treatment of malaria. The World Health Organization (WHO) recommends the use of ACTs for the treatment of uncomplicated malaria. In India, the combination of artesunate and sulphadoxine-pyrimethamine (AS + SP) is used for treatment of *P. falciparum* malaria. Within a few years after

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introduction of the ACT, *P. falciparum* developed resistance against artemisinin and first time reported from Western Cambodia [9]. Now it has spread throughout Southeast Asian countries including North East region of India [7,10–13]. Currently, *in vivo* therapeutic efficacy studies (TES) are considered the gold standard for determining antimalarial drug efficacy [14]. However, WHO also recommends that TES results be complimented using molecular marker studies [15]. SP resistance in *P. falciparum* has been linked to single nucleotide polymorphisms (SNPs) A16 V, C50R, N51I, C59R, S108 N/T and I164L in dihydrofolate reductase (*pfdhfr*) and SNPs S436A/F, A437G, K540E, A581G and A613S in dihydropteroate synthase (*pfdhps*) [16]. Accumulation of SNPs typically starting with S108 N leads to gradual reduction of susceptibility to SP [17] and quintuple mutation in *pfdhfr* & *pfdhps* genes strongly correlated with SP resistance [18]. Recently polymorphisms in the propeller region of *P. falciparum* kelch protein (*k13* gene) have been found to be associated with artemisinin resistance [19]. Therefore, we aimed to assess the therapeutic efficacy of ACT and mutation analysis in *pfdhfr*, *pfdhps* and *k13* genes to keep track of resistance in ACT.

Material and methods

Study site

The study was carried out in Balaghat and Anuppur district, Madhya Pradesh during 2012–2014. Balaghat is located at 21°48'N 80°11'E. Birsa and Baihar CHCs of district Balaghat were undertaken for the study. Anuppur district is situated in the north eastern part of Madhya Pradesh. It is located at 23.1°N 81.68°E. Pushprajgarh CHC was undertaken for the present study. Both Balaghat and Anuppur districts are highly malarious districts in the state contributing 9.6% and 3.5% malaria respectively. Birsa and Baihar community health centre (CHC) in Balaghat is a forest area inhabited by Baiga ethnic tribal group. There are 302 villages (256844 populations) bordering Chhattisgarh state and this CHC is considered as hot spot of malaria endemic region [20]. In Anuppur district, Pusprajgarh CHC is highly malarious block (contributing 56% district malaria) and more than 75% [21] population are tribal. The inhabitants are mostly illiterate (overall literacy about 30% according to census 2011), scantily clothed and work mainly in the forest nursery as labourers and other casual workers. The villages are surrounded by reserved forest of Sal (*Shorea robusta*) with difficult hilly terrain with numerous hillocks. There are several perennial streams and tributaries which provide numerous breeding sites for mosquito [22]. Both *P. falciparum* and *P. vivax* prevail in this area with preponderance of *P. falciparum*, *P. malariae* is also reported from the study area [21]. *Anopheles culicifacies* and *An. fluviatilis* are the major malaria vector and the study area are under two round of Alphacypermethrin spray as vector control [20,21].

Ethical statement

The study protocol was approved by the Institutional Ethical Committee (IEC) of National Institute for Research in Tribal Health (NIRTH), Jabalpur. Written consents were obtained from patients prior to their enrolment. The consent form was provided and explained in detail to the patient, either directly or to their parents/guardians (in case of children aged up to 18 years). All the consent forms were also signed by the patient or by a relative/family member in the presence of an appropriate witness.

Sample size

Assuming a 5% treatment failure rate to ACT, a 95% confidence level and a precision estimate of 5%, 73 patients were estimated as a minimum for inclusion into the study. With a 20% loss to follow-up and withdrawals during the 28-day follow-up period, 88 patients were planned to be included in the study from each site.

Enrollment and sample collection

Patients presenting with fever or history of fever and symptoms of malaria were screened for malaria parasites by microscopy during July to December every year. Fifty-six villages in Balaghat and thirty-two villages in Anuppur district were selected for this study and this study was carried out for 3 years in same villages. Peripheral smear positive for *P. falciparum* malaria and fulfilling the enrollment criteria were enrolled in the study. The demographic information (age, gender, body temperature) were recorded. ACT (AS + SP) was given orally over a three-day period as per national guideline and therapeutic efficacy was determined following standard protocol [14]. Two to three drops of finger prick blood was also blotted on to 3 MM filter paper (Whatman International Ltd., Maidstone, United Kingdom) at the time of enrollment and during the follow-up for molecular study.

Microscopic blood examination

Parasite counts were done on Giemsa-stained thick films and the numbers of parasites per 200 WBC were counted by light microscopy. Parasite density, expressed as the number of asexual parasites per μL of blood, was calculated by dividing the number of asexual parasites by the number of white blood cells counted, and then multiplying it by an assumed white blood cell density (typically 6000 per μL). When the number of asexual parasites is less than 100 per 200 white blood cells in follow-up smears, counting was done against at least 500 white blood cells. A blood slide sample was considered negative when examination of 1000 white blood cells or 100 fields containing at least 10 white blood cells per field revealed no asexual parasites. The presence of gametocytes on the day the patient was enrolled or on the day of follow-up was also recorded.

Inclusion and exclusion criteria

Patients enrolled in the study were selected on the basis of the following criteria; patients with *P. falciparum* mono-infection, an axillary temperature ≥ 37.5 °C, with parasitaemia in the range of 1000–50,000 asexual parasites per μL , able to take medicines orally and absence of severe malnutrition. Absence of sign of severe malaria was among the major criteria that were used to select representative samples according to WHO 28 day *in vivo* protocol [14].

The exclusion criteria includes; mixed infection of *P. falciparum* with other Plasmodium species, clinical and laboratory manifestation of severe sign of malaria, history of antimalarial ingestion in the two weeks preceding presentation, concomitant presence of other febrile conditions (e.g. acute respiratory infection, measles, severe diarrhea and others), severe malnutrition, children with body weight < 5 kg and persistent vomiting. Pregnant and lactating women were also excluded from the study.

Quality assurance of microscopy

Blood smears of enrolled patients including follow-up smears were examined by two independent microscopists (one at each study site and another at the NIRTH laboratory, Jabalpur). If any discordance was found, third reading was performed by another senior microscopist. Quantification of parasitaemia was also performed by two independent microscopists with a third reading performed by a senior microscopist if the difference between the first two readings varied by more than 25%.

Therapeutic efficacy study

Artesunate and Sulphadoxine-Pyrimethamine Tablets (ACT combi pack manufactured by ZEST Pharma) of each age group was obtained from NVBDCP central government supply through district malaria office Balaghat and administered by a medical officer. Clinical examination of the enrolled patients was performed and case record form was completed for each patient. Clinical observation was recorded daily for the first four days (0–3 days) and during subsequent follow-up on day 7, 14, 21 and 28. Drug doses were administered under supervision. Patients were observed for an hour after administration of the treatment. Full drug doses were re-administered if the patients vomited study drug within 20 min [23].

Classification of responses to treatment

On the basis of parasitological and clinical outcome of treatment with ACT (AS + SP), patients were classified according to the WHO definition of therapeutic responses: Early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF) and Adequate Clinical and Parasitological Response (ACPR) [14].

Molecular study

Genomic DNA was isolated by Chelex-100 method. Briefly, 1 ml of 1x PBS and 50 μL of 10% saponin was added to each tube containing dried blood spots and vortexed for 20–30 s. The tubes were incubated overnight at 4 °C and after incubation centrifuged for 30 s at 800 rpm. Supernatant was discarded and again 1 ml of 1x PBS was added without saponin to each tube and vortexed. The tubes were then incubated at 4 °C for 30 min and centrifuged at 800 rpm for 1 min. Supernatant was discarded, 100 μL of sterile water and 50 μL of 20% Chelex were added to each sample and incubated at 95 °C for 12 min. After incubation, tubes were centrifuged at 15000 rpm for 5 min and the solution was transferred to the new tube. Tubes were again spin at 15,000 rpm for 10 min and supernatant (DNA) was transferred into another tube and stored at -20 °C for further experiment.

PCR amplification of the *pfdhfr* gene

The extracted DNA was used for PCR amplification of the *dhfr* gene spanning codons 15–170. The PCR primers and conditions are given in the supplementary Table 1 for 742 bp fragment, a primary PCR and the nested PCR (542 bp). The primary PCR was performed in a volume of 25 μL with 0.2 U of *Taq* DNA polymerase, 0.2 mM each dNTP, 1 μM each primer, and 1.5 mM MgCl_2 . The reaction was allowed to proceed for 40 cycles with denaturation at 94 °C for 30 s, annealing at 45 °C for 45 s, and extension at 72 °C for 45 s. Final extension was at 72 °C for 10 min. The nested PCR was performed with a final MgCl_2 concentration of 1.5 mM for 35 cycles. Other nested PCR conditions were the same as those described for the primary PCR. The PCR products were resolved on a 1.5% agarose gel.

PCR amplification of the *pfdhps* gene

The PCR amplification was carried out for the *dhps* gene spanning codons 425–640. The primary PCR was set up for the amplification of *dhps* gene 1167 bp and nested PCR 735 bp (supplementary Table 1). The primary PCR was performed in a volume of 25 μL with 0.22 U of *Taq* DNA polymerase, 0.2 mM each dNTP, 0.4 μM each primer, and 1.5 mM MgCl_2 . The reaction was allowed to proceed for 35 cycles after an initial denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 90 s. Final extension was at 72 °C for 10 min. The nested PCR 653 bp was performed with annealing at 50 °C for 30 cycles. Other nested PCR conditions were the same as those described for the primary PCR. The PCR products were resolved on a 1.5% agarose gel.

Nucleotide sequencing

The PCR products were purified from the agarose gel by using HiYield™ gel/PCR DNA extraction kit, as per the manufacturer's recommended protocol. The gel-purified product was used with the ABI Big Dye Terminator Ready

Figure 1. Flow chart showing over all patients screening, enrollment and follow-up status.

Reaction Kit Version 3.1 for the sequencing PCR. The sequencing PCR was performed in a volume of 20 µl with 1 µl to Terminator Ready Reaction Mix (TRR), 3.2 pmol of gene specific primer, and 5X sequencing buffer. Cycling conditions for the sequencing PCR included 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 4 min. The sequences were analyzed using software Bioedit Sequence Alignment Editor v.7.0.5.2 and using the online sequence alignment tool ClustalW.

k13 propeller gene amplification and sequencing

The *k13* propeller gene was amplified by the nested PCR method described by Ariey et al. [19] with some modifications. The cycling conditions and primer sequence were given in supplementary Table 1. An aliquot of the PCR products was analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide to confirm amplification. The nucleotide sequence of *k13* gene of *P. falciparum* isolate from the study site has been submitted in GenBank under Accession number KX121050, KX121052.

Data analysis

Data from both clinical and parasitological assessments for each participant were entered into the WHO standardized Microsoft excel data collection sheet. This form was used for both data management and analysis. All data was independently entered double blind. Data were analysed by estimation of difference in proportion according to a 95% confidence interval.

Results

Therapeutic efficacy study

Overall, 1616 patients were screened for malaria parasite during a period of 3 years. Out of these 44.4% (718/1616) were found positive for malaria parasite with 89% (638/718) *P. falciparum* mono infection. A total 311 patients were enrolled who fulfil the enrolment criteria as well as consented for the study. Therapeutic efficacy was determined in 76% (237/311) cases, loss to follow-up in 18% (56/311) and withdrawal from study in 6% cases (18/311) (Figure 1). The loss of follow up was high (18%) because of the populations are labourer and they moved out for wage earning and we couldn't trace them during the follow-up. Patients who had not taken the complete observed treatment were withdrawn from the study.

Most of the patients (46%) were in 5–8 year age group and 68% patients having parasitaemia between ≥ 1000 to 1999 per µL blood (Table 1). Adequate clinical and parasitological response was more than 99% with a single case of late parasitological failure was recorded. Year wise treatment outcome, enrollment, loss of follow-up and withdrawal is shown in Table 2. The parasite clearance was rapid during the study period and complete clearance of parasitaemia within 48hrs of administration of drug were observed in 97.4% (231/237) (Table 2). One case, late parasitological failure had temperature of 36.9 °C and parasite density of 80 parasite/µL of blood (8% of day 0 parasitaemia) on day21. Molecular genotyping of *msp1*

Table 1. Demographic and baseline characteristics of patients completed the therapeutic efficacy study.

S.No	Characteristics		2012 <i>n</i> (%)	2013 <i>n</i> (%)	2014 <i>n</i> (%)	Total <i>n</i> (%)
1	Sex	Male	44 (57)	42 (56)	39 (46)	125 (52.7)
		Female	33 (43)	33 (44)	46 (53)	112 (47.3)
2	Age	1–4 Year	14 (18.2)	09 (12)	03 (3.6)	26 (11)
		5–8 Year	45 (58.4)	28 (37.3)	36 (42.3)	109 (46)
		9–14 Year	11 (14.3)	28 (37.3)	38 (44.7)	77 (32.5)
		≥ 15 Year	07 (9.1)	10 (13.3)	08 (9.4)	25 (10.5)
3	Parasitemia (Asexual parasite/µl)	≥ 1000 –1999	43 (55.8)	48 (64)	70 (82.4)	161 (68)
		2000–4999	12 (15.6)	16 (21.3)	09 (10.6)	37 (15.4)
		5000–9999	14 (18.2)	07 (9.3)	00 (0)	21 (9)
		$\geq 10,000$	08 (10.4)	04 (5.3)	06 (7)	18 (7.6)
4	Fever at the time of enrolment (≥ 37.5 °C)	Yes	04 (5)	16 (21.3)	25 (29.4)	45 (19)
		No	73 (95)	59 (78.7)	60 (70.6)	192 (81)
5	History of fever	Yes	69 (90)	74 (98.7)	01 (1.2)	144 (61)
		No	08 (10)	01 (1.3)	84 (98.8)	93 (39)
6	Previous antimalarial drug intake	Yes	00 (0)	00 (0)	00 (0)	00 (0)
		No	57 (74)	75 (100)	85 (100)	217 (91.6)
		Unknown	20 (26)	00 (0)	00 (0)	20 (8.4)

Note: *n*: number, µl: micro liter.

Table 2. Year wise treatment outcomes and parasite clearance time with AS + SP study from 2012–2014.

S.No.	Outcome of treatment (28 days)		2012	2013	2014	Total
			<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
1	Primary classification	ACPR	77 (100.0)	74 (98.7)	85 (100.0)	236 (99.6)
		ETF	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
		LCF	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
		LPF	0 (0.0)	1 (1.3)	0 (0.0)	1 (0.4)
		Total analysis	77 (73.3)	75 (62.5)	85 (98.8)	237 (76.2)
		Loss to follow-up	26 (24.8)	30 (25.0)	0 (0.0)	56 (18.0)
		Withdrawal	2 (1.9)	15 (12.5)	1 (1.2)	18 (5.8)
		Total	105 (100.0)	120 (100.0)	86 (100.0)	311 (100.0)
2	Parasite clearance (hours)	≤24	51 (66.2)	64 (85.3)	74 (87.1)	189 (79.7)
		>24–48	22 (28.6)	9 (12.0)	11 (12.9)	42 (17.7)
		>48–72	2 (2.6)	1 (1.3)	0 (0.0)	3 (1.3)
		>72	2 (2.6)	1 (1.3)	0 (0.0)	3 (1.3)

and *msp2* of failure sample showed the same genotype as on day0 and classified as recrudescence.

Molecular analysis of *pf dhfr* and *pf dhps* genes

DNA was isolated from all the enrolled cases ($n = 311$) and attempted to analyse for *dhfr* mutation encoding pyrimethamine resistance and *dhps* mutations associated with sulphadoxine resistance. A total 232 (75%) isolates were analyzed for *dhfr* gene and 216 (69%) isolates were analyzed for *dhps* gene.

A total 232 isolates were analyzed for the *dhfr* gene covering the codon 11–169, over the three year period i.e. 85 samples from 2012, 67 samples from 2013 and 80 samples from 2014. The presence of five *pf dhfr* mutation codon (A16 V, N51I, C59R, S108 N/T and I164L) known to be involved in pyrimethamine resistance. Over all 72% parasite isolates were harboring the mutant genotype while only 28% with wild type genotype. Majority of them were double mutant (43.5%) followed by single mutant (27.2%) and emerging 1.3% triple mutant (Table 3). Year wise analysis revealed that double mutant alleles were increased during the subsequent year but it was not significant statistically. While triple mutant was totally absent in the year 2012 and emerged in 2013 (1.5%) and subsequently increased to 2.5% in 2014 (Table 3). All five codon (A16 V, N51I, C59R, S108 N/T and I164L) were found mutant and among the mutant genotype. Almost

80% isolates were mutant at codon S108 N followed by codon C59R (57%) and N51I (32%) while codon A16 V and I164L found only in 2% isolates. A total 8 haplotype was recorded including 5 single point mutation, 2 double mutants with combination of codon N51I/S108 N and C59R/S108 N, and a triple mutant (C59R/S108 N/I164L). Double mutant combination of C59R/S108 N was found more prevalent in comparison to other combination (Figure 2). Interestingly, N51I/S108 N combination was increasing over the year from 6 to 19% (Figure 2), while codon N51I/C59R combination was not observed in the study.

A total 216 isolates (80, 65 and 71 in 2012, 2013 and 2014 respectively) were analyzed for the *dhps* gene covering 430–601 codon. The presence of five *pf dhps* mutations (S436F/A, A437G, K540E, A581G and A613S/T) known to be involved in sulfadoxine resistance. A total 22% isolates were harbouring the mutant genotype and 78% wild type genotype. Single mutant was recorded among 18% isolates while double mutant was found only in 4% isolates (Table 3). Year wise analysis revealed that double mutant alleles was totally absent in the year 2012 and emerged in 2013 (3.1%) and increased in 2014 up to 8.5% (Table 3). Of these five codon, four (S436F/A, A437G, K540E and A581G) were found mutant and among these 87% isolates were mutant at codon K540E followed by codon A437G (19%), S436A (6%) and A581G (4.3%). A total seven haplotype was recorded including four single

Table 3. Year wise distribution of samples and point mutation in *P. falciparum dhfr*, *dhps* and *k13* gene.

Gene	Year	No. of samples analyzed	Type of alleles							
			Wild type		Single mutant		Double mutant		Triple mutant	
			<i>N</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
<i>dhfr</i>	2012	85	25	29.4	28	32.9	32	37.6	0	0
	2013	67	18	26.9	14	20.9	34	50.7	1	2
	2014	80	22	27.5	21	26.3	35	43.8	2	3
	Total	232	65	28.0	63	27.2	101	43.5	3	1
<i>dhps</i>	2012	80	68	85.0	12	15.0	0	0.0	0	0
	2013	65	51	78.5	12	18.5	2	3.0	0	0
	2014	71	50	70.4	15	21.1	6	8.5	0	0
	Total	216	169	78.2	39	18.1	8	3.7	0	0
<i>k13</i>	2012	39	26	66.7	1	2.6	12	30.8	0	0
	2013	45	28	62.2	1	2.2	16	35.5	0	0
	2014	51	29	56.9	0	0.0	22	43.1	0	0
	Total	135	83	61.5	2	1.5	50	37.0	0	0

Figure 2. (a) Mutation rates in the *P. falciparum* *dhfr* codon among the yearly isolates and (b) Mutation rates in the *P. falciparum* *dhps* codon among the yearly isolates.

point mutation, three double mutants with combination of codon S436A/K540E, A437G/K540E and A581G/K540E. Double mutant at codon A437G/K540E was found to be more prevalent in comparison to other combination (Figure 2). Interestingly, A581G/K540E combination was observed in 2014 among 17% isolates (Figure 2).

P. falciparum k13 propeller mutation

A total of 186 samples (adequate amount of blood samples available) were used to analyze the mutation in *k13* gene with sequencing of propeller region of *k13* gene. Out of these, 135 isolates were successfully sequenced and analyzed (Table 3). Sequences were analyzed from codon 427–702 covering propeller region of *pfk13* gene. Out of these, 39% (52) isolates were with non-synonymous mutation. Double mutation at codon M579T and N657H was recorded in 37% (50) isolates, while only two isolates had single mutation at codon M579T. Synonymous substitutions were not observed in the study. The double mutants were increased from 31% to 43% during the period of three years but the difference was not significant statistically.

Discussion

Artemisinin-based combination therapy has been implemented since 2010 in India for the treatment of uncomplicated malaria. Combination therapy has ability to slow the emergence and spread of drug resistance. It offer the choice of short acting drug as artemisinin and its derivatives reduces most of the parasite biomass during their initial rapid action and long acting partner can usually eliminate the small number of remaining parasite [24]. Therefore, the long acting partner drug in ACT is having important challenge in successful malaria treatment program. The present study of 28-day efficacy of AS+SP in *P. falciparum* malaria patients remained most efficacious except one case which was a late treatment failure among all the age groups and in the area of perennial malaria transmission [20]. Very few studies were carried out from India to monitor the efficacy of ACT. During 2009–2010

(implementing time of ACT in the whole country) the efficacy of AS + SP was 99% [7] and subsequently its efficacy reduced mainly from North-eastern region. Saha et al. in 2012 found about 9% treatment failure cases from bordering areas of North-eastern region of West Bengal [15] and in 2014 Mishra et al. reported low efficacy in North-eastern states ranging from 77–82% [8]. This could be due to high level of triple and quadruple *dhfr* and *dhps* mutation in this region [25–29], as mutations in *pfdhfr* and *pfdhps* is considered one of the important markers for the emerging resistance against SP. In this study, one case (5-year-old child) of treatment failure on day 21 was recorded with low parasite density (80 parasite/ μ L on day 21). The genotyping of this case was found to be *dhfr* triple mutant at codon C59R/S108 N/I164L and double mutant (S436A/K540E) *dhps* gene. Although the *k13* genotype was found to be wild type. In the present study, the prevalence of *dhfr* mutant genotype is high and is associated with pyrimethamine resistance. Mutation at codon S108 N is most prevalent followed by codon C59R, which is similar to studies conducted at different part of India [25,28–31]. Single mutant I164L was reported for the first time from Central India in 2013 during our study which may be a cause of concern as it is linked to high levels of pyrimethamine resistance. Pathak et al. and Tonya et al. did not observe this mutation in this region [30,31]. Majority of the isolates (44%) were double mutant (either S108 N/C59R or S108 N/N51I) in the *dhfr* codon may result in partial resistance to pyrimethamine as such partial mutation can inhibit the pyrimethamine efficacy up to 10-fold [32]. Double mutant S108 N/C59R was most prevalent in our study and this combination was also found the most prevalent genotype from the study carried out in different part of India [6,28,30,31,33]. Emerging triple mutant C59R/S108 N/I164L in our study was observed for the first time in 2013. Whereas, previous study carried out from this area did not found any triple mutant [30,31]. While in the other north-eastern states of India, triple mutation is most prevalent and increasing from 10.9% in 2004 [25] to 22.5% in 2014 [28]. This is directly associated with the SP resistance resulting either in treatment failure or delayed parasite clearance.

Resulting, the AS+SP combination of ACT treatment was replaced by Artemether-Lumefantrine in north-eastern states.

In case of *pf dhps*, mutations at various codon (S436F/A, A437G, K540E, A581G and A613S/T) is associated with sulfadoxine resistance. In most of the reports from India, single mutation at codon S436A and A437G are more prominent and established [25,28]. In the present study, we found mutation at codon K540E (87%) to be most prevalent followed by codon S436A from Central India while previous study did not found any mutation at codon K540E and A581G [31]. Importantly three double mutant haplotype with combination S436A/K540E, A437G/K540E and A581G/K540E was observed in this study while no double mutant genotype was found in the samples studied in 2010 [31]. Although it is still a good indication that there was no triple mutant till 2014.

White suggested that a strong indicator for SP treatment failure is the quintuple mutation in three *pf dhfr* codon (S108 N/T, N51I & C59R) and two *pf dhps* A437G & K540E codon [34]. The prevalence of mutation in *pf dhfr* codon S108 N and *pf dhps* codon S436F and A437G among Indian isolates are key point mutations which are associated with other mutations responsible for various level of resistance [35,36]. Despite the mutations at various codons of the *pf dhfr* and *pf dhps* genes, the efficacy (99%) of the AS+SP remained successful may be due to absent of rarity of quintuple mutant genotype associated with higher level of SP resistance in this study.

Mutations in *k13 propeller* were identified as an important molecular marker for detection of emerging resistance against artemisinin and its derivatives. Although more than 100 non-synonymous mutations have been reported from worldwide [13,37–40] but till date only 4 point mutations i.e. Y493H, R539T, I543T and C580Y are confirmed to be associated with artemisinin resistance [19]. Other point mutations that include 13 different codon which have been associated with late parasite clearance [41–45]. Although in the present study we found 39% mutant isolates but none of these point mutations were found to be associated either with resistance or responsible for delayed parasite clearance. Previous studies from India found very limited number of polymorphism at codon G533A, S549Y, A578S, R561H, F446I [13,33] but out of these only few point mutations are associated with delayed parasite clearance. Bharti et al. also reported a very limited number of polymorphism at codon 579 from the Central Indian region [46]. But in the present study we found high number of mutant isolates at codon 579 which comes in between the point mutations either confirmed associated with artemisinin resistance (580) or associated with delayed parasite clearance (578).

The limitation of the study is that we couldn't sequence all the samples for *k13* gene as only 186 (very

less quantity) DNA samples were available for the *k13* analysis and no DNA was available for repeat sequencing.

Therefore more information is required to confine the role of other mutations in the artemisinin resistant phenotype. Based on this study the AS+SP is highly efficacious in this region for the treatment of uncomplicated *P. falciparum* malaria. However, the mutation responsible for metabolism of partner drug (*dhfr* and *dhps* gene combinations) is spreading. The evolving of triple mutation in the *dhfr* gene indicating that this parasite subpopulation acquiring the mutation pattern which is associated with the treatment failure [47]. This is a matter of concern that the situation arrived in the north-eastern part of the country due to triple and quadruple mutant alleles may also spread in this area in the near future.

Conclusion

The first line therapy for *P. falciparum* malaria recommended by the National programmes i.e. AS+SP remain efficacious in the Central India. Increasing the mutation in *dhfr* and *dhps* gene showing the development of antifolate resistance is recorded in this area but no functional mutation in *k13* showed the efficacy of artemisinin. Combination of molecular and *in vivo* efficacy should be monitored at regular interval to find out the emergence of resistance.

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Disclosure statement

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