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Therapeutic efficacy of artemisinin combination therapies and prevalence of S769N mutation in *Pf*ATPase6 gene of *Plasmodium falciparum* in Kolkata, India

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ABSTRACT

Objective: To study the *in vivo* efficacy of these two ACTs in the treatment of *Plasmodium falciparum* (*P. falciparum* malaria) in Kolkata and to determine the prevalence of mutant S769N codon of the *Pf*ATPase6 gene among field isolates of *P. falciparum* collected from the study area. **Methods:** A total of 207 *P. falciparum* positive cases were enrolled randomly in two study arms and followed up for 42 days as per WHO (2009) protocol. A portion of *Pf*ATPase6 gene spanning codon S769N was amplified and sequenced by direct sequencing method. **Results:** It was observed that the efficacy of both the ACT regimens were highly effective in the study area and no mutant S769N was detected from any isolate. **Conclusions:** The used, combination AS+SP is effective and the other combination AM+LF might be an alternative, if needed.

1. Introduction

Malaria, particularly due to *Plasmodium falciparum* (*P. falciparum*) is a leading cause of disease and death worldwide. Approximately 5% of the World's population is at risk and an estimated 225 million confirmed cases and 781 000 deaths have been reported in 2009[1]. The emergence and spread of multidrug-resistant *P. falciparum*, particularly to the previous mainstay antimalarial drugs chloroquine (CQ), and sulphadoxine-pyrimethamine (SP) is further worsening the situation. To deal with the resistance of *P. falciparum*, World Health Organization (WHO) now advocates artemisinin combination therapies (ACTs) as the first-line treatment for uncomplicated falciparum malaria in all malaria endemic areas[2]. Replacing ineffective, failing treatments (CQ and SP) with ACTs the morbidity and mortality associated with malaria has been reduced[3-5].

In India, about 2 million cases of malaria are reported annually and till 2009 CQ was the first line treatment for *P. falciparum*. Resistance to CQ was first reported in 1973 from Diphu area of Karbi Anglong district of Assam[6] and then has spread in various parts of the country[7-9]. Therapeutic efficacy studies done in India between 1978 and 2007 documented a failure to chloroquine beyond cut off level of 10%[10]. In an effort to counteract the increasing resistance of *P. falciparum* to CQ National Vector Borne Disease Control Programme has recently introduced ACT, a combination of artesunate + sulphadoxine-pyrymethamine (AS + SP), as the first line agent for the treatment of uncomplicated *P. falciparum* malaria throughout India. Among the other combinations of ACTs, artemether + lumefantrine (AM+LF),

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not included in the National Drug Policy, but licensed for marketing in India is in frequent use by private practitioners.

Although artemisinin and its derivatives are the most potent and rapidly acting antimalarials, its resistance has been reported in murine models of malaria[11]. More recently, *in vivo* and *in vitro* artemisinin resistance has been documented among human falciparum malaria infections in South East Asia[12,13]. A recent *in vivo* randomized, three-arm open label prospective trial of ACTs by Calcutta School of Tropical Medicine in Jalpaiguri district of West Bengal, India has showed a therapeutic failure of 9.5% and 4.1% in AS+SP and AM+LF study arms respectively (unpublished data). Studies provide compelling evidences that artemisinins act by selectively inhibiting *Pf*ATPase6 protein, the only SERCA-type Ca^{2+}-ATPase in the *P. falciparum* genome, believed to be the primary target for artemisinins[14,15]. A subsequent study in French Guyana showed that S769N *Pf*ATPase6 mutation was associated with raised artemether IC_{50}, and suggested that the mutation may be used as a molecular marker for monitoring artemisinin resistance[16]. The present study was aimed to evaluate the *in vivo* therapeutic efficacy of two commonly used combinations of ACTs *i.e.* AS+SP and AM+LF and to investigate the polymorphism at codon S769N of the *Pf*ATPase6 gene among *P. falciparum* field isolates collected from Kolkata, India.

2. Materials and methods

2.1. Study site and design

The study was carried out at the Malaria Clinic attached to the Department of Protozoology of the Calcutta School of Tropical Medicine, Kolkata, India from October, 2010 to February, 2011, where the annual parasite index in 2008 was 10.69. In Kolkata, malaria transmission is seasonal (July-December) with predominance of falciparum malaria.

The study was a randomized, two-arm open label prospective trial, for evaluation of clinical and parasitological responses of two ACTs (AS+SP and AM+LF) for treatment of uncomplicated *P. falciparum* malaria, based on the therapeutic efficacy protocols of WHO[17]. The study protocol was approved by the Institutional Ethics Committee of the Calcutta School of Tropical Medicine.

2.2. Screening and enrolment of patients

The febrile patients from the surrounding areas attending the Malaria Clinic of the Calcutta School of Tropical Medicine were screened for malarial parasite by examining Giemsa stained thick and thin peripheral blood smears. All patients with confirmed *P. falciparum* mono-infection were explained about the study protocol and requested to participate in the study. Those who fulfilled the following inclusion criteria as per WHO protocol[17] were enrolled after obtaining written, informed consent.

All patients with confirmed *P. falciparum* infection (asexual parasites 1 000-100 000/ μL), with fever (axillary temperature ≥37.5 ℃) or history of fever in preceding 24 h and fulfilling other inclusion criteria were explained about the benefits and risks of the study. Pregnant or lactating mother and children under 5 kg bodyweight were excluded. Patients with other febrile conditions, danger signs[17] or severe malaria were also excluded. Recruited patients were randomized into two study groups by using "Simple Random Sampling without Replacement".

2.3. Treatment of enrolled patients

The therapeutic efficacy of two different regimens of ACTs was evaluated in the present study. The tested ACTs were AS+SP, AM+LF. AS and SP were supplied by NVBDCP, AM+LF were procured from M/S Themis Medicare Limited, India. All the patients were treated as per WHO guidelines[2] for the treatment of Malaria according to their body weight. In AS+SP group, artesunate 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 AM+LF group (a co-formulated 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 hours. The number of tablets per dose according to body weight; one tablet for 10-15 kg, two tablets for 15-25 kg, three for 25-35 kg and four for those weighing over 35 kg. Primaquine (PQ) 0.75mg/Kg single dose was given on day 1. Study team directly observed and documented administration of each dose of medication.

2.4. Follow up and study endpoints

The day, the patient was enrolled and received the first dose of medicine was designated as day 0. Thereafter, the schedule calls for clinical re-assessment were done on day 1, 2, 3, 7, 14, 21, 28, 35 & 42. Patients were advised to return on any day during follow-up period if symptoms recurred without waiting for schedule visit day. During the follow-up visits the axillary temperature was recorded. In addition, thorough clinical examination was carried out to identify the presence of any danger signs of severe and complicated malaria. On the event of occurrence of any such complications the patient was withdrawn from the study and was treated as per existing standard of care for that condition. Blood was collected in EDTA vial on day 0 and any other day on reappearance of parasite for molecular biology studies.

According to the WHO 42-day follow-up protocol[17] for assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated falciparum malaria, the follow-up completed patients met one of the following

clinical endpoints: (1) early treatment failure, (2) late treatment failure, which included late clinical failure and late parasitological failure and (3) adequate clinical and parasitological response .

2.5. Statistical analysis

The data was entered into a standard data entry programme designed by Global Malaria Programme and analyzed by Kaplan-Meier survival curve according to WHO standard procedures (http://www.who.int/malaria/resistance). Statistical software "R" (version 2.13.1) was used to calculate the Z-test value for the comparison of the efficacy of the two study arms and the 95% confidence interval was calculated by Dimension Research calculator (http://www.dimensionresearch.com/resources/resources overview.html).

2.6. Laboratory examination

2.6.1. Microscopy and parasite count

Parasite counts were done on Giemsa-stained thick films and the number of parasites per 200 WBC was counted, assuming a WBC count to be 8 000/μL, parasitaemia was calculated and expressed as per μL of blood. A thick smear was diagnosed as negative on initial review if no parasites were seen in 100 oil immersion fields and 10% of positive and negative slides were crosschecked. Females in child bearing age group were subjected to human chorionic gonadotropin test (TestPack® +Plus™ hCG Urine, Abbott, USA).

2.6.2. DNA extraction and PCR genotyping

Genomic DNA of *P. falciparum* was isolated from 200 μL EDTA blood using QiaAmp DNA minikit as per manufacturer's instructions (Qiagen, Hilden, Germany). Isolated DNA was stored at -20 ℃ and an aliquot was used as the DNA source for molecular biological study.

The recrudescence and re-infection of *P. falciparum* was differentiated by using msp 1 (block 2), msp 2 (block 3) marker genes. The polymorphic repetitive regions of these genes were amplified by nested PCR using the oligonucleotide primers, as described elsewhere[18,19]. All amplification reactions were carried out in a final volume of 20 μL which included 2 μL of DNA template (genomic DNA for the primary reactions and the product of the primary reaction for the secondary amplification). Oligonucleotide primers were used at final concentrations of 0.3 μM in both primary as well as secondary reactions. The reaction mixture contained PCR Buffer, 0.2 mM concentration of each of the four deoxynucleoside triphosphates, and 0.75 U of Ampli*Taq* polymerase (Perkin Elmer, Branchburg, NJ, USA). The reactions were carried out in the presence of 2 mM $MgCl_2$ for all oligonucleotide combinations except the msp 2 nested PCR, for which a concentration of 1.5 mM $MgCl_2$ was used. Amplification was performed using a Veriti 96 well Thermal Cycler (Perkin Elmer, Branchburg, NJ, USA) under the following conditions: an initial denaturation at 94 ℃ for 2 min followed by 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 54 ℃ for primary PCR (50 ℃ for msp 2 nested and 59 ℃ for msp 1 nested) for 1 minute, extension at 72 ℃ for 2 min. The final extension was done at 72 ℃ for 5 min. The PCR products were stored at 4 ℃ until further analysis was done.

Nested PCR products were analyzed by electrophoresis using 2% agarose gels (performed in TBE buffer). All the distinguishable allelic variants for each marker paired samples were loaded side by side. The gels were stained with Ethidium Bromide and visualized under UV illumination and documented by Gel-Doc system. Gel photographs were analyzed by visual comparison of DNA fragments on base line and recurrent samples.

2.6.3. Nested PCR-sequencing assay of PfATPase6

We analyzed *Pf*ATPase6 S769N mutation, which was thought to be associated with artemisinin resistance[14,15], among study isolates. The fragment of *Pf*ATPase6 in which S769N is located, was amplified by nested PCR method. The primary amplification was performed by using the oligonucleotide primers *Pf*ATPase6-P1, 5′-TTTATTTTCATCTACCGCTATTGTATGTGG-3′ and PfATPase6-P2, 5′- GCATTTATACATCCTTCTCGTTAATCTAAT-3′, as described elsewhere[20], in a reaction mixture of total volume 20 μL which consisted of 3 μL of genomic DNA, 0.3 μM of each primer pair, 0.2 mM of each deoxynucleoside triphosphate (dATP, dTTP, dGTP, dCTP), 2.5 mM $MgCl_2$, PCR buffer, and 1.2 unit of *Taq* DNA Polymerase (Perkin Elmer, Branchburg, NJ, USA). Amplification was performed under the following conditions: initial denaturation at 94 ℃ for 3 min, followed by 35 cycles of denaturation at 94 ℃ for 45 sec, annealing at 46 ℃ for 1 min, and extension at 72 ℃ for 1 minute and a final extension period at 72 ℃ for 10 min. The nested amplification was done by using the oligonucleotide primers *Pf*ATPase6-N1, 5′-CACCTGTACAATCATCAAATAAGAAGG-3′ and PfATPase6-N2, 5′- CTTCTAATTTATAATAATCATCTGTATTC-3′, as described elsewhere[20], in a total volume 50 μL with similar reaction mixture described above and using primary product as template. Amplification was performed under the following conditions: initial denaturation at 94 ℃ for 3 min, followed by 35 cycles of denaturation at 94 ℃ for 1 min, annealing at 50 ℃ for 1 min, and extension at 72 ℃ for 1 minute and a final extension period at 72 ℃ for 10 min. Secondary PCR products were resolved by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. Sequencing reactions were carried out with the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit on a 3730 XL genetic analyzer (Perkin Elmer, Branchburg, NJ, USA) as specified by the manufacturer's protocol. The

sequences of the amplicons were analyzed using the Bioedit Sequence Alignment Editor version 7.0.5.2. The sequences were then aligned using the online sequence alignment tool ClustalW (available at: http: //www.ebi.ac.uk/clustalw).

3. Results

3.1. In vivo therapeutic efficacy

Patients were screened between October, 2010 and February, 2011 at the study site. A total of 2 176 patients with fever were screened for malarial parasite, of whom 351 were positive for *P. falciparum*; 189 for *Plasmodium vivax (P. vivax)*; and 26 had mixed infections. Among the 351 *P. falciparum* cases, 207 patients were enrolled into the study and were randomized into two study arms. In AS+SP study arm 102 patients and in AM+LF study arm 105 patients were recruited (Figure 1). The demographic data and clinical parameters of the study groups are summarized in Table 1.

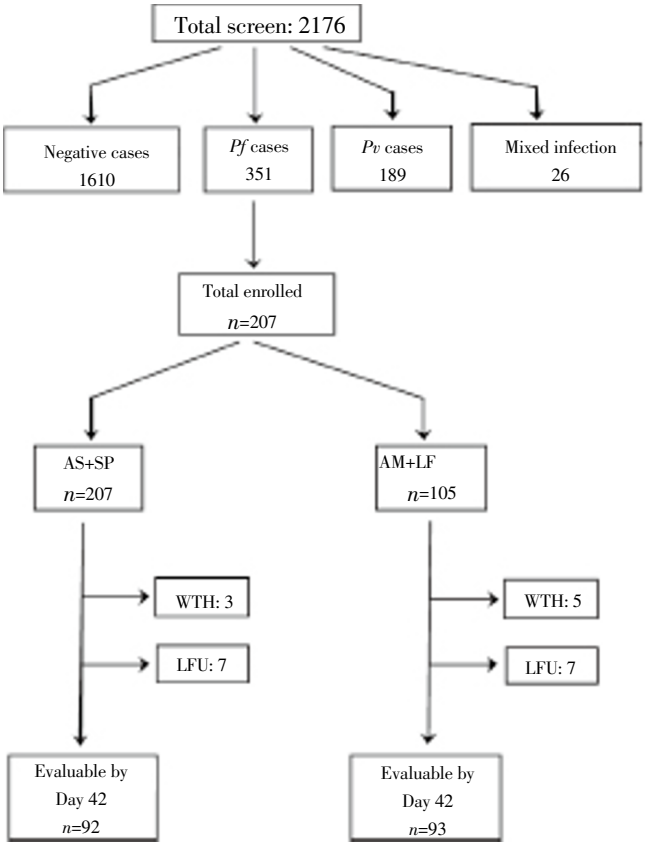

Figure 1. Profile of patients screened, enrolled and completing study protocol.
* WTH: Withdrawn, LFU: Loss to Follow-up.

In AS+SP arm, out of 102 patients, three patients withdrew (one on day 1, one on day 2 and one on day 7) their consent from the study and seven patients were lost to follow-up due to movement away from site and could not be traced. End point was reached in 92 cases. No treatment failure case was recorded, so all the cases were classified as ACPR. Therefore, cure rate of AS+SP by per protocol analysis was 100% (95% *CI* 96.1 – 100.0) (Figure 2).

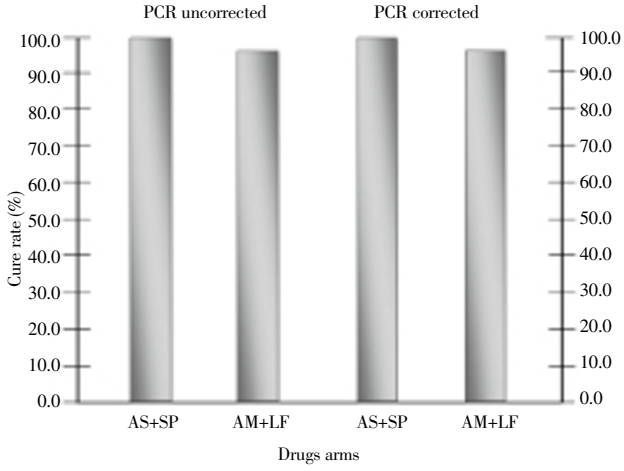

Figure 2. PCR uncorrected and PCR corrected cure rates by per protocol analysis.

In AM+LF arm, out of 105 recruited patients, five patients withdrew (three on day 1 and two on day 7) their consent, seven patients were lost to follow-up due to movement away from the site and 93 cases completed 42 days follow-up. Out of 93, one patient came back with fever and asexual form of *P. falciparum* was detected on day 26. The msp-1 and msp-2 genotyping of the treatment failure case showed that the recurring parasitaemia was due to recrudescence. Therefore, PCR corrected cure rate of AM+LF was 98.9% (95% *CI* 94.2 – 100.0) (Figure 2).

Statistical software "R" (version 2.13.1) was used for two sample tests for proportion. Both the combinations were highly effective and no significant difference was observed between the efficacy of AS+SP and AM+LF ($Z = 0.997$, $P = 0.159$).

3.2. Analysis of PfATPase6 mutation

The complete DNA sequence of the *Pf*ATPase6 gene is 4 049-basepairs (bp), and it is located in chromosome 1. The gene contains three exons and three introns. A 1 793-bp fragment spanning the coding region of exon 1 of the *Pf*ATPase6 gene was amplified by primary PCR. In the nested reaction, a 645-bp fragment was amplified from the primary amplicon which contain the S769N codon. The nested PCR products were then sequenced.

Out of 207 isolates, 100 samples (50 samples of AS+SP arm and 50 samples of AM+LF arm) were targeted for sequencing assay which were successfully performed in 92 isolates (45 in AS+SP arm and 47 in AM+LF arm). No S769N mutation was detected in any of the analyzed samples (Figure 3).

Table 1

Baseline characteristics of the study patients.

Study Arms	Characteristics						
	Sex no. (%)	Weight(kg)	Age category no. (%)	Age(year)	Temperature (℃)	Haemoglobin (g/dL)	Parasite count (no/ μ L)
AS+SP	Male:67 (67.7)	47.9±12.3	5–15: 29 (28.4)	30.3±15.2	37.8±0.21	11.84±1.46	8469.0±15259.2
(*n* = 102)	Female:35 (34.3)	(25–77)	Adult: 73 (71.6)	(9–68)	(37.5–38.6)	(9.9–15.0)	(1040–90000)
		95% *CI*: 45.51–50.29		95% *CI*: 27.35–33.25	95% *CI*: 37.76–37.84	95% *CI*: 11.56–12.12	95% *CI*: 5507.72–11430.28
AM+LF	Male:62 (59.1)	48.2±13.9	5–15: 35 (33.3)	29.5±16.1	37.7±0.39	11.04±1.15	8176.0±13655.1
(*n* = 105)	Female: 43	(17–77)	Adult: 70 (66.7)	(6–63)	(37.5–40.0)	(8.5–14.0)	(1000–80000)
	(40.9)	95% *CI*: 45.54–50.86		95% *CI*: 26.42–32.58	95% *CI*: 37.63–37.77	95% *CI*: 10.82–11.26	95%*CI*: 5564.15–10787.85

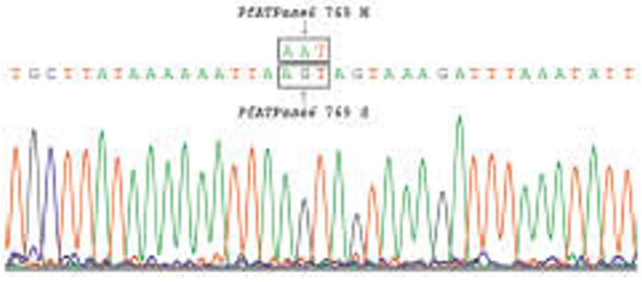

Figure 3. Sequencing image of nested PCR product indicating the polymorphism at codon 769 of *Pf*ATPase6 gene.

4. Discussion

Due to the increasing CQ resistance of *P. falciparum* reported by different studies from various parts of the country[10], Government of India has included ACT (AS+SP) in National Drug Policy for treatment of all uncomplicated *P. falciparum* in 2010. Previously AS+SP was used in all primary health centers (PHCs) of 117 high risk districts and 256 chloroquine resistant PHCs of 48 districts for the treatment of falciparum malaria. Before the introduction of AS+SP, CQ resistant *P. falciparum* cases were treated by SP as the second line of treatment. However, SP resistance has developed quickly in other parts of the world following its widespread use[21-24]. During 2001–2007, twenty two studies were conducted in different parts of India and recorded SP failure rate 0.0%–56.7% with a median of 13.6%[25]. As SP is the partner drug of the recommended ACT, so the periodical monitoring of this combination will be helpful. Government of India has recently approved another ACT, AM+LF, for marketing but has not included in the National Drug Policy. AM+LF is a fixed dose combination and frequently used by private practitioners since 2006.

In this study, we found that both the combinations were well tolerated and produced rapid parasite and fever clearance. Majority of patients of both the study arms were free of parasites within 48 hours and cure rates were very high at the study site where chloroquine resistance is high (66%)[26]. The AS+SP combination was 100% effective against *P. falciparum* malaria. Studies from other parts of the country showed 0%–4% failure rate with this combination[25]. A recent study by Calcutta School of Tropical Medicine recorded a significant failure rate (9.5%) of AS + SP in Jalpaiguri district of the same state (unpublished data). Although the failure rate was just below the limit (10%) for drug policy change but it is alarming. So, the efficacy of this combination varies in different geographical regions of the same country. In AM+LF combination, we observed a case of treatment failure on day 26, so therapeutic efficacy of this combination is 98.9%. Similar finding was reported from India[27]. By using nested PCR sequencing assay, no *Pf*ATPase6 S769N mutation was found in any *P. falciparum* isolates collected from the study area. The finding is consistent with the results of the in vivo efficacy study. However, single case of treatment failure may be due to inadequate blood levels due to low absorption or altered pharmacokinetics of the drug.

Both the combinations of ACTs tested in this study were effective for the treatment of uncomplicated falciparum malaria in India. AM+LF might be an alternative choice to AS+SP to the policy makers, if needed.

Conflicts of interest

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

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