The Association of K76T Mutation in Pfcrt Gene and Chloroquine Treatment Failure in Uncomplicated Plasmodium falciparum Malaria in a Cohort of Nigerian Children

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Abstract: The aim of this study was to evaluate the association of K76T mutation in Pfcrt gene and chloroquine treatment failure following reports that the efficacy of chloroquine in the treatment of uncomplicated falciparum malaria in Africa is seriously compromised by high levels of drug resistance. The occurrence of mutation on codon 76 of Plasmodium falciparum chloroquine resistance transporter (Pfcrt) gene has been associated with development of resistance to chloroquine. We investigated the association of K76T mutation in Pfcrt gene in malaria-infected blood samples from a cohort of Nigerian children with uncomplicated falciparum malaria treated with chloroquine and its association with clinical (in vivo) resistance. The Pfcrt T76 allele was very significantly associated with resistance to chloroquine (Fischer exact test: p = 0.0001). We conclude that K76T mutation in Pfcrt gene is significantly associated with chloroquine resistance and that it could be used as a population marker for chloroquine resistance in this part of the country.

Key words: Pfcrt gene mutation, treatment failure, chloroquine resistance, uncomplicated malaria, pre-school children, Nigeria

INTRODUCTION

Malaria is the most important parasitic disease of man (Barnes and White, 2005) and of great public health importance. Despite treatment and control efforts the disease continues to threaten the lives of more than half of the world’s population. For example, in the year 2002, malaria was transmitted in 88 countries putting 3 billion people at risk of infection (Hay et al., 2004). In 2001, malaria was the eighth highest contributor to the global loss of Disability-Adjusted Life Years (DALYs) and second in Africa (Snow et al., 2004). Each year an estimated 3 million people die from malaria and 500 million to 5 billion clinical episodes of the disease occur worldwide warranting anti-malarial treatment (Breman et al., 2004; Snow et al., 2005).

The clinical and epidemiological disease burden of malaria is generally documented in terms of morbidity and mortality (mainly infants, childhood and maternal), anaemia and neurocognitive impairment. An increasing recognition of the relationship of malaria and economic development is now also emerging (Malaney et al., 2004). Malaria causes annual economic loss of US $12 billion (WHO, 2002). Although four Plasmodium species infect humans, severe malaria and malaria-related deaths are almost entirely attributable to falciparum malaria (WHO, 2000) and children under five years of age are the worst affected.

Chloroquine was the primary drug used for treatment in most sub-Saharan countries including Nigeria. But resistance to the drug which has emerged and spread to all parts of the country (Ohanewaju and Johnson, 2001) has rendered the drug almost useless. This development forced a change in antimalarial treatment policy replacing chloroquine with artemisinin-based drugs for first line treatment of uncomplicated malaria.

Antimalarial resistance in P. falciparum parasites results in an enormous public health burden because of prolonged or recurrent illness and progression to severe illness, which is associated with increased hospitalization and deaths (Trape, 2001; Oluwes et al., 2002). The efficacy of chloroquine is thought to lie in its ability to interrupt haematin detoxification in malaria parasites as they grow in their host’s erythrocytes (Slater, 1993; Sullivan et al., 1996; Dorn et al., 1998), thus poisoning the parasite. Chloroquine-resistant P. falciparum survives by
reducing accumulation of the drug in the digestive vacuole. However, the mechanism by which this happens has not been determined (Wellums and Plowe, 2001). Leading proposals include involvement of digestive vacuole pH or changes in the flux of chloroquine across the parasite's cytoplasmic or digestive vacuole's membrane (Krogstad et al., 1987; Sanchez et al., 1997; Bray et al., 1998; Dzekunov et al., 2000).

Although significant progress has been made in understanding the molecular basis of chloroquine resistance (CQR) many aspects of it remain unclear (Basco and Ringwald, 1998; Cooper et al., 2005). Studies of the mechanism of CQR in P. falciparum for the past 18 years have associated the phenomenon with point mutations at amino acid positions 86, 184, 1034, 1042 and 1246 in Plasmodium falciparum multi-drug resistance (PMDR1) gene and amino acids at positions 72, 74, 75, 76, 97, 220, 271 and 326 in Plasmodium falciparum chloroquine resistance transporter (PfCRT) gene in South-East Asian, South American, West and East African strains (Mehlotra et al., 2001; Lopes et al., 2002; Sidhu et al., 2002).

The PfCRT gene has been identified close to the candidate gene 2 (C2) on chromosome 7 on a region previously associated with CQR (Fidock et al., 2000). The PfCRT K76T mutation appears to be critical for development of CQR (Fidock et al., 2000). Not only has the absence of this key mutation explained the chloroquine susceptible phenotype but the K76T mutation has consistently been selected in clinical chloroquine treatment failures (Djinde et al., 2001; Dorsey et al., 2001). The PfCRT K76T allele may therefore be considered eligible to be a population marker for surveillance of chloroquine resistant falciparum malaria in community studies.

However, the use of molecular markers in general and more specifically the use of polymorphism in the PfCRT gene at codon 76 for predicting the level of in vivo parasite resistance to chloroquine has not yet been validated with extensive data from clinical and field settings (Talisuna et al., 2002). Molecular markers of drug resistance in P. falciparum could prove useful in defining the intensity of resistance in the individual patient and the extent and severity of the problem in communities (Negasa et al., 2003), which could serve as bedrock for evidence-based anti malarial policies.

The molecular basis of resistance of P. falciparum to chloroquine in northern Nigeria has not been extensively studied and molecular markers have not been used in unraveling the phenomenon. The monitoring of CQ resistance in various parts of Nigeria is therefore of critical importance for the Malaria Control Programme. Molecular techniques offer several advantages over classical traditional microscopy and in vitro and in vivo antimalarial drug efficacy studies.

We therefore investigated the association between the K76T mutation (also known as Lys-76-Thr) in PfCRT in parasite isolates and parasitological and clinical failure in pre-school children with uncomplicated falciparum malaria in Sokoto, Nigeria. The major aim was to ascertain whether the presence of the mutant codon 76 in individual parasite isolates is associated with in vivo parasitological and clinical failure to CQ at the individual patient and community level.

**MATERIALS AND METHODS**

**Study site malaria epidemiology:** The epidemiology of malaria in Sokoto has been described previously by Umar et al. (2007).

**Patients’ recruitment:** Patients for the study aged 2-59 months were recruited at the outpatients' clinic of the Department of Paediatrics, Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto, Nigeria and the University Health Center, permanent site. The study was conducted from November 2005 to July 2006. The patients presented with signs and symptoms suggestive of simple uncomplicated malaria as defined by WHO (2000). Other inclusion criteria were fever during the preceding 48 h, P. falciparum monoinfection with a parasite density of 2,000-200,000 ability to return for stipulated follow-up visits and verbal consent or signing of an informed consent form by a parent/guardian of the child to participate in the study.

**Exclusion criteria:** children less than 2 months old or older than 5 years, children with sickle cell disease, jaundice, tonsillitis, otitis media, measles, severe malnutrition, abscesses, severe vomiting, recent history of convulsion, or those who had taken any antimalarial drug 2 weeks previously or those whose urine tested positive to Dill and Glazko’s test for chloroquine.

The study was reviewed and approved by the ethical committee of UDUTH and by Management of the University Health Center.

**Sample size and study design:** A sequential sampling scheme was used. Since the actual prevalence of clinical failures due to treatment with chloroquine was not known in the area we assumed it to be 20%, aiming at a precision of 10% and confidence level 95%, a minimum sample size of 61 was required.
Clinical and physical examinations including weighing (kg), taking of axillary temperature, age and gender were performed and the information entered into proforma data sheets. Two milliliters of blood were also taken into EDTA-containing tubes for parasite specie identification, parasite density count, estimation of hematological parameters and DNA extraction. Each child was treated with 10 mg chloroquine base (chloroquine phosphate® syrup, NAFDAC No. 04-0289, Emurz Pharmaceutical Industries, Isolo, Lagos) per kg body weight. The drug was administered at the clinic on day 0 under the supervision of the consultant paediatrician. Patients who vomited were given another dose. Parents or guardians of patients were asked to stay at the clinic for at least one hour before going home, after being instructed to give child another dose (10 mg kg⁻¹ body weight) on day 1 and half dose (5 mg kg⁻¹ body weight) on day 2. They were also instructed to return to the clinic for follow-up visits on days 3, 7 and 14 for clinical assessment of the child’s condition and subsequent collection of samples for parasite count, PCV and hemoglobin estimation. They were also instructed to return to the clinic even before the third day if the child’s condition deteriorated.

Patients’ follow-up: The patients were followed up on an outpatient’s basis on days 3, 7 and 14. The clinical condition, body temperature and presence of other symptoms of malaria were assessed. On day 3, children with symptoms of malaria or who have not improved remarkably were administered rescue treatment: sulphadoxine-pyrimethamine, amodiaquine or Coartem® (Artemether + lumefantrine, Norvatis Pharmaceutical Corp, New York, USA).

Parasite specie identification and count: Twenty microliters of blood was collected from the EDTA-anticoagulant bottle and thick and thin smears were made on pre-labelled slides in accordance with the method described by Hendricks and Mathews (1991). Thin smears were used for the identification of the malaria parasite species as described by Fleck and Moody (1998).

Thick smears were used for parasite count. The number of asexual forms of the parasite was counted against 200 leucocytes and then multiplied by 8000 according to Slutsker et al. (1994).

Parasite DNA extraction and purification: Sub-sampling: A subset of 20 Plasmodium falciparum-infected blood samples comprising 10 from children who failed treatment with CQ (CQ resistant parasites) and 10 randomly selected from children who were cured with CQ (CQ susceptible parasites) was selected for a case-controlled study to assess the association between clinical resistance to CQ and the presence of the P.falc Lys-76-Thr mutation in the parasites.

DNA extraction was performed according to the method of Warhurst et al. (1991) and Vaidyakosol et al. (1995). Briefly, the vials containing the malaria-infected blood were taken out of cold storage (-20°C) and thawed at 37°C for 3 min. About 100 μL of blood was then mixed with 150 μL of cold phosphate buffered saline (PBS, pH 7.4) and centrifuged for 5 min at 6000 g to recover the parasites and unlysed erythrocytes. The pellet was re-suspended in 2 mL cold PBS (containing 0.15% saponin) and incubated at 37°C for 5 min. After lysis, centrifugation was repeated at 6000 g for 5 min to recover the parasites. The pellet was immediately suspended in 25 μL of 4x lysis buffer (40 mM Tris pH 8.0 with HCl, 80 mM EDTA pH 8.0, 2% SDS, 2 mg mL⁻¹ proteinate K and 100 mg mL⁻¹ Ribonuclease A). Sterile distilled water was added to a final volume of 100 μL and the mixture gently shaken before incubation for 1 h at 50°C. Next, 300 μL of distilled water was added to the mixture and DNA extracted by adding an equal volume (400 μL) of phenol-chloroform-isooamyl alcohol (v/v/v 25: 24: 1). This step was repeated twice. The supernatant was then treated with an equal volume of chloroform-isooamyl alcohol (v/v 24: 1). The DNA was precipitated by the addition of an equal volume of 0.3 M sodium acetate and cold ethanol (stored at -20°C) by inversion of the tubes 5-6 times. The DNA was spooled in a rod, dried under vacuum and re-suspended in 300 μL Tris EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA was washed with 1 mL 70% ethanol in a microcentrifuge tube and left to dry for 15-20 min at 37°C and redissolved in 20 μL T.E. buffer (pH 8.0). Aliquot portion of the genomic DNA extracted from each sample was used immediately for Polymerase Chain Reaction (PCR) and the rest was stored at -20°C.

Nested mutation specific PCR for the detection of PfCr Lys-76-Thr mutation: Five microliter of genomic DNA, 200 μM of each dNTP, 1 μM of CRTPl primer (5'-CGG TTA ATA ATA AAT ACA CGC AG-3'), 1 μM of CRTp2 primer (5'-CGGAT TTAC AAAAAACTAT AGTTACC-3'), 2.5 units of taq polymerase, in reaction buffer (500 mM KCl, 100 mM Tris-Cl (pH8.3), 15 mM MgCl₂), all in a final volume of 50 μL in a PCR tube were transferred into the thermal cycler and PCR performed with the following conditions: initial denaturation at 95°C for 3 min, 45 cycles at 95°C for 30 sec, annealing at 56°C for 30 sec, extension at 70°C for 60 sec and final extension at 60°C for 5 min. This was to amplify 537 base pair region around the K76T mutation.
Two mutation specific diagnostic PCRs were mounted, each using CRTD1 (5'-TGTATGT CAT GTG TTT AAA CTT-3') coupled with allele specific primers CRTF4m (5'-GTT CTG CAA GAA AAT G-3'), CRTF4w (5'-GTT CTT TTA GCA AAA ATCT-3') for detection of resistant T76 genotype or sensitive K76 genotype respectively. All other conditions were the same as in the previous PCRs.

**Nested PCR and restriction digestion for detection of Pfcrt Lys-76-Thr mutation:** A second nested PCR was performed in a 25 μL volume reaction utilizing internal primers CRTD1 (5'-TGTATGT CAT GTG TTT AAA CTT-3') and CRTD 2(5'-CA A TAT AGT TAC CAA TTT T3') flanking the K76T mutation. Five microliters of the secondary amplification products (145 base pair) was mixed with 0.5 units of restriction enzyme, Apol and incubated for 6 h at 50°C as instructed by the manufacturer.

The digestion reaction was then analyzed by electrophoresis on a 2% agarose gel. Apol digests the sensitive type allele, resulting in two fragments of 100 and 34 base pairs but does not digest the resistant allele (Frean et al., 1992). D4-2 strain DNA, HB-3 strain DNA and water were used as mutant type allele, wild type allele and negative controls respectively.

**Data analysis:** Data obtained from the in vivo study were analyzed using descriptive statistics. The data on the presence or absence of mutation of codon 76 of the Pfcrt gene were collated with the clinical responses. The clinical responses of the patients (S, RI, RII or RIII) were correlated with the Lys-76-Thr mutation using Fisher's exact test as described by Adagu and Warhurst (1999) and Duraisingh et al. (2000) and analysed using a 2x2 contingency table with the aid of Graph pad Instat 3.0 for Windows software (Graph pad software, San Diego, California, USA). A p-value of <0.05 was considered indicative of a statistically significant association.

**RESULTS**

A total of 63 children aged 2-59 months were recruited into the study but only 36 (56.3%) completed the study. Data from those who did not complete the study were excluded from analysis. The baseline data of the study participants are shown in Table 1. Mean weight of the study subjects was 11.0±4.02 kg. Males constituted 21.0 (58.3%) of the study population and females 15.0 (41.7%), thus there were more males than females in the study population (male: female ratio 1.4:1.0). At presentation, 28 (77.8%) of the children were febrile (axillary temperature > 37.5°C). The Geometric Mean Parasite Density (GMPD) was 9.048±8.39 per μL of blood. The mean Haemoglobin was 10.65±1.52 g dL⁻¹ while the mean Packed Cell Volume (PCV) was 32.07±4.42%.

The results of the in vivo test for sensitivity to chloroquine are shown in Table 2. Twenty-six (72.2%) of the cases exhibited adequate clinical and parasitological responses to chloroquine and were classified as sensitive. Ten (27.8%) of the cases presented various degrees of clinical and parasitological resistance (R1, RII, RIII) (Table 3).

The Pfcrt T76 allele was predominantly present in the CQ resistant isolates with a frequency of 10, but it was also present once in the CQ susceptible isolates. The Pfcrt K 76 genotype was absent in all the CQ resistant isolates (Table 4). Pfcrt T76 allele was very significantly associated with resistance to chloroquine (Fisher exact test: p = 0.0001) (Table 5). A representative PCR and restriction digestion gel for the detection of K76T mutation in Pfcrt gene is shown in Fig. 1.
Table 4: Frequency of Pfct alleles in chloroquine sensitive and resistant isolates from children

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Clinical outcome</th>
<th>Pfct allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>ACR/Sensitive</td>
<td>T76</td>
</tr>
<tr>
<td>002</td>
<td>ACR/Sensitive</td>
<td>K76</td>
</tr>
<tr>
<td>003</td>
<td>ACR/Sensitive</td>
<td>T76</td>
</tr>
<tr>
<td>004</td>
<td>ACR/Sensitive</td>
<td>K76</td>
</tr>
<tr>
<td>005</td>
<td>ACR/Sensitive</td>
<td>K76</td>
</tr>
<tr>
<td>006</td>
<td>ACR/Sensitive</td>
<td>K76</td>
</tr>
<tr>
<td>007</td>
<td>ACR/Sensitive</td>
<td>K76</td>
</tr>
<tr>
<td>008</td>
<td>ACR/Sensitive</td>
<td>K76</td>
</tr>
<tr>
<td>009</td>
<td>ACR/Sensitive</td>
<td>K76</td>
</tr>
<tr>
<td>010</td>
<td>ACR/Sensitive</td>
<td>K76</td>
</tr>
<tr>
<td>012</td>
<td>RR/Resistant</td>
<td>T76</td>
</tr>
<tr>
<td>013</td>
<td>RR/Resistant</td>
<td>T76</td>
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<tr>
<td>014</td>
<td>RR/Resistant</td>
<td>T76</td>
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<td>015</td>
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<td>016</td>
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<td>017</td>
<td>RR/Resistant</td>
<td>T76</td>
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<td>019</td>
<td>RR/Resistant</td>
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<tr>
<td>020</td>
<td>RR/Resistant</td>
<td>T76</td>
</tr>
<tr>
<td>020</td>
<td>RR/Resistant</td>
<td>T76</td>
</tr>
</tbody>
</table>

Table 5: Association of T76 allele with in vivo chloroquine resistance

<table>
<thead>
<tr>
<th>Association</th>
<th>2x2</th>
<th>p-value</th>
<th>Statistic (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfct T76</td>
<td>10</td>
<td>0.0001</td>
<td>100.0</td>
<td>90.0</td>
</tr>
<tr>
<td>And CQR</td>
<td>0</td>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
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Fig 1: DNA Electrophoresis gel (2% agarose) showing the electrophoretic pattern of the amplification products of Pfct gene after digestion with Apol. Lanes 1 and 13 are DNA 100 bp markers, lanes 2 and 5 HE-3DNA and sample with K76 genotypes, lane 6 control (water), lanes 3 and 4, 7-12 are samples with T76 genotypes.

DISCUSSION

Chloroquine resistance by *P. falciparum* parasites is a growing obstacle in the treatment of malaria. Initially, both the biochemical and genetic basis of resistance in *P. falciparum* were controversial and attempts to elucidate the exact mechanism continued (Ereij et al., 1998; Cooper et al., 2005). The landmark findings of mutations at certain codons on the Pfct gene (Djimde et al., 2001) and their association with in vivo chloroquine resistance in a large sample study in Mali might have helped resolve the controversy.

CQ resistance was initially localized to mutations in the *P. falciparum* multi-drug resistance gene (Pfmdr) on chromosome 5 (Foote et al., 1990). However, linkage analysis mapped the resistant determinant to a segment on chromosome 7 where Pfct is located (Wellens et al., 1990; Sidhu et al., 2002). As the samples of this study were obtained from pre-school children, to whom few studies had devoted attention, it presented a unique opportunity to assess the prevalence of these molecular markers in the non-immune population in a highly endemic malaria area with comparatively less intensity of CQ resistance than the western and eastern parts of the country. Resistance emerges de novo through spontaneous mutations or gene duplications, which are thought to be independent of drug selection pressure, but the mutations are then selected for and spread as a result of drug pressure which provides a selective advantage to resistant parasites (Barnes and White, 2005).

A hallmark of malaria in children under five years of age is the absence of immunity (premunition), which however, is gradually acquired after the age of five. Immunity is a major factor in the resolution of malaria infection (Marsh, 1992). The implication of this is that non-immune children can easily contract malaria infection and clear their parasitaemia without drug intervention. When left untreated, the illness may persist for a long time and if treated with an ineffective drug an opportunity is provided for resistant strains to arise and spread within the community. In the study of Malian children frequently infected with different *P. falciparum* strains, Djimde et al. (2001) found that the ability to clear resistant parasites after treatment improves with age. Among children less than 10 years old, 32% of infections with CQ resistant parasites cleared after CQ treatment, whereas 66% of older children and young adults showed such clearance.

Frequent in vivo clearance of CQ resistant parasites with the K76T mutation also occurred in other studies, even when resistant parasites have been more prevalent for many years, as in Uganda (Dorsey et al., 2001), Mozambique (Mayor et al., 2001) and Cameroon (Lasco and Ringwald, 2001). In populations of Northern Nigeria not receiving drug treatment, age-related immunity was evident in the increase of daily spontaneous clearance rates (0.2% in children <5 years old to 0.5% in children >9 years old) (Molyneaux and Granicca, 1980). Thus, pre-existing immunity is a potent factor in the efficacy of antimalarial therapy.

Our results indicate a predominant role for mutation (K76T) in the *Plasmodium falciparum* chloroquine resistance transporter (*Pfct*) gene. In a study of CQ resistant parasites from Zaria, Adag and Warhurst (1999) found high prevalence of mutations in the *Pfmdr* gene but *Pfct* gene was not investigated. There were no previous reports on the association of mutation of the *Pfct* gene and CQ resistance of *P. falciparum* isolates.
from Northern Nigeria. But in Mali, Pfert T76 was strongly associated with CQ treatment failure (Djimde et al., 2001).

The predictive value of Pfert T76 for CQ resistance in vivo depends on the frequency of the mutation in the local parasite population which is abrogated once it reaches 100%, suggesting additional factors that account for clinical CQ resistance (Babiker et al., 2001; Dorsey et al., 2001; Mayor et al., 2001).

The amino acid 76 mutation on the Pfert gene appears to be critical for the development of chloroquine resistance (Mayor et al., 2001; Cooper et al., 2002). How can this be explained? Amino acid 76 of Pfert is located in the first transmembrane segment of the molecule and may affect properties of its channel or transporter function. The substitution of lysine with threonine introduced an amino acid with different chemical property owing to the hydroxyl group. This resulted in replacement of a positive charge with a negative charge which may be critical to the mechanism of CQR. Mutations which alter charge within or near transmembrane domains have been shown to produce marked shifts in properties of transporters in other eukaryotic systems (Egger et al., 2000; Pajor et al., 2000).

Possible explanations for the role of Pfert mutations in CQR include (i) mutation alters proton flux across the digestive vacuole (DV) membrane, thereby lowering vacuolar pH to decrease the concentration of soluble haeme and reduce the formation of toxic drug/haematin complexes (Dzahunov et al., 2000) and (ii) mutation directly decreases drug influx or enhance drug efflux through Pfert at the DV membrane (Siddhu et al., 2002). In distinguishing between these possibilities, the result with quinine may be relevant. Quinine (QN) has been shown to target haematin and interfere with haemazoin formation in a manner similar to CQ (Dorn et al., 1998, Hawley et al., 1998). Cooper et al. (2002) introduced two mutations (K76N and K76I) on position 76 resulting in enhancement of QN susceptibility by the K76I mutation and the reduction of the QN response by the K76N mutation and concluded that the results could not be explained by a simple, steady state PIII effect. The results instead suggest stereo-specific interactions between QN and Pfert that produce enhanced sensitivity, in the case of the K76I mutation or reduced sensitivity in the case of K76N or K76T mutations. This view is consistent with studies that have demonstrated the structural specificity of CQR, i.e. the comparable responses of both CQR and CQS parasite lines to certain CQ analogues that have length variation in the alkyl side (De et al., 1996; Ridley et al., 1996) or to tert-butyl amodiaquine analogues (O’Neill et al., 1997).

In vivo resistance, as determined by persistent or recurrent parasitaemia after treatment (WHO, 1973) or by inadequate therapeutic response (early or late treatment failure) (WHO, 1996), depends not only on the innate ability Plasmodium falciparum parasites to cope with chloroquine but also on host factors that affect parasite survival. Among these factors are drug uptake, distribution and metabolism. Unfortunately, our study design has not allowed us study the effect of these factors on the clinical response to CQ observed in our study population.

In conclusion, this study demonstrated high prevalence and highly significant association of the K76T mutation in the Pfert gene with CQ resistance. If this finding is validated in another study in the same setting with a higher population size, then the prevalence of T76 genotypes may be used for population based surveillance of CQR in this region. If sentinel sites are established it would enable health authorities to monitor the sensitivity of malaria parasites to drugs in current use by regularly assessing the levels of molecular markers in various communities.

REFERENCES


