AAC Journals.ASM.org

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

Pabitra Saha,[a] Subhasish K. Guha,[a] Sonali Das,[a] Shrabanee Mullick,[a] Swagata Ganguly,[a] Asit Biswas,[b] Dilip K. Bera,[a] Gaurangadeb Chattopadhyay,[d] Madhusudan Das,[c] Pratip K. Kundu,[a] Krishnangshu Ray,[a] and Ardhendu K. Maji[a]

Calcutta School of Tropical Medicine, Kolkata, India[a]; Department of Health and Family Welfare, Government of West Bengal, Swasthya Bhawan, Salt Lake City, Kolkata, India[b]; Department of Zoology, University of Calcutta, Kolkata, India[c]; and Department of Statistics, University of Calcutta, Kolkata, India[d]

In India, chloroquine has been replaced by a combination of artesunate and sulfadoxine-pyrimethamine (AS-SP) for uncomplicated *P. falciparum* malaria. Other available combinations, artemether-lumefantrine (AM-LF) and artesunate-mefloquine (AS-MQ), not included in the national program, are widely used by private practitioners. Little is known about the therapeutic efficacy of these artemisinin combinations and the prevalence of molecular markers associated with antimalarial drug resistance. A total of 157 patients with *P. falciparum* monoinfection were recruited and randomized into three study groups (AS-SP, AM-LF, and AS-MQ). All patients were followed up for 42 days to study the clinical and parasitological responses according to the WHO protocol (2009). We assessed the polymorphism of the *pfATPase6*, *pfcrt*, *pfdhfr*, and *pfdhps* genes by the DNA-sequencing method. The PCR-corrected therapeutic efficacies of AS-SP, AM-LF, and AS-MQ were 90.6% (95% confidence interval [CI], 0.793 to 0.969), 95.9% (95% CI, 0.860 to 0.995), and 100% (95% CI, 0.927 to 1.00), respectively. No specific mutational pattern was observed in the *pfATPase6* gene. All isolates had a K76T mutation in the *pfcrt* gene. In the *pfdhfr-pfdhps* genotype, quadruple mutation was frequent, and quintuple mutation was documented in 6.3% of *P. falciparum* isolates. The significant failure rate of AS-SP (9.5%), although within the limit (10%) for drug policy change, was due to SP failure because of prevailing mutations in *pfdhfr*, $I_{51}R_{59}N_{108}$, with *pfdhps*, G_{437} and/or E_{540}. The efficacy of this ACT needs periodic monitoring. Artemether-lumefantrine and artesunate-mefloquine are effective alternatives to the artesunate-sulfadoxine-pyrimethamine combination.

Malaria remains one of the major global public health problems, with an estimated 225 million confirmed cases and 781,000 deaths in 2009 (34). *Plasmodium falciparum* is the most virulent infectious species of malaria parasite in tropical and subtropical countries. Chloroquine (CQ) has been in use as the major antimalarial drug for the past few decades in all areas where malaria is endemic. The widespread use of CQ has led to the emergence of CQ-resistant parasites. CQ resistance in *P. falciparum* malaria, first reported from Southeast Asia and South America in the late 1950s, has spread throughout the world. In India, CQ-resistant *P. falciparum* was first reported from the Diphu area of the Karbi Anglong district of Assam in 1973 (23), followed by its spread to various parts of the country. Due to the emergence of resistant malarial parasites, particularly *P. falciparum* against different antimalarial drugs, malaria treatment has moved into the era of artemisinin-based combination therapy (ACT). Recently, the National Vector Borne Disease Control Program (NVBDCP) of India has introduced ACT, a combination of artesunate-sulfadoxine-pyrimethamine (AS-SP), as the first-line agent to treat uncomplicated *P. falciparum* malaria. Two other ACTs consisting of artemether-lumefantrine (AM-LF) and artesunate-mefloquine (AS-MQ), not included in the national program but readily available in the market, are in frequent use by private practitioners.

In the late 20th century, candidate molecular markers for antimalarial drug resistance were identified by cloning and sequencing parasite genes homologous to those known to mediate resistance in other organisms (4, 5, 14). Based on differences in DNA sequence between sensitive and resistant parasite clones, point mutations (12, 13, 22) were evaluated for their association with *in vitro* resistant phenotypes. Molecular markers for resistance were assessed in clinical trials (6, 7, 17) to establish their association and ability to predict treatment failure *in vivo*.

In *P. falciparum*, two candidate genes have been found to be potentially associated with artemisinin resistance. Mutations and increased copy numbers of the *pfmdr1* gene were associated with reduced *in vitro* susceptibility to the artemisinins (9, 24) and reduced *in vivo* clinical efficacy of AM-LF in Africa (25). *P. falciparum ATPase6* was shown to be a target for the artemisinin drugs in *Xenopus* oocytes (10). A single amino acid change, L263E, in *pfATPase6* was associated with resistance to artemisinin in this model (10, 28). Mutation of S769N in *pfATPase6* of *P. falciparum* isolates from French Guiana was associated with decreased *in vitro* sensitivity to artemether (16). However, it is unclear whether mutations in *pfATPase6* are associated with *in vivo* artemisinin resistance (1). The dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*) genes are associated with SP resistance. The quintuple mutation (*pfdhfr*, I51, R59, N108, and *pfdhps*, G437

Received 8 October 2011 **Returned for modification** 5 December 2011
Accepted 31 January 2012
Published ahead of print 6 February 2012
Address correspondence to Ardhendu K. Maji, ardhendu_maji@yahoo.com.

doi:10.1128/AAC.05388-11

and E540) is considered a relevant molecular marker of SP treatment failure (17).

A recent *in vivo* therapeutic efficacy study showed a high rate of resistance of *P. falciparum* to different antimalarial drugs (CQ, 66%; SP, 16%; CQ-SP, 8%) in Jalpaiguri (20). Considering the SP resistance status in the study area and the frequent use of two other combinations of ACTs, the present study was designed to evaluate the *in vivo* efficacies of three different regimens of ACTs against *P. falciparum* malaria and polymorphism of the *pfATPase6*, *pfcrt*, *pfdhfr*, and *pfdhps* genes in tea gardens of Jalpaiguri District.

MATERIALS AND METHODS

Study site and design. The study was performed at Turturi Primary Health Centre (PHC) under Alipurduar Block II of Jalpaiguri District, West Bengal, India, from November 2009 to February 2010, where the annual parasite index (API) (total number of slides positive for parasites in a year × 1,000/total population) in 2008 was 2.8. In Jalpaiguri, malaria transmission is seasonal, with a peak from September to January and predominance of *P. falciparum* malaria (78.07%; West Bengal Government data). The study area is surrounded by several tea gardens and inhabited by different tribal communities.

The study was a randomized, three-arm, open-label, prospective trial for evaluation of clinical and parasitological responses of three ACTs for treatment of uncomplicated *P. falciparum* malaria, based on the therapeutic efficacy protocols of the WHO (32). The study protocol was approved by the Institutional Ethics Committee of the Kolkata School of Tropical Medicine.

Patient screening and enrollment. Febrile patients from surrounding tea gardens and local villages attending the outpatient department (OPD) of the Turturi PHC were screened for malarial parasites by examining Giemsa-stained thick and thin peripheral blood smears (PBS). All patients with confirmed *P. falciparum* monoinfection were informed about the study protocol and requested to participate in the study. Those who fulfilled the inclusion criteria according to the WHO protocol (32) were enrolled after written informed consent was obtained.

Inclusion criteria. Patients over 6 months of age with *P. falciparum* monoinfection (asexual parasite count, 1,000 to 100,000/μl), an axillary temperature of ≥37.5°C or history of fever during the past 24 h, and ability to swallow oral medication were included in the study. Those having symptoms of severe falciparum malaria, a febrile condition due to diseases other than malaria, or history of regular medication that might interfere with antimalarial pharmacokinetics and pregnant women and lactating mothers were excluded.

Treatment. The therapeutic efficacies of three different regimens of ACTs were evaluated in the present study. The tested ACTs were AS-SP, AM-LF, and AS-MQ. AS and SP were supplied by NVBDCP and AM-LF and MQ were procured from M/S Themis Medicare Limited, India. The recruited patients were randomized by using simple random sampling without replacement (SRSWR) into three study groups, each receiving one of the above-mentioned drug regimens. The study was designed in such a way that patients below 5 years of age were not enrolled in the AS-MQ study arm, as local health officials objected to enrolling them due to a paucity of safety data about mefloquine in Indian children. All the patients were treated according to WHO guidelines (31) for the treatment of malaria according to their body weight. In the AS-SP group, artesunate at 4 mg/kg body weight once daily for 3 days and a single dose of SP (25/1.25 mg base/kg body weight) on day 0 were administered. In the AM-LF group (coformulated tablets containing 20 mg of artemether and 120 mg of lumefantrine), a total of six doses were administered at 0, 8, 24, 36, 48, and 60 h. The numbers of tablets per dose were as follows: one tablet for 10 to 15 kg of body weight, two tablets for 15 to 25 kg, three for 25 to 35 kg, and four for those weighing over 35 kg. In the AS-MQ group, artesunate was administered in a dose of 4 mg/kg once daily for 3 days and mefloquine was dosed at 25 mg base/kg over 2 days on day 1 (15 mg/kg) and day 2 (10 mg/kg). All recruited patients were admitted to the PHC for the first 4 days for monitoring of the clinical and parasitological response to the treatment. Primaquine (PQ) in a single dose of 0.75 mg/kg was given on day 1. Study investigators directly observed and documented the administration of each dose of medication. In case of vomiting within 30 min of administration, a full dose was repeated.

Follow-up schedule. The day, the patient was enrolled and received the first dose of medicine was designated day 0. Thereafter, the schedule called for clinical reassessment on days 1, 2, 3, 7, 14, 21, 28, 35, and 42. Patients were advised to return on any day during the follow-up period if symptoms recurred and advised not to wait for a scheduled visit day. During the follow-up visits, all patients were examined both clinically and parasitologically. In the event of occurrence of any complications, the patient was withdrawn from the study and treated according to the existing standard of care for that condition.

Study endpoints and statistical analysis. Therapeutic outcomes were classified according to WHO guidelines (32) into early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), and adequate clinical and parasitological response (ACPR). The data were entered into a standard data entry program designed by the Global Malaria Program and analyzed with a Kaplan-Meier survival curve according to WHO standard procedures (http://www.who.int/malaria/resistance). The free statistical software R (version 2.13.1) was used to calculate the *Z*-test value for the comparison of the efficacy of the three study arms, and Fisher's exact test was done to verify the correlation between *dhfr-dhps* haplotypes and the AS-SP treatment failure cases.

Laboratory examination. (i) Microscopic blood examination and parasite count. To calculate the parasite count, the number of parasites per 200 white blood cells (WBCs) was determined by experienced microscopists using a light microscope. Assuming a WBC count to be 8,000/μl of blood, the parasitemia was calculated and expressed per μl of blood.

(ii) p-LDH test. All microscopically confirmed *P. falciparum*-positive cases were screened for a *P. vivax*-specific p-LDH (*Plasmodium* lactate dehydrogenase) test (by using an rapid diagnostic test that contained monoclonal anti-*P. falciparum* HRP-II-specific and anti-*P. vivax* p-LDH-specific antibodies [M/S Tulip Group, Goa, India]) to detect any mixed infection.

(iii) DNA isolation and *msp1* and *msp2* genotyping assays. The genomic DNA of the parasite was isolated from the blood samples collected on day 0 and in all episodes of malaria during the 42-day study period using a QIAamp DNA Blood Minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Genotyping of *P. falciparum* parasites was performed with a nested PCR assay based on the amplification of *msp1* and *msp2* as described in detail previously (11, 26). In the primary reaction, the oligonucleotide primers used spanned the entire genetic segments, i.e., block 2 for *msp1* and block 3 for *msp2*. In the nested reaction, separate primer pairs targeted the respective allelic types of *msp1* (K1, MAD20, and RO33) and *msp2* (FC27 and 3D7).

The amplified products from the nested reaction were separated by electrophoresis on a 2% high-resolution agarose gel (SeaKem LE Agarose; Lonza, Rockland, ME) in 1× TBE buffer (100 mM Tris, 100 mM boric acid, 5 mM EDTA [pH 8.0]) and visualized with UV light following staining with ethidium bromide. The fragment size was estimated in relation to a 100-base-pair DNA ladder (Invitrogen Corporation, Carlsbad, CA) both by the naked eye and in a GelDoc Xr system with Quality One analysis software version 4.4.1 (Bio-Rad).

Before treatment, the mean multiplicity of infection (MOI) was calculated as the quotient of the total number of *P. falciparum* genotypes and the number of positive PCR samples for each marker *msp1* and *msp2* gene.

In patients with recurrent parasitemia, blood samples on day 0 and on the day of parasite reappearance were used to distinguish between recrudescence and new infection. A recrudescent infection was defined as one that showed a match in size of at least one allele for both the *msp1* and *msp2* genes on day 0 and on the day of recurrent parasitemia.

(iv) Polymorphism of the *pfATPase6*, *pfcrt*, *pfdhfr*, and *pfdhps* genes. Five pairs of primers were used to span 4,068 bp of the *P. falciparum* *pfATPase6* gene as described previously (15). A part of the *pfcrt* gene

TABLE 1 Baseline characteristics of the study patients

Characteristic	Value		
	AS-SP ($n = 53$)	AM-LF ($n = 52$)	AS-MQ ($n = 52$)
Sex [no. (%)]			
Male	31 (58.49)	27 (51.92)	32 (61.54)
Female	22 (41.51)	25 (48.08)	20 (38.46)
Weight (kg)			
Mean	30.58	23.23	34.90
Range	09–65	06–52	14–60
SD	14.90	13.88	13.32
95% CI	26.56–34.59	19.46–27.00	31.28–38.52
Age category [no. (%)]			
< 5 yr	06 (11.32)	19 (36.54)	0
5–15 yr	27 (50.94)	25 (48.08)	28 (53.85)
Adult	20 (37.34)	08 (15.38)	24 (46.15)
Age (yr)			
Mean	16.45	10.62	17
Range	02–55	01–55	05–50
SD	14.18	10.33	10.75
95% CI	12.64–20.26	7.81–13.43	14.08–19.92
Temperature (°C)			
Mean	38.00	37.96	37.9
Range	37.60–39.3	37.6–39.3	37.5–39.3
SD	0.45	0.40	0.36
95% CI	37.88–38.12	37.85–38.07	37.81–37.99
Hemoglobin (g/dl)			
Mean	11.84	11.04	12.21
Range	9.9–15.0	8.5–14.0	9.0–14.8
SD	1.46	1.15	1.36
95% CI	11.45–12.23	10.73–11.35	11.84–12.58
Parasite count (no/μl)			
Mean	15,371	14,538	12,380
Range	1,040–70,000	1,040–51,680	1,000–44,000
SD	15,402.25	12,375.19	10,266.47
95% CI	11,224–19,518	11,174–17,902	9,589–1,5171
Gametocytes on day 0 [no. (%)]	3 (5.66)	1 (1.92)	2 (3.85)

covering single-nucleotide polymorphisms (SNPs) at codons 72 to 76 was amplified as described previously (19).

Portions of the *pfdhfr* gene spanning codons 16, 51, 59, 108, and 164 and the *pfdhps* gene spanning codons 436, 437, 540, 581, and 613 on day 0 and the day of recurrence were amplified by two rounds of PCR using primers described previously (2, 3).

Direct sequencing of the PCR products was done on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences were analyzed using Bioedit software.

RESULTS

Enrollment and demographics. A total of 2,544 patients with fever were screened for malaria parasites, 479 of which were positive for *P. falciparum* and 83 for *P. vivax*, while 21 had mixed infections. Among the 479 *P. falciparum*-positive cases, 157 subjects were enrolled in the study and randomized into three study arms. The demographic data and clinical parameters of the study groups are summarized in Table 1.

***In vivo* therapeutic efficacy outcomes.** Out of a total of 157 recruited patients, 4 withdrew from the study voluntarily, 1 was lost to follow-up, and 152 (53 in the AS-SP, 49 in the AM-LF, and 50 in the AS-MQ groups) completed the 42-day follow-up period (Fig. 1).

Of the 53 subjects recruited in the AS-SP group, all completed the 42-day follow-up. The non-PCR-corrected incidence of failure (LPF+LCF) of AS-SP was 9.5% (5/53). *msp1* and *msp2* genotyping was done for 5 apparent therapeutic failures to differentiate between new infection and recrudescence, and all were classified as recrudescence. Therefore, the PCR-corrected cumulative incidence of failure of AS-SP for *P. falciparum* was 9.5% (95% confidence interval [CI], 0.04 to 0.212), and the therapeutic efficacy was 90.6% (95% CI, 0.793 to 0.969) (Table 2).

Among the 52 patients in the AM-LF group, 3 withdrew before day 42 and 49 completed the study. The crude therapeutic efficacy of AM-LF was 95.9% (47/49), and crude therapeutic failure was 2 (4.1%). Based on *msp1* and *msp2* genotyping of the parasite on day 0 and the day of recurrence, the two cases were classified as recrudescence. The PCR-corrected 42-day cumulative incidence of failure of AM-LF therapy for *P. falciparum* was 4.1% (95% CI, 0.010 to 0.149), and therapeutic efficacy was 95.9% (95% CI, 0.860 to 0.995), which was higher than for the AS-SP group (Table 2).

In the AS-MQ study arm, out of 52 subjects, 1 withdrew voluntarily and 1 was lost to follow-up. Fifty patients completed the 42-day follow-up. One came back to the clinic with fever on day 26, and the ring form of *P. falciparum* was detected in a peripheral blood smear, but genotyping could not be done to differentiate between reinfection and recrudescence, as parasite DNA could

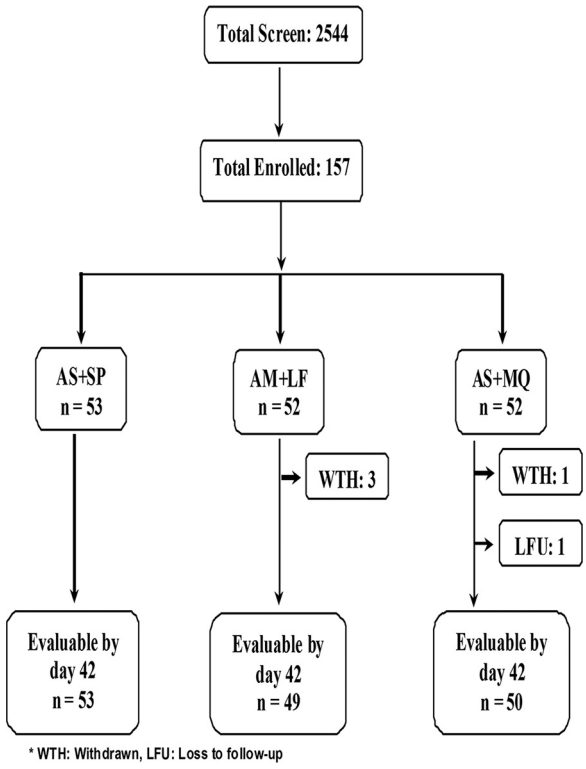

FIG 1 Profile of patients screened, enrolled, and completing the study protocol.

TABLE 2 Therapeutic efficacies of three study arms among patients with uncomplicated malaria in Jalpaiguri, India

Clinical outcome	AS-SP[a]			AM-LF[a]			AS-MQ[a]		
	n	%	95% CI	*n*	%	95% CI	*n*	%	95% CI
Non-PCR corrected									
Treatment failure	5	9.5	0.04–0.212	2	4.1	0.010–0.149	1	2.0	0.003–0.134
ETF	0	0	0.0–0.067	0	0	0.0–0.073	0	0	0.0–0.071
LCF	3	5.7	0.012–0.157	2	4.1	0.005–0.140	1	2.0	0.001–0.106
LPF	2	3.8	0.005–0.130	0	0	0.0–0.073	0	0	0.0–0.071
ACPR	48	90.6	0.793–0.969	47	95.9	0.860–0.995	49	98.0	0.894–0.999
PCR corrected									
Treatment failure	5	9.5	0.04–0.212	2	4.1	0.010–0.149	0	0	0.0–0.073
ETF	0	0	0.0–0.067	0	0	0.0–0.073	0	0	0.0–0.073
LCF	3	5.7	0.012–0.157	2	4.1	0.005–0.140	0	0	0.0–0.073
LPF	2	3.8	0.005–0.130	0	0	0.0–0.073	0	0	0.0–0.073
ACPR	48	90.6	0.793–0.969	47	95.9	0.860–0.995	49	100	0.927–1.00

[a] AS-SP, $n = 53$; AM-LF and AS-MQ, $n = 52$ for non-PCR corrected and $n = 49$ for PCR corrected.

not be amplified. This combination was 98% (95% CI, 0.894 to 0.999) effective, with failure to successfully PCR correct (Table 2). Importantly, no ETF was recorded in this group.

The statistical software R (version 2.13.1) was used for two sample tests for proportion. The difference in efficacy between AS-MQ (98%) and AS-SP (90.5%) was not statistically significant ($Z = 1.61$; $P = 0.05$). Similarly, no significant difference in efficacy was noted between AS-MQ (98%) versus AM-LF (95.9%) ($Z = 0.604$; $P = 0.27$) and AS-SP (90.5%) versus AM-LF (95.9%) ($Z = 1.06$; $P = 0.14$).

Fever and parasite clearance. In 27 (50.9%), 25 (47.1%), and 1 (1.9%) recipients of AS-SP, fever subsided by days 1, 2, and 3, respectively, while parasite clearance took place in 27 (50.9%), 17 (32.1%), and 9 (16.9%) by days 1, 2, and 3, respectively.

In the AM-LF group, fever subsided in 18 (34.6%), 32 (61.5%), and 2 (3.9%) patients by days 1, 2, and 3, respectively, with parasite clearance in 8 (15.4%), 30 (57.7%), and 14 (26.9%) by days 1, 2, and 3, respectively.

Fever subsided in 23 (42.2%) and 29 (55.8%) participants by days 1 and 2, respectively, while the parasites were cleared in 20 (38.5%), 22 (42.3%), and 10 (19.2%) by days 1, 2, and 3, respectively, among patients who received AS-MQ.

Side effects. Dizziness, headache, nausea, vomiting, and muscle pain were common in all the treatment arms, particularly among the patients who received AS-MQ (Table 3). These symptoms were treated symptomatically.

Allelic diversity and MOI of *P. falciparum*. Among the 157 samples from day 0, *msp1* and *msp2* genotyping was done successfully in 154 isolates. At the *msp1* locus, 13 alleles of the K1 family, 7 of the MAD20 family, and 10 of the RO33 family ranging from 110 to 530 bp, 150 to 250 bp, and 160 to 410 bp, respectively, were detected. For the *msp2* locus, 17 FC27 and 15 3D7 alleles ranging from 175 to 750 bp and 170 to 690 bp, respectively, were noted. The family distributions for K1, MAD20, and RO33 at *msp1* were 41.9%, 17.9%, and 40.2%, respectively, while for FC27 and 3D7 at *msp2* they were 49.0% and 51.0%, respectively. The frequencies of individual *msp1* genotypes were low, with 16 genotypes less than 10% and 14 greater than 10%, while at *msp2*, 25 genotypes were less than 10% and 7 more than 10%. The mean MOI was 3.10 (Table 4).

Sequencing of full-length *pfATPase6*. Sequencing of the full length of the *pfATPase6* gene was done successfully in 142 isolates. It revealed only sporadic point mutations compared to the wild-type sequence. Mutations at codons L263E and S769N, which have been proposed to confer artemisinin resistance, were not detected. While nonsynonymous mutations were detected at codons E431K (19%), K649E (25.4%), and N683K (5.6%), synonymous mutations were noted at codons G468G (2.8%), N483N (4.9%), and I898I (26.8%) (Table 5).

Mutations in *pfcrt*, *pfdhfr*, and *pfdhps*. The K76T mutation in the *pfcrt* gene was present in all the parasite strains ($n = 139$). The Southeast Asian haplotype (SVMNT) was predominant (86.3%) over the South American haplotype (CVIET) (13.7%) (Table 5).

The *pfdhfr* gene was sequenced successfully in 138 isolates that covered codons 16, 51, 59, 108, and 164. Mutations at codons C59R (100%) and S108N (95.7%) were more frequent than others. Four different haplotypes were recorded, among which $A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$ was the most prevalent (84.8%) (mutated amino acids are in boldface) (Table 5).

Sequencing of the *pfdhps* gene was successful in 126 isolates that covered codons 436, 437, 540, 581, and 613. Six different *pfdhps* genotypes were noticed, of which the $\mathbf{A}_{436}A_{437}\mathbf{E}_{540}A_{581}A_{613}$ (44.4%) and $S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$ (33.3%) haplotypes were common (Table 5). No changes were observed in the *pfdhfr* and *pfdhps*

TABLE 3 Side effects

Adverse effect	No. of patients[a]		
	AS-SP ($n = 53$)	AM-LF ($n = 52$)	AS-MQ ($n = 52$)
Muscle pain	33	41	45
Headache	26	22	43
Dizziness	12	16	23
Nausea	21	18	34
Vomiting	5	2	43
Anorexia	19	3	11
Diarrhea	1	0	2
Itching	7	0	0
Abdominal pain	2	1	9
Cough	3	2	2

[a] Number of patients having symptoms at least once during ACT treatment in Jalpaiguri.

TABLE 4 Genetic diversity of *msp1* and *msp2* of 154 *P. falciparum* isolates (day 0) from Jalpaiguri District, India

Parameter	Value							
	mspI				*msp2*			
	K1	MAD20	Ro33	Total	FC27	3D7	Total	Total
No. of distinct bands	13	7	10	30	17	15	32	62
No. (%) of isolates having alleles of the corresponding family	136 (88.3)	90 (58.4)	150 (97.4)		154 (100)	148 (96.1)		
% of bands assigned to the corresponding family	41.9	17.9	40.2		49.0	51.0		
Mean MOI (±SD)	2.67 (±1.15)	1.73 (±0.92)	2.32 (±0.96)	2.30	3.94 (±1.49)	4.27 (±1.29)	4.10	3.10

genotypes of pretreatment and recrudescent samples from five treatment failure cases in AS-SP recipients.

Association between *pfdhfr* and *pfdhps* haplotypes and therapeutic outcomes of three ACT regimens. Sequencing of both the *pfdhfr* and *pfdhps* genes was done in 126 isolates. Quadruple mutations were found in 55 (43.6%), and quintuple mutations were recorded in only 8 (6. 3%) cases (Table 6). Although the sample size was small, a trend of association was observed between quadruple (*pfdhfr*, $\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$, and *pfdhps*, $\mathbf{G}_{437}$) ($P = 0.0316$) and quintuple (*pfdhfr*, $\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$, and *pfdhps*, $\mathbf{A}_{436}\mathbf{E}_{540}$) haplotypes ($P = 0.01107$) with AS-SP resistance, as analyzed by Fisher's exact test. A triple *pfdhfr* mutation ($\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$) was common in both the haplotypes. Thus, the *pfdhfr* $\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$ triple mutant was significantly associated ($P = 0.000198$) with AS-SP resistance, along with either a *pfdhps* $\mathbf{G}_{437}$ or $\mathbf{E}_{540}$ mutation.

TABLE 5 Polymorphism of *Pfcrt*, *PfATPase6*, *Pfdhfr* and *Pfdhps* genes in study population

Candidate gene[a]	Occurrence of mutation		
	n	%	95% CI
Pfcrt ($n = 139$)			
Haplotypes			
$\mathbf{S}_{72}V_{73}M_{74}N_{75}\mathbf{T}_{76}$	120	86.3	80.62–92.04
$\mathbf{C}_{72}V_{73}\mathbf{I}_{74}\mathbf{E}_{75}\mathbf{T}_{76}$	19	13.7	7.96–19.38
PfATPase6 ($n = 142$)			
Synonymous mutation			
G468G	4	2.8	0.1–5.54
N483N	7	4.9	1.37–8.49
I898I	38	26.8	17.56–31.74
Nonsynonymous mutation			
E431K	27	19.0	12.56–25.46
K649E	36	25.4	18.19–32.51
N683K	8	5.6	1.84–9.42
Pfdhfr ($n = 138$)			
A16V	0	0	0–2.13
N51I	13	9.4	4.55–14.29
C59R	138	100.0	97.87–100.69
S108N	132	95.7	92.25–99.05
I164L	8	5.8	1.9–9.7
Genotype			
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}S_{108}I_{164}$	6	4.3	0.95–7.75
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$	117	84.8	78.79–90.77
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$	8	5.8	1.9–9.7
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$	7	5.1	1.41–8.73
Pfdhps ($n = 126$)			
S436A	63	50.0	41.27–58.73
A437G	42	33.3	25.1–41.56
K540E	64	50.8	42.06–59.52
A581G	7	5.6	1.56–9.56
A613S	0	0	0–2.34
Genotype			
$S_{436}A_{437}K_{540}A_{581}A_{613}$	6	4.8	1.04–8.48
$\mathbf{A}_{436}A_{437}K_{540}A_{581}A_{613}$	7	5.6	1.56–9.56
$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	42	33.3	25.1–41.56
$S_{436}A_{437}\mathbf{E}_{540}A_{581}A_{613}$	8	6.3	2.09–10.61
$S_{436}A_{437}K_{540}\mathbf{G}_{581}A_{613}$	7	5.6	1.56–9.56
$\mathbf{A}_{436}A_{437}\mathbf{E}_{540}A_{581}A_{613}$	56	44.4	35.76–53.12

[a] Mutated amino acids are in boldface.

DISCUSSION

Artemisinin-based combination therapy has been recommended by WHO to treat uncomplicated *P. falciparum* cases in all countries where malaria is endemic (30, 31, 33), as artemisinin and its derivatives reduce most of the parasite biomass during their initial rapid action and an effective long-acting partner drug can usually eliminate the small number of remaining parasites. Five combinations are currently recommended: AM-LF, AS-MQ, AS-SP, dihydroartemisinin-piperaquine, and artesunate-amodiaquine (34). The combination AS-SP was introduced by the Government of India in 2010 as the first-line agent to treat all diagnosed uncomplicated *P. falciparum* malaria.

In the present study, a treatment failure rate of 9.5% (95% CI, 0.04 to 0.212) was recorded for AS-SP. A previous study showed that a significant proportion of *P. falciparum* malaria in the study area was resistant to SP alone (20), with a record of both ETF and LTF, but in the present study, not a single case of ETF was observed in any of the three study arms. Perhaps this was due to early clearance of the parasite by the artemisinin component of the combination therapy. Dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) are two essential enzymes in the folate biosynthesis pathway and are inhibited by pyrimethamine and sulfadoxine, respectively. The mutations at *pfdhfr* $\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$ and *pfdhps* $\mathbf{G}_{437}$ and $\mathbf{E}_{540}$ are associated with SP treatment failure (17, 27). In the present study, a quadruple haplotype (*pfdhfr* $\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$ and *pfdhps* $\mathbf{G}_{437}$) and a quintuple haplotype (*pfdhfr* $\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$ and *pfdhps* $\mathbf{A}_{436}\mathbf{E}_{540}$) were noted to be significantly associated with AS-SP failure ($P = 0.0316$ and $P = 0.01107$, respectively). In both the haplotypes, a triple *pfdhfr* $\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$ mutation was commonly associated with AS-SP failure ($P = 0.000198$). However, it is not possible to postulate that the *pfdhfr* $\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$ mutation alone was responsible for AS-SP failure, as we did not come across any isolate having this genotype exclusively without a *pfdhps* $\mathbf{G}_{437}$ and/or $\mathbf{E}_{540}$ mutation.

Currently, 11 countries are using AS-SP as a first- or second-line treatment. AS-SP treatment failure rates remained low in

TABLE 6 Association between *dhfr-dhps* mutations and *in vivo* therapeutic efficacy outcomes of three ACT regimens in the treatment of *P. falciparum* malaria in Jalpaiguri District, India

Pfdhfr-Pfdhps genotype[a]	No. of patients					
	AS-SP ($n = 43$)		AM-LF ($n = 41$)		AS-MQ ($n = 42$)	
	ACPR	TF[b]	ACPR	TF	ACPR	TF
Double mutant						
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$	2	0	3	0	1	0
Triple mutant						
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}A_{437}K_{540}A_{581}A_{613}$	3	0	2	0	2	0
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	10	0	7	0	12	0
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}\mathbf{E}_{540}A_{581}A_{613}$	2	0	3	0	3	0
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}\mathbf{G}_{581}A_{613}$	3	0	2	0	2	0
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}S_{108}I_{164}$-$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	2	0	2	0	2	0
Quadruple mutant						
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	1	2	1	0	1	0
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}A_{437}\mathbf{E}_{540}A_{581}A_{613}$	14	0	19	0	15	0
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	1	0	0	0	1	0
Quintuple mutant						
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}A_{437}\mathbf{E}_{540}A_{581}A_{613}$	0	2	0	0	0	0
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$\mathbf{A}_{436}A_{437}\mathbf{E}_{540}A_{581}A_{613}$	0	1	0	2	3	0

[a] Mutated amino acids are in boldface.
[b] TF, treatment failure.

studies conducted in South American countries (Colombia, Ecuador, and coastal areas of Peru), the Middle East and Central Asia (Afghanistan, the Islamic Republic of Iran, Pakistan, Tajikistan, and Yemen), South Asia (India and Sri Lanka), and eastern Africa (Somalia and the Sudan), including countries in which AS-SP was used as a first-line treatment, where the median treatment failure rate was 0 to 1.5% (34). The high clinical efficacy of this combination may be partially due to the rarity of the *pfdhfr* and *pfdhps* quintuple mutant (36). In contrast, high failure rates of this combination have been observed in several African countries with high SP resistance.

AM-LF remains highly effective in most parts of the world, with the exception of Cambodia (34). In India, two cases of AM-LF-resistant *P. falciparum* were reported from Orissa (29). A treatment failure rate of 4.1% (95% CI, 0.010 to 0.149) was recorded in the present study with AM-LF.

The artesunate-mefloquine combination was first introduced in Thailand to treat *P. falciparum* malaria after the spread of mefloquine resistance. Presently, eight countries are using this combination as a first- or second-line treatment. High therapeutic efficacy of this combination has been recorded in different parts of the world, except in Cambodia and Thailand (35). No such record is available from India. In this study, only a single treatment failure was observed. However, it could not be determined whether it was due to reinfection or recrudescence, as we failed to genotype the strain.

As the pharmacokinetics and molecular markers associated with LF and MQ were not studied, the observed failure of *P. falciparum* cases treated with AM-LF (4.1%) could not be explained.

pfATPase6 has been suggested to be a specific target of the artemisinin drugs (10). Decreased *in vitro* sensitivity to artemether of field isolates of *P. falciparum* was reported from French Guiana (but not in Cambodia) and was found to be associated with the S769N mutation in the *pfATPase6* gene (10, 16). In this study, no consistent pattern of *pfATPase6* mutations was noted. Mutations at codons L263E and S769N, associated with artemisinin resistance, were not detected, although *in vivo* failure rates of 9.5% and 4.1% with AS-SP and AM-LF, respectively, were documented. Similar observations were made by Dondorp et al. (8) with *in vivo* artemether monotherapy.

Genotyping of day 0 samples showed that both the allelic variation and mean MOI of *msp2* were higher than those of *msp1* and that the frequencies of most of the alleles (41/62) were below 10%. The *P. falciparum* population of the study area was genetically diverse, with 30 *msp1* and 32 *msp2* alleles. As the frequencies of most of the *msp1* and *msp2* alleles were low, the probability of new infection with a genotype identical to that in pretreatment infection was very rare. In the present study, all 7 (5 in the AS-SP arm and 2 in the AM-LF arm) recurrent parasitemias were classified as recrudescence. We used the agarose gel electrophoresis method for genotyping. The method is limited by its inability to discriminate among DNA fragments with small differences in molecular weight. Recently, various researchers (18, 21) have used capillary-based genotyping assays, which have a highly accurate fragment-sizing capacity.

The PCR-corrected failure rate of the recommended ACT (AS-SP) in *P. falciparum* malaria was 9.5%, which was just below the cutoff level for drug policy change (10%) recommended by WHO. Considering the observed failure rate of AS-SP and its association with a quadruple haplotype (*pfdhfr* $\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$ and *pfdhps* $\mathbf{G}_{437}$) and a quintuple haplotype (Pfdhfr $\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$ and Pfdhps $\mathbf{A}_{436}\mathbf{E}_{540}$) in the study area, the efficacy of this combination should be closely monitored periodically. The efficacies of the other two combinations were superior to that of AS-SP, which may attract policy planners. However, further studies are needed in other parts of the country to have a better understanding and knowledge of the resistance pattern of artemisinin combination therapy.

ACKNOWLEDGMENTS

We are grateful to the Department of Health and Family Welfare, Government of West Bengal, India, for funding the project. We especially thank all the patients who participated in this study and the staff of the Turturi PHC. We are grateful to the Director, Kolkata School of Tropical Medicine, for his kind permission to publish the data.

We have no conflicts of interest concerning the work reported in this paper.

A.K.M., S.K.G., and D.K.B. conceived and designed the study protocol; P.S., S.M., S.G., S.D., P.K.K., and A.B. performed the clinical assessment and the in-vivo therapeutic efficacy study; P.S., A.K.M., G.C., and M.D. performed the PCR and sequencing analysis and interpretation of data; A.K.M., P.S., S.K.G., G.C., K.R., and D.K.B. drafted the manuscript. All the authors read and approved the final manuscript.

REFERENCES

1. **Afonso A, et al.** 2006. Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes atp6 (encoding the sarcoplasmic and endoplasmic reticulum $Ca2^{+}$-ATPase), tctp, mdr1, and cg10. Antimicrob. Agents Chemother. **50**:480–489.
2. **Ahmed A, et al.** 2004. *Plasmodium falciparum* isolates in India exhibit a progressive increase in mutations associated with sulfadoxine-pyrimethamine resistance. Antimicrob. Agents Chemother. **48**:879–889.
3. **Andriantsoanirina V, et al.** 2009. *Plasmodium falciparum* drug resistance in Madagascar: facing the spread of unusual *pfdhfr* and *pfmdr-1* haplotypes and the decrease of dihydroartemisinin susceptibility. Antimicrob. Agents Chemother. **53**:4588–4597.
4. **Brooks DR, et al.** 1994. Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, Plasmodium falciparum, with differing resistance to sulfadoxine. Eur. J. Biochem. **224**:397–405.
5. **Bzik DJ, Li WB, Horii T, Inselburg J.** 1987. Molecular cloning and sequence analysis of the *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene. Proc. Natl. Acad. Sci. U. S. A. **84**:8360–8364.
6. **Djimdé A, et al.** 2001. A molecular marker for chloroquine-resistant falciparum malaria. N. Engl. J. Med. **344**:257–263.
7. **Djimdé A, Doumbo OK, Steketee RW, Plowe CV.** 2001. Application of a molecular marker for surveillance of chloroquine resistant falciparum malaria. Lancet **358**:890–891.
8. **Dondorp AM, et al.** 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. N. Engl. J. Med. **361**:455–467.
9. **Duraisingh MT, et al.** 2000. The tyrosine-86 allele of the *pfmdr1* gene of *Plasmodium falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. Mol. Biochem. Parasitol. **108**:13–23.
10. **Eckstein-Ludwig U, et al.** 2003. Artemisinins target the SERCA of *Plasmodium falciparum*. Nature **424**:957–961.
11. **Falk N, et al.** 2006. Comparison of PCR-RFLP and Genescan-based genotyping for analyzing infection dynamics of Plasmodium falciparum. Am. J. Trop. Med. Hyg. **74**:944–950.
12. **Fidock DA, et al.** 2000. Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol. Cell **6**:861–871.
13. **Foote SJ, et al.** 1990. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in Plasmodium falciparum. Nature **345**:255–258.
14. **Foote SJ, Thompson JK, Cowman AF, Kemp DJ.** 1989. Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. Cell **57**:921–930.
15. **Imwong M, et al.** 2010. Exploring the contribution of candidate genes to artemisinin resistance in *Plasmodium falciparum*. Antimicrob. Agents Chemother. **54**:2886–2892.
16. **Jambou R, et al.** 2005. Resistance of *Plasmodium falciparum* field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6. Lancet **366**:1960–1963.
17. **Kublin JG, et al.** 2002. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of Plasmodium falciparum malaria. J. Infect. Dis. **185**:380–388.
18. **Liljander A, et al.** 2009. Optimization and validation of multi-coloured capillary electrophoresis for genotyping of Plasmodium falciparum merozoite surface proteins (msp1 and 2). Malar. J. **8**:78.
19. **Mittra P, et al.** 2006. Progressive increase in point mutations associated with chloroquine resistance in Plasmodium falciparum isolates from India. J. Infect. Dis. **193**:1304–1312.
20. **Mullick S, et al.** 2011. Efficacy of chloroquine and sulphadoxine-pyrimethamine either alone or in combination before introduction of ACT as first line therapy in uncomplicated *P. falciparum* malaria in Jalpaiguri District, West Bengal, India. Trop. Med. Int. Health **16**:929–935.
21. **Mwingira F, et al.** 2011. *Plasmodium falciparum* msp1, msp2 and Glurp allele frequency and diversity in sub-Saharan Africa. Malar. J. **10**:79.
22. **Peterson DS, Walliker D, Wellems TE.** 1988. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc. Natl. Acad. Sci. U. S. A. **85**:9114–9118.
23. **Sehgal PN, Sharma MID, Sharma SL, Gogoi S.** 1973. Resistance to chloroquine in falciparum malaria in Assam state, India. J. Commun. Dis. **5**:175–180.
24. **Sidhu AB, et al.** 2006. Decreasing pfmdr1 copy number in Plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J. Infect. Dis. **194**:528–535.
25. **Sisowath C, et al.** 2005. In vivo selection of Plasmodium falciparum pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). J. Infect. Dis. **191**:1014–1017.
26. **Snounou G.** 2002. Genotyping of *Plasmodium* spp. Nested PCR. Methods Mol. Med. **72**:103–116.
27. **Talisuna AO, et al.** 2004. Two mutations in dihydrofolate reductase combined with one in the dihydropteroate synthase gene predict sulphadoxine-pyrimethamine parasitological failure in Ugandan children with uncomplicated falciparum malaria. Infect. Genet. Evol. **4**:321–327.
28. **Uhlemann AC, et al.** 2005. A single amino acid residue can determine the sensitivity of SERCAs to artemisinin. Nat. Struct. Mol. Biol. **12**:628–629.
29. **Valecha N, et al.** 2009. Therapeutic efficacy of artemether-lumefantrine in uncomplicated falciparum malaria in India. Malar. J. **8**:107.
30. **World Health Organization.** 2001. Antimalarial drug combination therapy. Report of a WHO technical consultation. World Health Organization, Geneva, Switzerland.
31. **World Health Organization.** 2006. Guidelines for the treatment of malaria. World Health Organization, Geneva, Switzerland.
32. **World Health Organization.** 2009. Methods for surveillance of antimalarial drug efficacy. World Health Organization, Geneva, Switzerland.
33. **World Health Organization.** 2010. Guidelines for the treatment of malaria, 2nd ed. World Health Organization, Geneva, Switzerland.
34. **World Health Organization.** 2010. World malaria report: 2010. World Health Organization, Geneva, Switzerland.
35. **World Health Organization.** 2010. Global report on antimalarial drug efficacy and drug resistance: 2000–2010. World Health Organization, Geneva, Switzerland.
36. **Zakeria S, et al.** 2010. High prevalence of the 437G mutation associated with sulfadoxine resistance among *Plasmodium falciparum* clinical isolates from Iran, three years after the introduction of sulfadoxine-pyrimethamine. Int. J. Infect. Dis. **14**(Suppl. 3):e123–e128.