Original Research Article

Prevalence of Mutant Alleles Responsible for Chloroquine Resistance among *Plasmodium falciparum* Isolates in North Central, Nigeria**.**

ABSTRACT

Introduction

Although chloroquine (CQ) has been officially replaced with artemisinin combination therapy (ACT) as first line drug for the treatment of malaria in Nigeria since 2005, a lot of people still believe that chloroquine is more effective chiefly because of the decline in the sensitivity of Plasmodium falciparum to ACT. Thus resulting into unofficial use of CQ for self medication. This study was conducted in order to survey the current status of chloroquine resistant strains of pfcrt and pfmdr1 in view of possible re-introduction of chloroquine for malaria treatment.

Method

DNA was extracted from one hundred (100) microscopically confirmed Plasmodium falciparum positive blood samples spotted on 3mm Whatmann filter paper. The detection of mutations in Plasmodium falciparum chloroquine resistance transporter (Pfcrt) and Plasmodium falciparum multidrug resistance (Pfmdr1) genes was performed by nested polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP).

Results

*Results showed the presence of mutant alleles of Pfcrt and Pfmdr1 in 60% and 41% of the samples respectively. However, there was no significant correlation in the prevalence of mutant alleles (**T**76/**Y**86) in relation to gender (p = 0.59/ 0.08) and age (p=0.59/0.93) of participants respectively.*

Conclusion

The observed high prevalence of chloroquine resistance despite thirteen years of withdrawal calls for serious concern

Keywords: chloroquine, malaria, *Plasmodium falciparum*, mutation, mutant allele

1. INTRODUCTION

Malaria poses a significant challenge to the health and well being of populations living in malaria-endemic regions. Although the number of cases and deaths due to malaria has declined globally,

the disease is still responsible for about 200 million cases leading to 500,000 deaths annually [1]. The burden of malaria is heaviest on African nations (and particularly Nigeria) where 90% of global deaths due to it occur [2]. Efforts to effectively control malaria have in the recent decades been hampered by emergence of resistant strains of *Plasmodium*. The development and spread of resistance to *Plasmodium falciparum*, the most virulent *species* of malaria parasite is one of the greatest challenges to malaria control today [3] CQ had historically played a key role in the management of the disease, being the mainstay of malaria therapy worldwide from 1940s up to the 1990s [4]. However, extensive use of CQ as a monotherapy for prophylaxis and chemotherapy led to the emergence of resistant strains of *Plasmodium falciparum* across many malaria-endemic countries prompting policy change which eventually brought about the withdrawal of CQ in favour of artemisinin combination therapy (ACT) in 2005 in Nigeria accordance to WHO recommendation. Nonetheless, CQ has remained available on the counter and is being dispensed without prescription [5].

Essential qualities of CQ which made it a drug of choice in the past decades include its low cost, low toxicity and high efficacy in clinical cases. Chloroquine acts by targeting the parasite haematin detoxification pathway in the digestive vacuole in a two-pronged attacks where it adsorbs onto growing hemozoin polymers and also binds to toxic haematin molecules generated as the parasite digests host haemoglobin resulting in accumulation of toxin haematin in the digestive vacuole [6].

Resistance to CQ is primarily mediated by the genetic replacement at codon 76 (K76T) of lysine with threonine in the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) which increases efflux of CQ from the digestive vacuole of the parasite thereby rendering the drug ineffective [7]. Polymorphisms in the *Plasmodium falciparum* multidrug resistance gene-1 (*pfmdr*1) which encodes the P-glycoprotein homolog, modulates chloroquine resistance in mutant pfcrt-harbouring parasites in vitro and impact on the sensitivity multiple antimalarial drugs.

Changes in pfmdr1 sequence alter transport of multiple drugs in and out of the parasite food vacuole with individual polymorphisms leading to opposite effects on different drugs. Mutations at pfmdr1 have been linked to decreased sensitivity to chloroquine and amodiaquine [8]. Drug efficacy studies are critical in guiding drug policy as monitoring and evaluation are also essential. CQ has been replaced with ACT since 2005 in Nigeria. This study was therefore conducted in order to assess the sensitivity of CQ given the time interval since its withdrawal, and also in the light of emerging resistance to the current drug of choice.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

Study Design

This is a cross-sectional descriptive study and a subsection of a larger study conducted between 2014 and 2016 aimed at characterizing the genetic diversity of *Plasmodium falciparum* in Niger State, North Central, Nigeria

Study Area

The study was conducted at Swashi Clinic and Maternity, Niger State, Nigeria

Ethical Clearance

Ethical clearance was obtained from ethical review committee of University of Ilorin Teaching Hospital while informed consent of all participants was obtained before sample collection.

Sample Collection

Blood samples of all patients aged 1 to >30 years with fever or history of fever 48 hours prior to presentation were collected on microscope slides and on 3MM Whatmann's filer paper. Patients with microscopically confirmed *falciparum* parasitaemia were recruited into the study.

Laboratory Analysis

Microscopy

Thick and thin blood films on were stained with Giemsa stain and examined under compound microscope for the asexual stage of *P. falciparum*. Parasite density was determined by counting the number of asexual parasite against 200 leucocytes taking the total leucocytes count to be 8,000/ul of blood.

DNA Extraction

DNA was extracted from microscopically confirmed *P. falciparum* blood samples spotted on filter paper by using Chelex Extraction method earlier described [9]

PCR for detection of Pfcrt and pfmdr1 genes

The detection of mutations responsible for chloroquine resistance was performed by amplifying sequences marking the *Plasmodium falciparum* chloroquine resistance transporter *(pfcrt)* and *Plasmodium falciparum* multidrug resistant 1 (pfmdr) genes using nested PCR followed by restriction fragment length polymorphism (RFLP) according to previously described procedures[10]

Primers used for pfcrt (K76T) and pfmdr1 (N86Y) primary amplifications included Crtp1, Crtp2, Mdr1 and Mdr2 and their sequencs are as shown in Table 1 and 2 respectively. The primary PCR components in a final volume of 15µl was buffer 10x, 25mM $Mgcl_2$, 50mM deoxynucleotide triphosphate (dNTPs), 10µm of each of the primer (Crtp1, Crtp2, Mdr1 and Mdr2), 5u/µl mM deoxynucleotide triphosphate (dNTPs), 10µm of Taq polymerase and 2 µl of DNA samples. The cycling protocol for pfcrt was as follows: initial

denaturation at 94°C for 3 minutes followed by 45 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 60°C for 1 minute and 72°C for 15 minutes. For pfmdr1 the cycling protocol was initial denaturation at 95°C for 5 minutes followed by 45 cycles of 95°C for 30 seconds, 45°C for 30 seconds, 65°C for 45seconds and 72°C for 15 minutes.

Nested PCR and RFLP for Pfcrt and Mdr1 mutation-specific detection

Secondary PCR was conducted by using the forward primer Crtd1 and the reverse primer Crtd2 (Table 1) 2µl of 10x dilution of primary PCR was used in a follow-up, nested, allele specific PCR amplifications to detect the codon for pfcrt K76T. The PCR stages for these diagnostic amplifications were initial denaturation at 94°C for 3 minutes, followed by 45 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 60°C for 1 minute and a final extension at 72°C for 15 minutes. Purified genomic DNA from *P. falciparum* clones 3D7 (Chloroquine sensitive) and Dd2 (chloroquine resistant) were used as positive controls, and water and uninfected blood spots on filter paper were used as negative controls.

After amplification, 20 µl of the amplicons was incubated overnight at 50°C with mutation-specific restriction enzyme *Apo I*. In the PCR products, the DNA sequence was cleaved at the wild-type (76K) codon

site (if present) into two fragments, while the mutant alleles (76T) codon found in chloroquine-resistant *P. falciparum*) were not cut. The digested products were separated by electrophoresis in a 2% agarose gel containing ethidium bromide, and DNA was visualised by ultraviolet transillumination.

Similarly, amplification of codon 86 of the pfmdr1 gene was carried out using the following primers: Mdr1 and Mdr2 for the primary PCR reactions and Mdr3 and Mdr4 for the secondary reactions (Table 2) after which restriction with *ApoI* or *Afl III* was done. DNA fragments were compared by size and with the PCR products generated from genomic DNA of the 3D7 and Dd2 strains (used as references for susceptible and resistant genotypes, respectively). Thermocycling conditions for pfmdr1 secondary reaction were initial denaturation at 95°C for 3 minutes, followed by 25 cycles at 95°C for 30 seconds, 45°C for 30 seconds, and 65°C for 45 seconds and a final extension at 72°C for 15 minutes.

Table 1: PCR Primer Sequences for Amplication of Codon 76 of Pfcrt

Primer Name	**Sequence 5' - 3'**
Pfcrt-P1 (Forward Primer)	CCG TTA ATA ATA AAT ACA CGC AG

Pfcrt-P2 (Reverse Primer)	CGG ATG TTA CAA AAC TAT AGT TAC C
Pfcrt-D1 nested1 (Forward Primer)	TGT GCT CAT GTG TTT AAA CTT
Pfcrt-D2 nested 2 (Reverse Primer)	CAA AAC TAT AGT TAC CAA TTT TG

TABLE 2: PCR Primer Sequences for Amplication of Codon 86 of Pfmdr

Primer Name	**Sequence 5' - 3'**
Mdr1 (Forward primer)	GCG CGC GTT GAA CAA AA A GAG TAC CGC GTG
Mdr2 (Reverse primer)	GGG CCC TCG TAC CAA TTC CTG AAC T CAC
Mdr3 (Forward primer)	TTT ACC GTT TAA ATG TTT ACC TGC
Mdr4 (Reverse primer)	CCA TCT TGA TAA AAA ACA CTT CTT

Data analysis

Data were subjected to statistical analysis by using MS-Word, Chi-square and Fisher's exact test. Statistical significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

RESULTS

Prevalence of Molecular Markers *P. falciparum* Resistance to Chloroquine

100 isolates of *Plasmodium falciparum* were typed with markers of chloroquine resistance (pfcrt and pfmdr 1) at codons **K**76**T** and **N**86**Y**. Whereas the prevalence of wild (sensitive) alleles **N**86 of pfmdr1 (59.0%) was higher than the mutant alleles **Y**86 (41.0%), the prevalence of mutant alleles **T**76 (60.0%) of pfcrt was considerably higher than that of the wild type alleles **K**76.

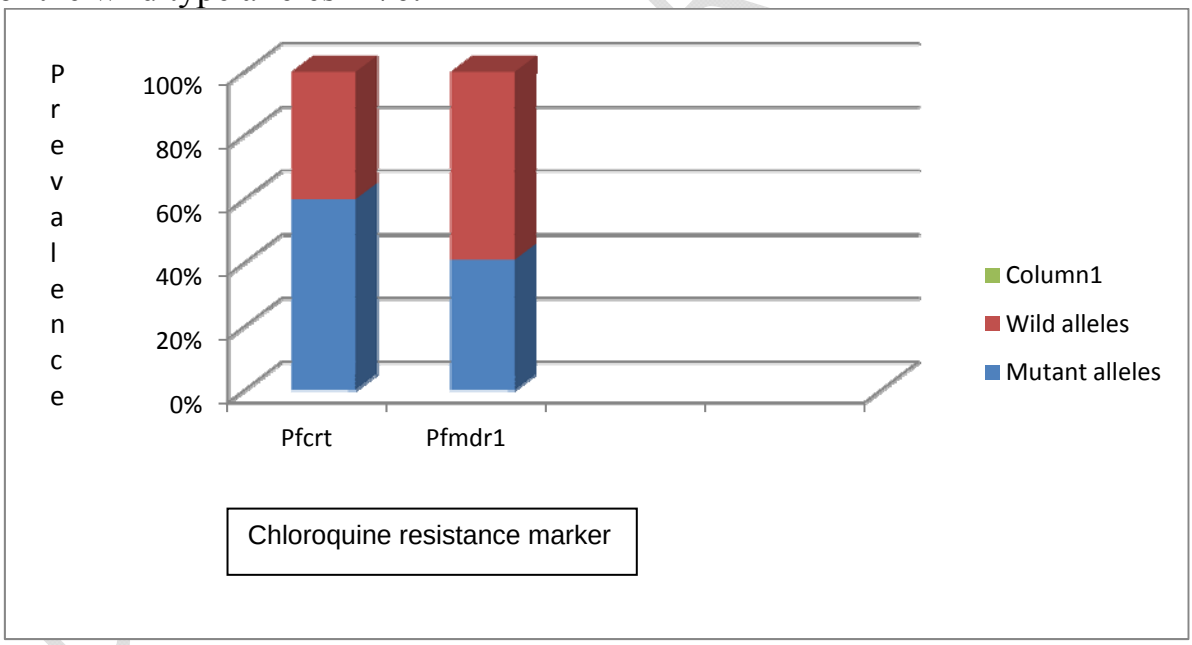

Fig. 1: Prevalence of Molecular Markers *P. falciparum* Resistance to Chloroquine

Prevalence of Drug-resistance marker by gender

Of the 100 isolates that were successfully typed by drug resistance markers, 44 were males while 55 were females. Thus females were more represented and also recorded higher higher prevalences of mutant

alleles at the two codons , however, this was not statistically significant ($p>0.05$)

Table 3: Prevalence of Drug-resistance markers stratified by gender

Drug Resistance Marker	Males (44)	Females (56)	p-Value
T76	26 (59.1%)	34 (60.7%)	0.77
Y86	15 (34.1%)	26 (46.4%)	0.08

Prevalence of Molecular Markers Associated with *P. falciparum* Resistance to Chloroquine by age

The age of 100 participants for drug resistance typing ranges from 1year to above 30 and were grouped into four: 1-10years, 11-20, 21-30 and greater than 30. The age groups 1-10 and 11-20 were more represented being 62% and 24% respectively. The least representation was observed in the age groups 21-30 and > 30 being 9% and 5% respectively.

Prevalence of mutant alleles by age showed an increasing rate of mutation with age in most of the codons but analysis of data by age categories did not discover any statistical difference ($p>0.05$).

Table 4: Prevalence of Molecular Markers Associated with *P. falciparum* Resistance to Chloroquine by age

		No(%) of mutant alleles	
Age Group	% Examined	**T**76	**Y**86
1-10	62	36 (58.1%)	25 (40.3%)

11-20	24	15 (62.5%)	10 (41.7%)
21-30	09	06 (66.7%)	04 (44.4%)
>30	05	03 (60.0%)	02 (40.0%)
Total	100	60 (60.0%)	41 (41.0%)
p-Value		0.582332	0.4531

DISCUSSION

Chloroquine resistance has been associated in vitro with point mutations in two genes, pfcrt and pfmdr1, which encode the *P. falciparum* digestive-vacuole transmembrane proteins and P-glycoprotein-mediated multidrug resistance (Pgh1), respectively. Identification of Pfcrt as the central determinant of chloroquine-resistant *P. falciparum* malaria provides a molecular marker that can be used for surveillance of resistance and to evaluate drug treatment and prophylaxis policies.

Analysis of well-characterized molecular markers of *P. falciparum* resistance to the 4-amino-quinolines revealed a high prevalence of resistant genotypes.

The **T**76 mutation which is associated with chloroquine resistant i.e the substitution of threonine (T76) for lysine (K76) at position 76(K76T) of the amino acid sequence in pfcrt which encodes a transporter protein of the *P. falciparum* digestive vacuole was found in 60.0% of samples with

241 *falciparum* malaria infection while and **Y**86 mutation (associated with
242 resistance to amodiaquine, mefloquine, halofantrine and lumefantrine)
243 that is the substitution of tyrosine (Y86) for asparagines (N86) at
244 position 86 (N86Y) was found in 41.0%.

245 The high prevalence of resistance observed in this study indicates a
246 pertinacity of resistance to chloroquine for more than a decade after the
247 change of malaria treatment policy in Nigeria. This result is consistent
248 with the findings from other parts of Nigeria and most regions of high
249 drug resistance[11-14]. The result is however in sharp contrast to findings
250 from Malawi where *plasmodium* became susceptible to chloroquine after
251 cessation of usage[15]. Also in Tanzania, chloroquine regained its
252 sensitivity after two and half years of withdrawal of drug pressure [16].

253 CQ was abandoned for artemisinin combination therapy in 2005 due to
254 widespread resistance and highly significant clinical failure across the
255 country. It is expected that the susceptibility of CQ will be restored
256 about 10 years after withdrawal as it happened in Malawi and Tanzania.
257 Persistence of CQ resistance witnessed in this study might not be
258 unconnected with poor drug control policy in the country which permits
259 the drug to be freely available for use outside government hospitals.

Cross-resistance between CQ and Amodiaquine may also be a contributory factor due to the similarities in their modes of action. Amodiaquine is currently a partner drug with artemisinin in the treatment of malaria in Nigeria. Although this drug remains effective in areas of substantial CQ resistance, the two drugs are chemically related and several clinical and in vitro reports have shown cross resistance between CQ and AQ or active metabolite of AQ[13].

The present study showed a predominance of wild type of *pfmdr1* N86 (59%), this could be considered as an indicator for low susceptibility of *P. falciparum* isolates to mefloquine, amodiaquine and quinine[17-18]. Mutations in Pfmdr 1 has also been associated with with decreased susceptibility to artemether and lumefantrine drugs separately [(19-22)]. However, mutations at pfmdr1 gene alone do not seem to be sufficient to explain *in vitro* resistance to antimalarial drugs.

This study did not discover any positive association in the prevalence of mutant alleles in relation to sex and age of the participants, although lower age had lower prevalence of mutant alleles **T**76 (58.1%) and **Y**86 (40.3%) this may not be unconnected with acquired immunity of the

adults. However, the differences were not statistically significant. This is consistent with findings of Atroosh *et. al*., (2012).

COMPETING INTERESTS

No competing interests

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