Original Article

Pfcrt mutant haplotypes may not correspond with chloroquine resistance

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Abstract

Introduction: Chloroquine resistance in *Plasmodium falciparum* is associated with mutations in *pfcrt* and *pfmdr1* genes. The frequency distribution of *pfcrt* K76T and *pfmdr1* N86Y mutations and their association with chloroquine susceptibility was studied in an endemic area along the Indo-Bangladesh border.

Methodology: A single-arm prospective study of clinical and parasitological responses in *P. falciparum* malaria patients to chloroquine was conducted *in vivo*. PCR-RFLP assay was used to detect *pfcrt* K76T and *pfmdr1* N86Y mutations in *P. falciparum*. The PCR products of *pfcrt* gene were sequenced, translated and aligned for haplotyping.

Results: Out of 63 cases, 44 (69.8%) responded adequately to chloroquine treatment. *Pfcrt* K76T mutation was recorded in 100% of the treatment failure cases, whereas *pfmdr1* N86Y mutation was found in 52.6% of the cases only. Early treatment failure (84.2%) occurred more frequently than late treatment failure (15.8%). Kaplan–Meier survival analysis showed that the probability estimate for treatment success after 7 and 15 days was 0.84 (95% CI = 0.72-0.92) and 0.70 (95% CI = 0.57-0.80), respectively. Sequence analysis of 72 to 76 *pfcrt* gene codons revealed the presence of two mutant (CVMNT, CVIET) and two wild (CVMNK, CVIEK) haplotypes. The mutant CVIET haplotype was predominantly distributed (42.1%).

Conclusions: The presence of mutations in *pfcrt* K76T and *pfmdr1* N86Y genes is not sufficient to explain the therapeutic efficacy of chloroquine to *P. falciparum*. Study suggests that *pfcrt* K76T mutant haplotypes are widely distributed and are spreading diligently, which needs to be taken into account in devising an antimalarial policy.

Key words: Chloroquine, *pfcrt*, *pfmdr1*, resistance, haplotype

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Introduction

The problem of antimalarial resistance in *P. falciparum* has been a great concern in various malaria control programs [1]. In India, resistance to widely used antimalarial chloroquine was first detected in Assam; since then, it spread into other parts of the country and has led to many malaria-related casualties. The tremendous pressure of chloroquine resistance in the northeast region has led to a switch-over to artesunate-based combination therapy (ACT) as the first line of treatment in uncomplicated malaria. Chloroquine has been the drug of choice for a long time. It acts by blocking the hemozoin formation mechanism in the malaria parasite. The exact mechanism of chloroquine resistance is unknown; however, it is believed that the malaria parasite, through some uncertain mechanism, effluxes chloroquine from food vacuoles to survive the drug pressure. Mutations in the *pfcrt* and *pfmdr1* genes of the *P. falciparum* malaria parasite have been implicated in decreasing antimalarial susceptibility**.** The K76T mutation, along with some other mutations such as C72S/R, M74I/T, N75E/D/K/I, K76T/I/N, I194T, and A220S mutation in the *pfcrt* gene, was found to be associated with chloroquine resistance [2]. Multiple studies in *falciparum* malaria-endemic areas have suggested that K76T mutant parasites are linked to chloroquine resistance in both *in vitro* and *in vivo* trials [3-7]. The K76T mutation has also been found to be associated with amodiaquine resistance and predictive treatment failure for both chloroquine and amodiaquine [8-10]. The association of the N86Y mutation in the *pfmdr1* gene in chloroquine resistance has been debated; some studies suggest that its presence along with *pfcrt* mutations can cause higher levels of chloroquine resistance [11-14]. At present, the molecular data conferring antimalarial resistance in *P. falciparum* is scant in northeast India. The present investigation was carried out to evaluate the therapeutic efficacy of antimalarial chloroquine

against the *P. falciparum* malaria parasite. The association of *pfcrt* K76T and *pfmdr1* N86Y mutations with *in vivo* chloroquine resistance was also assessed.

Methodology

Study sites

The study was carried out in Hrishyamukh Primary Health Centre of Belonia, a sub- division in Tripura state. The study area is located near the Indo-Bangladesh border and is characterized by a hot and humid climate. The population is mainly comprises ethnic tribes, and the economy is largely agrarian.

In vivo study of chloroquine sensitivity

A one-arm prospective study of clinical and parasitological responses after *in vivo* administration of chloroquine was conducted between July 2007 and September 2009, following the standard World Health Organization (WHO) protocol [15]. Adults and children (> 6 months of age) with fever or history of fever in the preceding 24 hours without any danger signs were enrolled in the study (n = 63). All the patients enrolled fulfilled the following criteria: a) *P. falciparum* mono-infection with parasitaemia with a range of 1,000-100,000 asexual parasites/μL of blood; b) absence of any danger signs, febrile illness, and anemia; c) availability for follow-up visits; d) absence of regular medication; e) no pregnancy or breastfeeding; and f) consent to participate in the study. The WHO standard protocol recommends 28 days of follow-up in the *in vivo* antimalarial trials, but a 14-day follow-up protocol was used in this study, as the follow-up of enrolled patients for a long duration was difficult and, at the same time, high failure rates were anticipated in the study area [16]. Post-treatment follow-up was done on days 2, 3, 7, and 14 of the drug administration for clinical and parasitological assessments. The treatment outcomes were classified as early treatment failure (ETF), late treatment failure (LTF), and adequate clinical and parasitological response (ACPR).

Diagnosis and collection of blood samples

Suspected malaria patients were diagnosed in the field using a rapid detection kit (Optimal-IT; M/S Diamed AG, Cressier, Switzerland). Both thick and thin blood smears were collected using the finger-prick method and stained with Giemsa stain for microscopic examination of the malaria parasite. The blood smears were collected on each follow-up day and used for parasite counts using microscopy. Parasitaemia was calculated by counting the asexual stages of the *P. falciparum* parasite against 200 white blood cells (WBC) and multiplied by 8,000 as an assumed average WBC count per microliter of blood. On the first day, a few drops of blood were collected on FTA papers, air dried, and stored in a desiccator at room temperature and subsequently in a -20°C deep freezer for molecular study.

DNA extraction, PCR assay, and sequence analysis

DNA was extracted from FTA cards using a Qiagen Blood DNA Mini Kit following the manufacturer's guidelines with minor modifications. PCR assay was used to amplify *pfcrt* and *pfmdr1* using primers described elsewhere [12]. PCR was performed in 25 μL reaction volume containing 5 μL of sample DNA, 2.5 mM $MgCl_2$, 200 M of dNTPs, 1 mM each forward and reverse primers, and 0.5 unit of Taq polymerase (Roche, Basel, Switzerland). Reaction conditions were similar to those described previously [12]. Nested PCR products of *pfcrt* and *pfmdr1* genes were further restriction digested using Apo I and Afl III enzyme, respectively (New England Biolabs, Ipswich, USA), for allelic identification. For the *pfmdr1* N86Y mutation, AflIII cleaves mutant type but not wild type, whereas for the *pfcrt* K76T mutation, ApoI cut the wild type but not the mutant gene [12,17]. The PCR products of *pfcrt* were purified using a gel extraction kit (Qiagen, Hilden, Germany) and sequenced using ABI BigDye Terminator ready reaction kit at BioLink India Pvt Ltd., (New Delhi, India) using previously described primers [12]. *Pfcrt* gene sequences obtained were translated using the online translation tool Expasy (http://web.expasy.org/translate), aligned using BioEdit software version 7.0.9.0, and analyzed at codons 72 to 76 to detect the haplotypes.

Data analysis

The Chi-square test was used to compare *pfcrt* and *pfmdr1* mutations, *in vivo* results with *pfcrt* K76T and *pfmdr1* N86Y mutations, different haplotypes, and treatment failures. The specificity and sensitivity of *pfcrt* and *pfmdr1* mutations in detecting chloroquine resistance were calculated using *in vivo* as the reference standard. A Kaplan–Meier survival analysis was performed to estimate the treatment success probability at different days.

Ethical clearance

The study was a part of the project sanctioned by the Ministry of Defence, Government of India, for management of malaria along Indo-Bangladesh border

areas. The project was approved by the institutional ethical committee (IEC), and informed written consent was obtained from the enrolled adults and the guardians of the child volunteers. The study aims and procedures were explained to the volunteers, and it was clearly stated that participation was free of cost. The enrolled patients were free to withdraw their names from the study at any time. All the treatments were provided under the direct supervision of local health authorities and observed for some time to ensure that there was no reaction or vomiting.

Results

Baseline information including age, sex, weight, and *P. falciparum* parasitaemia of the study volunteers is shown in Table 1. In the *in vivo* study, treatment failure was found in 19 (30.2%) cases, whereas 44 (69.8%) cases responded adequately to the chloroquine treatment. Clinical response to chloroquine in different age groups revealed that treatment failure cases were highest in the 0-5 year age group (47.4%). Chloroquine sensitivity and distribution of *pfcrt* and *pfmdr1* mutations are shown in Table 2. No significant difference was found between *pfcrt* and *pfmdr1* mutant allele frequencies according to age ($p > 0.2$) (Table 2). The *pfcrt* K76T mutation was recorded in all the treatment failure cases (n = 19) and in six ACPR cases ($p < 0.0001$). On the other hand, the *pfmdr1* N86Y mutation was found in only 52.6% (n = 10) of the treatment failure cases ($p = 0.6$). Out of 25 total cases with the *pfcrt* K76T mutation, 21 (84%) were under 11 years of age. Similarly, 22 (78.6%) belonged to the 0-11 year age group, in which *pfmdr1* N86Y was detected. Age distribution showed that both K76T and N86Y mutations were more prevalent among patients under 11 years of age as compared to older patients ($p < 0.0001$). The *pfcrt* K76T mutation was more sensitive and specific compared to the *pfmdr1* N86Y mutation in terms of chloroquine drug resistance; sensitivity and specificity of K76T was 1 and 0.86 as compared to 0.53 and 0.59 in the N86Y mutation. Among the treatment failure cases, ETFs (n = 16; 84.2%) were higher ($p = 0.001$; $\chi^2 = 10.5$; OR = 14.1) than LTFs (n = 3; 15.8%). The Kaplan–Meier survival analysis showed that the probability estimate for treatment success using chloroquine after 7 and 15 days was 0.84 (95% CI = 0.72-0.92) and 0.70 (95% CI = 0.57-0.80), respectively. Survival time (mean ± standard error mean) for treatment failure and success was 12.05±0.91 (95% CI = 10.27-13.84) and 3.64±0.22 (95% CI = 3.20-4.07), respectively. Overall comparison of treatment success distribution for different levels using log–rank (Mantel–Cox regression) was statistically significant ($\chi^2 = 44.1$; $p = 0.000$).

Table 1. Baseline characteristics of the study volunteers

Age (95% CI), range	9.08±1.0 (7.10-11.05)	02- 49
Male counts, %	42	66.7
Female count, %	21	33.3
Weight (95% CI), range	18.11±1.8 (14.50-21.72)	6 to 65
Parasitaemia (95% CI), range	2994.6±715.2 (1564.6-4424.5)	16 to 35,000

Table 2. Chloroquine sensitivity and *pfcrt* & *pfmdr1* mutation distribution among all age groups

Age groups	n (resistant)	*Pfcrt* (%)	*Pfmdr 1* (%)	p	Chi
All ages	63 (19 = 30.159%)	39.7	44.4	0.24	2.82
0-5	19 (9 = 47.368%)	52.6	47.4	0.93	0.14
6-11	32 (7 = 21.875%)	34.4	40.6	0.26	2.66
12 and above	12 (3 = 25%)	33.3	50	1.69	0.43

Table 3. Single nucleotide polymorphism (SNP) position on *pfcrt* gene fragment

Haplotypes	**72**	**73**	**74**	**75**	**76**	**Total counts (%)**
CVMNT	TGT	GTA	ATG	AAT	ACA	9 (23.7)
CVIET	TGT	GTA	ATT	GAA	ACA	16 (42.1)
CVMNK	TGT	GTA	ATG	AAT	AAA	10 (26.3)
CVIEK	TGT	GTA	ATT	GAA	AAA	3 (7.9)

Sequencing of *pfcrt* PCR products was performed in all the 19 treatment failure cases and an equal number of randomly selected susceptible cases. Sequence analysis of 72 to 76 *pfcrt* gene codons revealed the presence of CVMNT, CVIET, CVMNK, and CVIEK haplotypes (Table 3). CVMNK and CVIEK are wild type, while CVMNT and CVIET are mutant haplotypes of the *pfcrt* gene. The mutant CVIET haplotype was more common (42.1%) compared to the other three haplotypes, irrespective of their drug susceptibility status ($\chi^2 = 11.9$; $p = 0.007$). Furthermore, among the treatment failure cases, the mutant CVIET haplotype was more widely distributed (63.2%) than the other mutant CVMNT haplotype ($\chi^2 = 2.6$; $p = 0.05$).

Discussion

The areas along the Indo-Bangladesh international border have experienced tremendous antimalarial resistance for various reasons [18]. The present study suggests that *pfcrt* K76T and *pfmdr1* N86Y mutant parasites are frequent in the region. Most of the chloroquine treatments failed during the early treatment stage, indicating that chloroquine should no longer be used as drug of choice against *falciparum* malaria. Previous studies conducted in the same area found that chloroquine ETF cases were predominant compared to LTF cases [18]. However, some studies reported that LTF cases are considerably more common than ETF cases [19,20]. Treatment failure was highest in children. Studies have suggested that parasite density, anemia, age, body temperature, and immunity influence the treatment outcome in children [20-21].

In recent years, in addition to the conventional *in vivo* and *in vitro* methods, the molecular markers-based approach to study and elucidate antimalarial drug resistance has proved useful [22-26,29,30,32]. In this study, we described the distribution of *pfcrt* K76T and *pfmdr1* N86Y mutations in the study area and attempted to evaluate the correlation of these mutations with *in vivo* clinical outcomes. The association of *pfcrt* mutation with chloroquine resistance suggests that the K76T mutation is the most reliable molecular marker in chloroquine resistance [3,12,17]. K76T and N86Y mutations were found in 39.4% and 44.4%, respectively, of the study population; however, the distribution of these mutations in both chloroquine-susceptible and -resistant cases varies considerably. The K76T mutation was found in all the *in vivo* resistant cases; however, its presence was not exclusive to resistant cases and was also found in six ACPR cases. On the other hand, the N86Y mutation, though more frequent, was found in only 10 *in vivo* resistant cases.

A previous study suggested that the N86Y mutation appeared with more than one *pfcrt* haplotype and therefore was not very reliable in detecting chloroquine resistance [23]. *Pfcrt* haplotyping showed four types of haplotype. The wild type haplotypes CVIEK and CVMNK were limited to ACPR and could not be recorded in treatment failure cases. On the other hand, the mutant type haplotypes CVIET and CVMNT were found in all the treatment failure cases, of which CVIET was more frequently distributed. These results show that the mere presence of a mutant haplotype may not necessarily confer chloroquine resistance. The presence of haplotypes as molecular markers may correspond to the intrinsic characteristics of malaria parasites but does not conclusively lead to treatment failure [21,23-25]. Treatment failure may depend on host immunity and interactions of the host with the parasite and drug [21,24]. Mutant haplotype CVIET is more endemic in this region; however, few studies have suggested that multiple mutant *pfcrt* haplotypes have been observed in high malaria transmission regions [23,26,27]. The CVIET haplotype is expected to be observed in the northeastern states and might have been spread due to inbreeding of *P. falciparum* in the study area. In areas that have low levels of complex and multiclonal malaria infections, the inbreeding of malaria parasites having mutant genotypes could spread the antimalarial drug resistance at an extraordinary rate [28]. The prevalence of the SVMNT haplotype in highly malaria-endemic study areas indicates the wide spread of chloroquine-resistant *P. falciparum*, which might have evolved due to prolonged use of antimalarial amodiaquine in malaria chemotherapy [29-31]. Studies have shown that *P. falciparum* mutant *pfcrt* haplotypes have selective advantage in competitive mosquito infections by protecting immature gametocytes from chloroquine [32].

Since chloroquine treatment failure cases have been reported from many parts of India, including the northeastern region, the National Vector Borne Disease Control Programme (NVBDCP) of India has changed the antimalarial drug policy and replaced chloroquine with artesunate combined therapy (ACT) for treatment of uncomplicated *falciparum* malaria cases [33]. Artesunate-based combined therapy has been recommended as the first line of malaria treatment. However, shifting to artesunate combined therapy has not shown significant differences in the

overall malaria incidence in the region [34]. Effective implementation of the new drug regimen and improved health infrastructure at ground level could be useful in the area of investigation.

Conclusions

Molecular analyses of haplotypes associated with chloroquine drug resistance in *P. falciparum* isolates suggest that mutant type haplotypes of *pfcrt* are widely distributed in the study area. *Pfcrt* K76T mutant haplotypes, though detected in all the treatment failure cases, could not be sufficient in deciding chloroquine resistance. Similarly, the *pfmdr1* N86Y marker has a very limited role in determining chloroquine resistance. This study emphasizes that *pfcrt* mutant haplotypes are spreading diligently, which must be considered in the creation of an effective malaria treatment policy.

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