

Full Length Research Paper

Rural-Urban Differences in pfcrT T76 Allele Acquisition Among Children with *Plasmodium falciparum* Malaria in Nigeria

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Malaria caused by *Plasmodium falciparum* remains a public health problem in Nigerian children with treatment complicated by expansion of chloroquine resistant strains known to harbour a common K76T point mutation in their pfcrT alleles. Here, we report the outcome of a 2 – year (March 2000 – February 2002) molecular surveillance for pfcrT76 in children aged 6 months – 13 years with acute uncomplicated falciparum malaria in rural and urban Lagos, Nigeria. Rural-urban pfcrT76 acquisition of 48.7 vs. 73.7% and 67.3 vs. 74.6% due to monoclonal and polyclonal *P. falciparum* parasitaemia, respectively, were found in the two study years, suggesting unstable but increasing prevalence of pfcrT 76 allele acquisition in the rural area. Further analyses showed that acquisition of pfcrT76 allele was independent of sex but occurred more in 5 – year old children than older children in both populations. The impacts of K76T mutation in pfcrT gene and immunity on the clinical efficacy of chloroquine against acute uncomplicated malaria are discussed.

Key words: pfcrT T76 mutation, *Plasmodium falciparum* malaria, chloroquine resistance, Nigerian children.

INTRODUCTION

In Nigeria, malaria caused by *Plasmodium falciparum* remains a public health problem as it accounts for 25% of infant mortality, 30% of childhood mortality and represents a major hyperpyrexia factor in children and adults (Mosanya, 1997; Salako et al., 2001; Eleso et al., 1993). The magnitude of failure to control malaria chemotherapeutically with chloroquine has increased significantly in the past 5 years owing to increased trend of the incidence of RI, RII and RIII parasitological resistances in the six geopolitical zones in the country

(Lege-Oguntoye et al., 1989; Anita Obong et al., 1997; Ezedinachi et al., 1999). This clinical scenario has been blamed on the expansion and intensification of chloroquine resistance *P. falciparum* clones first reported from the Eastern part of the country in 1986 (CDC, 1987). Promotive factors include adulteration, self-medication and pre-treatment sub-therapeutic levels of chloroquine especially in children (Mockenhaupt et al., 2000). Genetic studies by Adagu and Warhurst (2001) have identified lysine (K) to threonine (T) point mutation in codon 76 of pfcrT gene, which encodes a putative chloroquine anion transporter protein PFCRT in strains that displayed reduced susceptibility to chloroquine *in vitro*. A recent study by Happi et al. (2004) identified pfcrT

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T76 as a genetic marker of recrudescence in children with acute uncomplicated falciparum malaria.

Meanwhile, the recently reported epidemiological wave of chloroquine resistance malaria with clinical outcome improved with chloroquine-chlorpheniramine in Lagos (Okonkwo et al., 1999) lacks genetic evidence regarding the impact of pfcrT K76 \rightarrow T mutation in terms of spread and pattern of acquisition in both rural and urban areas where malaria is endemic and transmission is perennial.

In the present study, we have reported the trend of acquisition of pfcrT76 allele in the last two years from the pre-treatment samples of children presenting acute uncomplicated *Plasmodium falciparum* at a Primary Health facility in Ijede, a rural settlement and Massey Street Children's Hospital in Lagos metropolis. The effects of age and sex on the acquisition of this allele were also investigated.

MATERIALS AND METHODS

Study population

One hundred and ninety six (196) children aged 6 months to 13 years presenting with acute uncomplicated *P. falciparum* malaria were enrolled in the study. The cohorts were recruited from a Primary Health Care Centre in Ijede (N = 91; M/F = 48/43), a rural settlement, located about 40 km from Lagos Mainland and Massey Street Children Hospital (N = 105; M/F = 54/51), located in a crowded part of Urban Lagos and provides secondary health service for an average of 300 to 400 sick children per day. In both sites, falciparum malaria is endemic with perennial transmission. The main transmitting vectors are *Anopheles gambiae* complex and *A. funestus* (Afolabi et al., 2001 Salako et al., 1990). The children were enrolled based on fever presentation or appearance of fever (body temperature 37.5°C) in the last 24 to 48 h, microscopic detection of absolute *P. falciparum* parasitaemia of 2000 asexual forms/ml of whole blood and absence of concomitant illness.

A written informed consent for each sick child was obtained from a parent or guardian as a criterion for enrollment into the study. The study was approved by the Hospital Management Board, Lagos, Nigeria and spanned from March, 2000 to February 2002.

Thin and thick blood film microscopy

Each sick child was finger-pricked using a sterile lancet and drops of whole blood collected on labeled grease-free slide were used to make thick and thin blood films for quantitation of parasitaemia and speciation.

The parasite density was determined by counting the number of asexual forms of *P. falciparum* microscopically (X1000 magnification) on 5% Giemsa-stained thick film slide against 200 – 500 leukocytes with the assumption that there are 8000 leukocytes per l of blood.

Two drops of blood from each patient were also blotted onto 3 MM Whatman filter paper air-dried, stored in air-tight container and subsequently used for extraction of *P. falciparum* DNA and genotyping of pfcrT locus.

Amplification of the pfcrT locus

Parasite genomic DNA was extracted from the blood-spotted and dried filter paper by methanol fixation and heat extraction according to Snounou et al. (1993). 5-10 l aliquot of the extracted DNA was further amplified *in vitro* by nested PCR in a primary reaction reaction volume of 20 l to generate a primary fragment (outer pfcrT locus = 260 bp) that was subsequently used as a template (1.0 l) for the secondary PCR to generate a secondary fragment (inner pfcrT locus = 134bp) (Djimde et al., 2001). Two sets of primer pair based on sequence homology to pfcrT gene (GenBank accession AF030694) from Roche Diagnostics, GmbH, Germany were used. They were CRT1 (CCGTTAATAATAAATACACGAG) and CRT2 (CGGATGTTACAAAATATAGTTACC) for primary PCR of 45 cycles (95°C, 30 s; 56°C, 30 s; 60°C, 60 s) and a final extension of 60°C, 5 min followed by the use of CRTD1 (TGTGCTCATGTGTTTAAACTT) and CRTD2 (CAAACTATAGTTACCAATTTTG) for secondary PCR of 30 cycles (95°C, 30 s; 56°C, 30 s; 60°C, 30 s) and a final extension of 65°C for 5 min. Each PCR began with a 3-min pre-PCR step at 95°C.

The resulting PCR products (10 l) were electrophoresed on a 2% agarose gel, stained with ethidium bromide (5 g/ml) and have their molecular weight determined by extrapolation using a 100-bp ladder of molecular weight markers (Invitrogen Life Technologies, Netherlands) under ultraviolet violet transillumination.

Restriction endonuclease digestion of pfcrT

5 l of secondary fragment representing the inner pfcrT locus (size = 134 bp) within which K76T point mutation exists was digested overnight with 0.5 U *AclI* at 37°C. Digestion to 34 and 100 bp fragments indicates wild type pfcrT allele with lysine (K) encoded by codon 76 of the gene, while the mutant pfcrT allele with threonine (T) at position 76 is uncut by the restriction enzyme (Djimde et al., 2001). PfcrT alleles from *P. falciparum* DD2 and HB3 strains were used as wild and mutant controls, respectively. Allelic frequency rate (R) was defined as the proportion of pfcrT T76 in year 2 to that in year 1 in each of the study populations.

Statistical analysis

Data were presented as proportions and stratified by age and sex. Disparity between proportions was evaluated using the STATCALC program of Epi-Info version 6 software (CDC, Atlanta, GA).

RESULTS

The data in Table 1 summarized the outcome of a 2-year molecular surveillance study of the acquisition of pfcrT T76, a chloroquine resistant gene marker by children with acute uncomplicated *P. falciparum* malaria in the two study populations. 19 of 39 (Ijede) and 31 of 42 (Massey Street Children's Hospital) pre-treatment blood samples were found to harbour the pfcrT T76 mutant allele of *P. falciparum* in the year 2000 – 2001, corresponding to frequencies of 48.7% and 73.8%, respectively (χ^2 Mantel-Haenszel= 4.4; P = 0.04). In the year 2001 – 2002, the allelic frequencies of the mutant allele were 67.3% and 74.6%, respectively (χ^2 Mantel-

Table 1. Parasitaemia profile and incidence of pfcrT76 allele acquisition in children with acute uncomplicated *Plasmodium falciparum* malaria in rural and urban Lagos, Nigeria.

		Study site					
		Ijede (Rural)		Massey Hospital (Urban)			
Period	N	n(%)pfcrT76 (+ve) [@]		N	n(%)pfcrT76 (+) [@]	χ^2	P
Year 1	39	19(48.7)		42	31(73.8)	4.4	0.04
Year 2	52	33 (67.3)*		63	47 (74.6)	1.7	0.2
Total	91	52 (57.1)		105	78 (74.3)	6.4	0.1

N = Pre-treatment sample size. Parasitaemia = 2080 – 24,420 asexual forms / l of blood; Geometric mean = 2138 / l. [@], Data represent number and percentage (in parenthesis) of cases positive by PCR-RFLP for pfcr T76 in both monoclonal (pfcr T76 band only) and polyclonal (pfcrK76/T76 bands seen) parasitaemia. Differences in proportions between and within groups on yearly basis were analyzed by chi-square test with Mantel -Haenszel modification. * P < 0.05 versus year 1 rural. Allelic frequency rates R = 1.38 /year in Ijede (rural) and 1.01 / year in for Massey Children Hospital (urban).

Table 2. Gender variation in the acquisition of pfcrT76 allele in rural and urban Lagos, Nigeria.

Rural	Sex			
	M	F	χ^2	P
Year 1	11(57.9)	8(42.1)	0.92	0.3
Year 2	18(54.5)	15(45.5)	0.54	0.5
Total	29(55.8)	23(44.2)	1.37	0.2
Urban				
Year 1	18(58.1)	13(41.9)	1.59	0.2
Year 2	26(55.3)	15(44.7)	0.54	0.5
Total	44(56.4)	34(43.6)	1.37	0.2

Figures in parenthesis represent percentages of pfcr T76 positive cases in the pre-treatment samples analyzed by PCR-RFLP.

Haenszel= 1.7; P = 0.2). Allelic frequency rates in Ijede (rural) and Massey Hospital (urban) were 1.38/year and 1.01/year, respectively.

Further stratification of the acquired pfcr T76 allele by age revealed no significant difference (P > 0.05) in both populations (Table 2). However, children of age \leq 5 years were found to acquire the allelic mutant more than older children in both populations (59.0% vs. 41.0%; rural = 61.5% vs. 38.5%; P < 0.05) Table 3. The parasitaemia characteristics of the pre-treatment blood samples revealed acquisition of the mutant allele in monoclonal infections (pfcr T76 only) and polyclonal infections (i.e. mixed infection due to wild, pfcr K76 and mutant allele parasitaemia) (Figure 1). The alleles were identified by PCR-RFLP of primary fragments as 100 bp (wild allele) and 134 bp (mutant allele) molecular size bands (Figure 2).

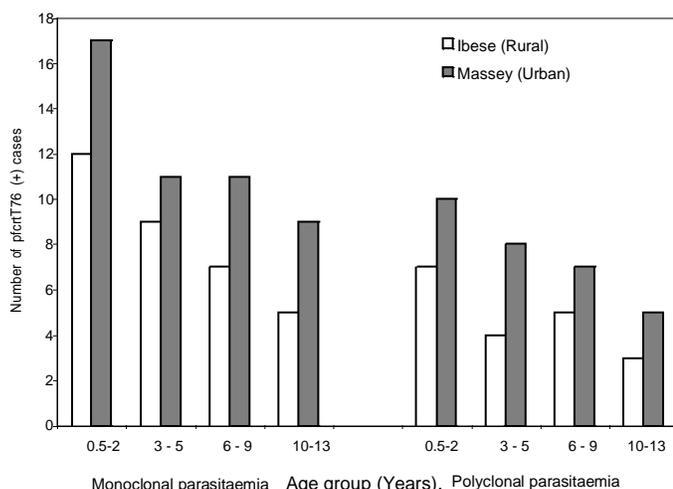


Figure 1. Age distribution of pfcr T76 allele due to monoclonal (single infections) and polyclonal (mixed infections) *Plasmodium falciparum* parasitaemia in the study population.

DISCUSSION

The present study has revealed increasing and high prevalence of pfcr T76 *P. falciparum* strains in rural and urban Lagos which is similar to observations in other parts of the country where chloroquine resistant strains have long emerged and now exhibiting expansion and intensification (Abdulahi et al., 2003; Happi et al., 2004). To our knowledge, this is the first report of pfcrK76T mutant *P. falciparum* strains in the study area.

The pfcr gene, which regulates intracellular parasite physiology and found crucial to parasite viability in infected erythrocyte (Waller et al., 2001) evolves from the K76 wild type to T76 mutant allele most likely as a result of parasite exposure to sub-therapeutic doses of chloroquine (Fidock et al, 2000). The national health policy that allows presumptive treatment of fever in children with chloroquine by care givers (i.e mothers, guardians etc) at home often provides wide ground for chloroquine abuse and under dosing (Ezedinachi et al,

Table 3. Comparison of pfcrT76 allele acquisition in less than 5-year old and older children for 2 years in rural and urban Lagos, Nigeria.

Age group	Ijede (Rural)	Massey Hospital (Urban)
< 5 years	32 (61.5)	46 (60.0)
> 5 years	20 (38.5)	32 (40.0)
Total	52 (100.0)	78 (100.0)
	2 = 5.5; P = 0.02	2 = 5.0; P=0.03

Figures represent number and percentages of pfcrT76 positive cases in the two age groups. Disparity between proportions was analyzed by chi-square test with Mantel-Haenszel modification. $P < 0.05$ indicates significance.

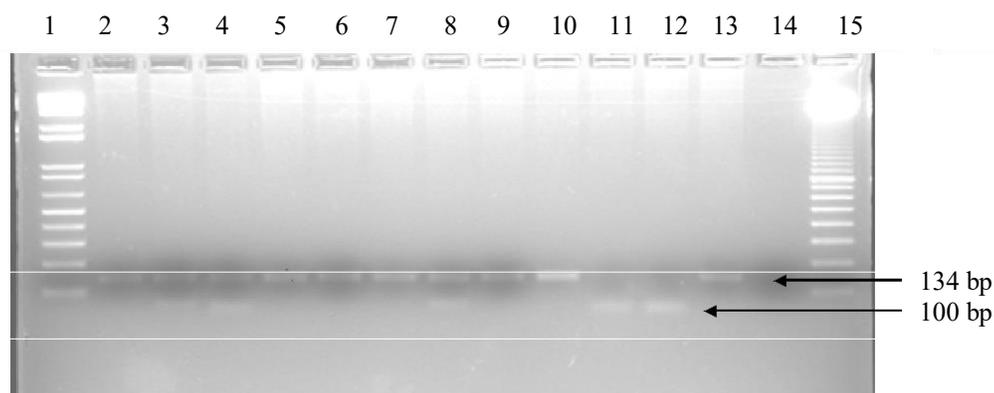


Figure 2. PCR-RFLP of secondary pfcr amplicon (134 bp) of *P. falciparum* strains recovered from the pretreatment blood samples of children with acute uncomplicated malaria. Lanes 1 and 15 = 100 bp ladder DNA markers. Lanes 2 – 11: Pretreatment *P. falciparum* infected blood samples. Lane 12: DD2 *P. falciparum* (wild pfcr K76 control). Lane 13: HB3 *P. falciparum* (mutant pfcr T76 control). Lane 14: uninfected blood sample (-ve control).

1997). This is because withdrawal of chloroquine especially in rural areas where microscopists are lacking and diagnosis is presumptive is often based on the clearance of clinical symptoms and not parasitaemia (Ramson-Kuti, 1989; Ezedinachi et al., 1997).

The significantly high incidence of pfcr T76 allele among ≤ 5 years old children in the study population agrees with the finding of May and Meyer (2003) who observed an inverse correlation between pfcr T76 acquisition and age of 100 Nigerian children with asymptomatic falciparum malaria in Abanla, a village with health post facility in Ibadan. The age pattern of acquisition of pfcr T76 observed in this study may therefore be a reflection of chloroquine abuse and also represent a strong predisposing factor to altered chloroquine clinical efficacy observed in this age group in the country (Mockenhaupt et al., 2000; Happi et al., 2004).

The observed 48.7 – 64.5% frequencies of pfcr T76 in rural Lagos is similar to the observations in the Sudan where pfcrT76 allelic frequency has been found to increase at a rate of 0.217 per year (Abdel-Muhsin et al.,

2004). In urban Lagos, the allelic frequency of 73.4 – 74.7 is comparable to 79% found in the pretreatment samples of malaria patients residing in a holoendemic region in Senegal (Thomas et al., 2002) and 82% found among malaria patients in Burkina Faso (Tinto et al., 2003), and is higher than 40 – 61.5% observed in traveler malaria cases (Labbe et al., 2003) but lower than 94% reported from an endemic area in Bangladesh (Van den Broek et al., 2004). Happi et al. (2003) had previously reported pfcr T76 allelic frequencies of 48% in pretreatment samples of children with acute uncomplicated malaria living in other parts of the country with similar malaria endemicity and transmission.

The clino-pathogenic consequences of pfcr T76 selection by endemic *P. falciparum* strains in a malarious area are manifold. From pathogenic perspectives, studies have shown that pfcr T76 acquisition favours severity and multiplicity of malaria infection (Ranjit et al., 2004). This agrees with previous reports that malaria complications such as anaemia (Hb < 5g/dl), and cerebral malaria are the major causes of death in Nigerian children with falciparum malaria and those from

other African countries where pfcr T76 *P. falciparum* clones are also a public health menace (Angyo et al, 1996; Olumese et al, 2002).

In clinical context, data concerning the pharmacological disposition of mutant *P. falciparum* strains to chloroquine and other heme binding antimalarials are grossly inconsistent. Whereas many *in vitro* work ranging from allelic exchange to complementation studies have effectively demonstrated the association of pfcr T76 with chloroquine resistance and hence drug failure (Fidock et al., 2000). *In vivo* studies have contrary findings. For instance, in the work of Happi et al. (2003), pfcr T76 alleles were found at comparable frequencies in the pretreatment samples of children that failed chloroquine and those with successful chloroquine treatment and in Gabon the allele was also observed in patients whose parasitaemia were cleared by chloroquine without recrudescence (Ranford et al., 1997). However, in an Ugandan study where 100% chloroquine failure rate was reported, the pre-treatment and recrudescence parasitaemia were due to pfcrT76 clones alone (Dossey et al., 2001).

The inconsistencies surrounding the relationship between pfcr T76 acquisition and chloroquine resistance *in vivo* have been attributed to the modulatory influence of other biological, environmental and genetic factors. These include variation in chloroquine uptake, distribution and metabolism in humans (Kallwarg and Harinasuta, 1992; Tett et al., 1996), mutations in other loci with pfcr gene to confer greater stability and increase gene expression (Waller et al., 2001), the co-existence of N86Y point mutation in pfmdr-1 gene located on chromosome 5 of *P. falciparum* as an indispensable requirement for *in vivo* resistance and levels of antimalarial immunity dependent on exposure to *P. falciparum* antigens (Chen et al., 2002) and antioxidant status (Metzger et al., 2001).

Acquired antimalarial immunity through previous parasite exposure has been found to induce chloroquine clearance of *P. chabaudi* parasitaemia in mice (Cravo et al., 2001). While vitamin A has been observed to upregulate CD36 expression, decline TNF- expression and promote phagocytic uptake of infected erythrocytes in animal models (Serghides and Kain, 2002). In several human studies, malaria has been associated with hypovitaminosis and vitamin A supplementation has been shown to reduce malaria morbidity and mortality in some clinical trials (Shankar et al., 1999).

Nigeria is also one of the countries in which micronutrient deficiency is a public health problem (Copper et al., 2002). However, there is paucity of information regarding the micronutrient profiles of the study population thereby weakening the possible understanding of the impact of antioxidant status on malaria in the study area.

Nevertheless, the fact that making or altering

antimalarial prophylactic and therapeutic policies in an endemic area in order to achieve effective malaria control is based on prior understanding of drug resistance profile has further made the present molecular surveillance study very important. This molecular method detects resistance genotypes within hours as against *in vitro* culture method, which has 2 – 3 days diagnostic time and *in vivo* clinical efficacy approach that requires at least 14 days for profiling of parasitological resistance types: RI, RII and RIII.

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