Chloroquine Resistant *Plasmodium falciparum* in Nigeria: Relationship between *pfcrt* and *pfmdr1* Polymorphisms, *In-Vitro* Resistance and Treatment Outcome

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Abstract: This study was designed to evaluate the association between polymorphisms in *pfcrt* and *pfmdr1* genes and *in-vitro* chloroquine (CQ) sensitivity in fresh isolates of *P. falciparum* and patients' treatment outcome. The modified schizont inhibition assay was used to determine *in-vitro* sensitivity of *P. falciparum.* Polymorphisms in *pfcrt* and *pfmdr1* genes were detected using nested PCR and RFLP techniques in 84 *P. falciparum* isolates obtained from patients with acute uncomplicated malaria.

Eighty five percent (71/84) and 15% (13/84) of the parasites were resistant and sensitive *in-vitro* to CQ respectively. Molecular analysis showed presence of mutant *pfcrtT76, pfmdr1Y86* and *pfmdr1F184* alleles in 60%, 33% and 14% of the isolates respectively. There was a significant association between *in-vitro* and *in-vivo* CQ resistance (p=0.029) and also between the presence of mutant *pfcrtT76+pfmdr1* Y86-Y184 haplotype and *in-vitro* (p=0.013) or *in-vivo* CQ resistance (p=0.024).

Overall results from this study demonstrates that the presence of *pfcrtT76+ pfmdr1* Y86-Y184 haplotype in Nigerian isolates of *Plasmodium falciparum* is predictive of *in-vitro* and *in-vivo* CQ resistance and therefore may be useful for monitoring resistance to this drug.

Key Words: *pfmdr1, pfcrt,* chloroquine-resistance, *in-vitro*, *in-vivo*, Nigeria.

INTRODUCTION

The emergence and spread of drug resistant malaria parasites in endemic regions has posed a great threat to usefulness of chloroquine (CQ) and sulphadoxine-pyrimethamine (SP), the cheapest and widely used antimalarial drugs. This widespread prevalence of antimalarial drug resistant parasites has led to strong calls for the introduction of artemisinin based combination therapies (ACTs) for treatment of malaria in most endemic areas, as it is expected to represent an effective approach in curbing the development of resistance by *Plasmodium falciparum* to currently available compounds [1, 2]. Presently, most malaria endemic countries in Africa including Nigeria have changed their first line antimalarial treatment from CQ or SP to amodiaquine combined with artesunate or the combination of artemether and lumefantrine. ACTs used in most malaria endemic countries have demonstrated high efficacy, protection against the development of resistance to each component and reduction of malaria transmission [3-5]. However, the relatively high of costs, dosing complexity and the limited experience of their use in sub-Saharan Africa may hamper the widespread deployment of these drug combinations [6]. In addition there are reports of the emergence of *P. falciparum* with reduced susceptibility to some of the ACTs [7].

This situation therefore points to the fact that other more affordable, effective and readily available combinations are still required. An earlier report [8] has demonstrated the return of CQ susceptible parasites in Malawi soon after the country switched from CQ to SP as the first line therapy for malaria. Furthermore, a recent clinical trial in Malawi also confirmed that CQ had excellent clinical efficacy, 12 years after it was removed from use [9, 10]. It is therefore necessary to understand the molecular basis of resistance to already available antimalarial drugs such as CQ, and explore the potentials of the information generated in improving the potency and rational for developing effective drug combinations.

The genetic and biochemical basis of CQ resistance in *P. falciparum* has been the subject of several research and has not been fully elucidated in field epidemiologic studies. *P. falciparum* resistance to CQ has been associated with lower drug accumulation in infected erythrocytes [11] or mainly to a mutation at position 76 (K76T) in the *Plasmodium falciparum* chloroquine resistance transporter *(pfcrt)* gene [12, 13]. Several other studies have suggested the implication of multidrug resistance-1 *(mdr1)* gene family in resistance to quinoline containing compounds in *P. falciparum* [14-16]. Five point mutations on codons 86, 184, 1034,1042 and 1246

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have been identified to influence the response of *P. falciparum* to CQ [14, 17]. Several reports have also shown that there is an association between polymorphisms in these two genes and resistance of *P. falciparum* to CQ *in-vitro* [12-14, 16, 18], or with CQ therapy failures in field studies [13, 18-26]. While most laboratory reports points to the involvement of these two genes in mediating resistance to CQ and perhaps other drugs, it is yet to demonstrate under field conditions how the allelic differences in *pfcrt* and *pfmdr1* genes correlate with the resistant phenotype in different malaria endemic areas of the world, or whether there are differences in resistance mechanisms by parasites in various regions of the world where malaria is endemic.

In this study, the involvement of polymorphisms in *pfcrt* and *pfmdr1* genes in *in-vitro* susceptibility of fresh isolates of *P. falciparum* as well as CQ treatment outcome in children with acute uncomplicated *P. falciparum* malaria in Southwest Nigeria was investigated. Point mutations in *pfcrt* and *pfmdr1* genes were found to be associated with both *in-vitro* and *in-vivo* CQ resistance. The potential implications of these findings in monitoring CQ resistance in areas where ACTs have been introduced for treatment of *P. falciparum* malaria are discussed.

STUDY DESIGN

Study Site

The study was carried out at the Malaria Research Laboratory, College of Medicine, University of Ibadan, Nigeria. Malaria in Ibadan is hyperendemic, transmission occurs year round but is more intense from April to October, during the rainy season.

Patients Selection, Treatment and Follow-Up

Children aged 6months to 12 years with acute symptoms of *P. falciparum* malaria infections were enrolled after clinical examination and microscopic confirmation of infections in a large clinical efficacy study. Informed consent for participation in the study was obtained from parents/guardians of children under the age of 10 years, while assent was obtained from each patient between the ages of 10-12 years. The Joint UI/UCH Institutional Review Committee (IRC) approved the study protocol. Each child was treated with standard dose of CQ (25mg/kg body weight over three days) and followed up for a period of 28 days according to World Health Organization (WHO) protocol [27]. Infection in each child was considered cured if no parasites appeared in the peripheral blood samples during the 28 days follow-up period after treatment. Children in whom parasites reappeared in their blood within the 28 days of follow up were classified as treatment failures and were re-treated with SP to cure the infection.

Determination of *In-Vitro* Susceptibility of Patient Isolates to CQ

In-vitro susceptibility of each patient isolate of *P. falciparum* to CQ was determined using a modification of the schizont inhibition assay [28]. Briefly, a template containing three-fold serial dilutions of the working CQ solution (9690nM) was prepared in a 96-well microtiter plate. Wells in row H served as controls without drug. Test plates were derived from each template by transferring 25µl of the drug dilutions to each plate. Two hundred microliters (200µl) of 1ml parasitized blood diluted in 19ml of culture medium (RPMI 1640+ HEPES and sodium bicarbonate) was transferred into each well of the plate. Plates containing parasites suspension with CQ in each well were incubated at 37^{o}C for 24 to 36 hours in a plexiglass chambers containing a gas mixture (5% O_2, 5% CO_2, 90% N_2). The final concentration of CQ in test plate ranged between 969nM and 1.3nM.

The assay was terminated when at least 60% of parasites in the control wells (Row H) were schizonts. Each well in a column of 96 well plate was harvested onto glass slides as thick smears, air dried and stained with Giemsa. Parasites development to schizont was determined by counting the number of schizonts against 200 white blood cells in each smear using x100 oil immersion objective of a light microscope. Concentration-response data were analyzed by a non-linear regression analysis. The 50% inhibitory concentrations (IC-50) for CQ were calculated using GraphPad Prism version 4.0 for windows software (GraphPad software, San Diego, LA, USA, www.graphpad.com).

According to the WHO criteria [29] of *in-vitro* CQ sensitivity of *P. falciparum*, a complete inhibition of parasite growth in wells with CQ concentration <107nM (55.5ng/ml) was considered as sensitive, parasite growth in wells with CQ concentration >107nM is defined as resistant and parasite growth in wells of 107nM CQ concentration but not in wells of higher concentration is defined as borderline sensitivity.

Parasite DNA Extraction

Parasite genomic DNA was extracted from blood samples collected on filter paper using the chelex extraction method according to the method of Plowe *et al.* [30].

Analysis of *P. falciparum* Point Mutations in *pfcrt* and *pfmdr1* Genes in Isolates Obtained from Patients

The lysine (K) to threonine (T) mutation at codon 76 of the *pfcrt* gene was detected by nested PCR followed by RFLP as previously described by Happi *et al.* [24]. DNA from two laboratory adapted *P. falciparum* clones, 3D7 (Wild type) and Dd2 (mutant) were used as negative and positve controls respectively.

The nested PCR and RFLP methods were also used to evaluate the *pfmdr1* N86Y, Y184F S1034C and N1042D point mutations as described previously [26, 31]. The K1 and 7G8 laboratory strains of *P. falciparum* presenting with different genotypes at the different codons analyzed were used as controls.

All amplifications were performed in a final volume of 25ul in a PCT- 200 Peltier thermal cycler (MJR Research Inc, MA, USA)

Determination of *Plasmodium falciparum* Clonal Profile in Infections

Previous studies [24-26, 32] on molecular analysis of *Plasmodium falciparum* malaria in Nigerian children following treatment with CQ, have demonstrated that the merozoite surface protein-2 (*msp-2*) was the most informative genetic

marker to evaluate parasites diversity and the complexity of *P. falciparum* infections in Ibadan, Nigeria. In this study, isolates from each *P. falciparum* infection were characterized on the basis of fragment sizes of alleles of *msp-2* after amplification by PCR. Infections were defined as polyclonal if samples from patients showed more than one allele of *FC27* or *IC1/3D7* families of *msp-2*. If an isolate had one allele at each of the families, the clone number was taken to be one. The complexity of infection was calculated as the mean number of distinct fragments of *FC27* and *IC1/3D7* per PCR-positive sample. Polymorphism in *msp-2* was also used to distinguish between recrudescence i.e. resistant infection and re-infection. A CQ resistant infection was defined as the occurrence of the same or a subset of the alleles at each of the families (*FC27 or IC1/3D7*) of *msp-2* in the pre and post-treatment samples. A lack of allelic identity in the two families of *msp-2* in matched pre and post-treatment samples indicated a newly acquired infection.

Data Analysis

Clinical response to treatment with CQ was expressed as cured (cleared) or resistant (failed). *In-vitro* profile of *P. falciparum* to CQ was defined as sensitive (S) or resistant (R). Mean values were determined as mean ± standard deviation (SD). The student *t*-test was used to compare mean values. The Fisher's exact test was used to assess a statistical association between *in-vitro* susceptibility profiles of patients' isolates of *P. falciparum* to CQ and point mutations in parasites *pfcrt*, *pfmdr1* genes and treatment outcome. *P* value < 0.05 was considered significant.

RESULTS

One hundred and twenty children with *P. falciparum* infection were recruited into the study. The mean age of the children was 5.41±2.94years (Range 6months-13years). *Plasmodium falciparum* blood sample was obtained from 84 children by venipuncture for evaluation of the association between *in-vitro* CQ susceptibility, markers of CQ resistance, and patients treatment outcome. The geometric mean of parasite density in the children at enrollment was 37,451 parasites/µl of blood (range: 7775 to 150,000 parasites/ µl). There was no significant difference between the age (P=0.82), and geometric mean parasites density (P=0.60) of the 84 children whose blood sample were obtained for *in-vitro* CQ susceptibility and 120 children that were enrolled in the main study.

Response of Infection to Treatment with Standard Regimen of CQ

Of the 84 children whose blood samples were obtained for the evaluation of the association between *in-vitro* susceptibility profile of parasites to CQ, molecular markers of CQ resistance and patients parasitological response to treatment, 62% (52 of 84) and 38% were classified as cured and treatment failures respectively after PCR correction by *msp-2* (Table **1**). Mean parasite clearance time (PCT) in the children cured with CQ was 3.03 ± 0.41 days, while the mean fever clearance time (FCT) was 2.12 ± 0.5 days after initiation of treatment. Parasites in the group of patients that failed treatment with CQ initially cleared but reappeared between 7 and 28 days (mean recrudescent time=15.56±6.03 days) after commencement of treatment. Infections in 26 out of 32 patients (81%) who failed treatment with CQ were classified as RI. Parasites in these patients initially cleared but reappeared between 14 and 28 days after commencement of treatment. RII level of resistance was observed only in six (19%) of the 32 patients who failed CQ therapy. Parasites in these patients cleared as well but reappeared by day 7 after initiation of treatment.

Parasites Population Structure and Complexity of Infections in Pre- and Post-Treatment Isolates

Matched sample pairs collected before and after treatment from all 32 patients who failed CQ treatment were successfully analyzed at the *msp-2* locus. Alleles were classified according to the size of PCR fragments.

Table 1. Demographic Characteristics of Patients at Enrollment and Patient Treatment Outcome to Chloroquine

	Treatment with CQ
No of patients/isolates	84
Sex	
Male (%)	43 (49%)
Female (%)	41 (51%)
Age (years)	
Mean±SD	5.41±2.94
Range	6mths-13years
Parasite count (ul^{-1})	
Geometric mean	37,451
Range	7775-150,000
In-vivo Treatment outcome	
Cured (%)	52 (62%)
Resistant (%)	32 (38%)

SD= Standard deviation.

Genotyping of these samples confirmed our previous report [30] of the presence of different allelic families of *msp-2* in parasite DNA derived from a single patient, indicating a polyclonal infection. All pre-treatment isolates were positive for the *msp-2 IC1* and/or *FC27* alleles and produced up to eight different fragment sizes (*IC1/3D7*: 390-1090 bp, *FC27*: 140-1100 bp). The estimated average number of genetically distinct parasite population as determined with *msp-2* in pre- and post-treatment isolates from these 32 patients was 5.3 and 3.8 respectively. There was a significant reduction (p=0.03) in the number of *msp-2* alleles in post-treatment isolates compared to pre-treatment isolates when infections recrudesced.

Detailed analysis of paired pre- and post-treatment isolates from these patients who failed treatment with CQ showed two categories of infection. The first group of infections consisting of 72% (23 of 32) of patient isolates that failed treatment with CQ, had identical paired PCR fragments at both *FC27* and *IC1* families of *msp-2*, indicating genuine recrudescent infections after treatment with CQ. The

second group of infections in the remaining 9 patients showed parasites similar to pre-treatment isolates and the presence of new parasite populations with different genotypes. However, infections in these patients were also considered as treatment failures.

Determination of *In-Vitro* CQ Susceptibility of Patient Isolates

Based on the WHO criteria [29] of *in-vitro* susceptibility of *P. falciparum* to CQ in 84 isolates with successful tests, 15% (13/84) had minimum inhibitory concentration (MIC) of CQ below 107nM therefore classified sensitive isolates. MIC of CQ >107nM was observed in 54% (45/84) of the isolates and classified as resistant isolates. Borderline isolates defined as MIC of 107nM were observed in 31% (26/84) of isolates. The mean CQ fifty percent inhibitory concentration (IC-50) for sensitive, borderline and resistant isolates was 6.28nM, 13.70nM and 64.25nM respectively.

Pfcrt and *pfmdr1* Polymorphisms Among Patient Isolates

Seventy-eight (78), 81 and 70 of the 84 samples were successfully amplified by PCR at locus 76 of *pfcrt,* loci 86 and 184 of *pfmdr1* respectively, and were considered for analysis (Table **2**). Sixty percent (60%) of the patients isolates successfully analyzed harbored the mutant *pfcrtT76* allele that has been associated with CQ resistance. Ten percent (10%) showed a mixed genotype, while 30% harbored the wild-type *pfcrtK76* allele. Analysis of post-treatment samples obtained from the patients who failed CQ treatment showed that isolates obtained from 58% carried the mutant *pfcrtT76* allele. None of the post treatment isolates harbored the wild-type *pfcrtK76* allele. Comparison of pre- and post-treatment samples obtained from patients who failed treatment with CQ, showed no increase in the prevalence of mutant *pfcrtT76* allele while mixed allele increased from 13% in pretreatment isolates to 42% in the post treatment isolates (Table **2**).

RFLP analysis of *pfmdr1* PCR products using *AflIII, Dra I, Dde I* and *Ase I* restriction enzymes were similarly performed [33] to detect *pfmdr1* N86Y, Y184F, S1034C and N1042D mutations respectively in isolates obtained from children with *P. falciparum* infection. Mutant *pfmdr1Y86* and *pfmdr1F184* alleles were present in 33% (27 of 81) and 14% (10 of 70) in the pre treatment isolates respectively. All isolates analyzed for S1034C and N1042D mutations harbored the wild type S1034 and N1042 alleles respectively, while 32% and 82% of the isolates harbored wild type *pfmdr1N86* and *pfmdr1Y184* alleles respectively (Table **2**). Mixed N86Y and Y184F allele were observed in 35% and 4% of the pre treatment isolates respectively. Analysis of post treatment isolates obtained from children who failed CQ treatment showed mutant *pfmdr1*Y86 and *pfmdr1*F184 alleles in 41% and 57% of the isolates respectively. The wild type *pfmdr1N86* and *pfmdr1Y184* alleles were present in 28% and 43% of the post-treatment isolates respectively. None of the post-treatment isolates harbored the mixed allele at codon 184 of *pfmdr1* gene. However 31% harbored mixed allele on codon 86 (Table **2**).

Polymorphisms in *pfmdr1* gene were clustered into 4 specific haplotypes (haplotypes I- IV). Haplotype I consisted of isolates with both mutant *pfmdr1*Y86 and *pfmdr1*F184 alleles (Y86-F184-S1034-N1042). Haplotype II consisted of isolates with wild type *pfmdr1*N86 and *pfmdr1*Y184 (N86-Y184-S1034-N1042), while haplotypes III and IV consisted of isolates with mutant *pfmdr1*F184 (N86-F184-S1034-N1042) and *pfmdr1*Y86 (Y86-Y184-S1034-N1042) respectively (Table **3**). In pre-treatment isolates, the *pfmdr1* haplotype I and II were observed in 7% and 17% of the isolates respectively. Haplotypes III and IV were observed in 12% and 64% of infections respectively (Table **3**). Comparative analysis of pre- and post-treatment isolates showed a significant and strong selection ($p=0.007$; $\chi^2=7.13$) of the *pfmdr1* haplotype I in post treatment samples obtained from patients who failed CQ treatment (Table **3**). Haplotypes II, III and IV were found in 4%, 19% and 46% of the post treatment isolates respectively (Table **3**).

Table 2. Frequency and Prevalence of *pfcrt* and *pfmdr1* Alleles (At Enrollment) in Peripheral Blood Obtained from Children with Acute Uncomplicated Malaria

Genes and Alleles	Frequency	Prevalence (%)
K76T *Pfcrt* (n=78)		
K76[a]	23	30
T76[b]	47	60
K76+T76	8	10
N86Y *pfmdr1* (n=81)		
N86[a]	26	32
Y86[b]	27	33
N86+Y86	28	35
Y184F *pfmdr1* (n=70)		
Y184[a]	57	82
F184[b]	10	14
Y184+F184	3	4

Pfcrt: *P. falciparum* CQ resistance transporter and *pfmdr1*: *P. falciparum* multiple drug resistance 1 gene
[a]-wild type/CQ Sensitive allele; [b]- Mutant/CQ Resistant allele.

Assessment of the combination of *pfcrt* alleles at codon 76 with *pfmdr1* haplotypes in pre and post-treatment samples obtained from patients showed that the wild type *pfcrtK76* allele+any *pfmdr1* haplotypes was only present in 27% (22/82) of the pre treatment isolates. Mutant *pfcrtT76*+ *pfmdr1* haplotype I (Y86-F184-S1034-N1042) was present in 2% (2/82) and 31% (8/26) of pre and post-treatment isolates respectively. Interestingly, the mutant *pfcrt*T76 allele combined with *pfmdr1* haplotype IV (Y86-Y184-S1034-N1042), was observed in 50% and 46% of all pre- and post-treatment isolates respectively. None of the post treatment isolates obtained from patients that failed CQ treatment harboured the wild type *pfcrtK76* allele+any *pfmdr1* haplotype (Table **4**).

Association between *In-Vitro* Chloroquine Susceptibility and Gene Polymorphisms

The *in-vitro* sensitivity profile according to WHO criteria showed that 45 out of 84 isolates were resistant to CQ. Correlation analysis between CQ *in-vitro* susceptibility and gene polymorphisms showed that there was a significant associa-

Table 3. Mutations Patterns and Prevalence in *pfmdr1* Gene in Isolates of *P. falciparum* Obtained from Children with Uncomplicated Malaria at Enrollment and after Treatment with CQ

*PFMDR1*CODONS							
Haplotypes	**86**	**184**	**1034**	**1042**	**Pre-Treatment (%) [n=70]**	**Post-Treatment (%) [n=26]**	**P Values (χ^2)**
I	**Tyr (Y)**	**Phe (F)**	Ser (S)	Asn (N)	5 (7%)	8 (31%)	0.007* (7.13)
II	Asn	Tyr	Ser	Asn	12 (17%)	1 (4%)	0.17
III	Asn	**Phe**	Ser	Asn	8 (12%)	5 (19%)	0.5
IV	**Tyr**	Tyr	Ser	Asn	45 (64%)	12 (46%)	0.1

Mutant allele in boldface; Tyr (Y)= Tyrosine; Asn (N)= Asparagine; Phe (F)= Phenylalanine; Ser (S)= Serine; * P value statistically significant.

Table 4. Prevalence of *pfcrt* and *pfmdr1* Polymorphisms in Pre-Treatment and Post-Treatment Isolates Obtained from Children Infected with *P. falciparum*

Alleles of Genes and Haplotype	Pre Treatment (%) n=82	Post Treatment (%) n=26
Pfcrt K76+any *pfmdr1* haplotype	22 (27)	(0)
PfcrtT76+pfmdr1 haplotype I	2 (2)	8 (31)
PfcrtT76+pfmdr1 haplotype II	12 (15)	1 (4)
PfcrtT76+pfmdr1 haplotype III	5 (6)	5 (19)
PfcrtT76+pfmdr1 haplotype IV	41 (50)	12 (46)

n= Number of isolates.

tion between *in-vitro* CQ resistance and mutant *pfcrtT76* (p=0.013; OR=3.553; 95%CI=1.278-9.875), wild-type *pfmdr-1Y184* (p=0.029; OR=0.203; 95%CI=0.05-0.818), *pfmdr1*-Y86-Y184-S1034-N1042 haplotype (p=0.004; OR=4.133; 95%CI=1.555-10.99), mutant *pfcrt*T76+*pfmdr1Y86* (p=0.028; OR=2.706; 95% CI=1.103-6.635), mutant *pfcrtT76+ pfmdr1*-Y86-Y184-S1034-N1042 (p=0.046; OR=2.464; 95%CI= 1.009-6.002). There was a strong association (p=0.011; OR=0.27; 95%CI=0.096-0.764) between the presence of the *pfcrt*K76 allele and CQ sensitivity despite the presence of any *pfmdr1* haplotype (Table **5**).

Association between Gene Polymorphisms and Patient Treatment Outcome

Univariate analyses showed that there was a significant association between CQ treatment failure and the presence of *pfmdr1*Y86-Y184 haplotype (p=0.022; OR=3.36; 95%CI= 1.157-9.759) or mutant *pfcrt*T76+*pfmdr1*Y86-Y184 haplotype (p=0.024; OR=2.864; 95%CI=1.138-7.209) (Table **6**).

Association between *In-Vitro* CQ Susceptibility and *In-Vivo* Treatment Outcome

The correlation between *in-vitro* susceptibility profiles of *P. falciparum* isolates obtained from patients and responses of infection to CQ showed that among the 32 patients who failed CQ treatment, 22 were resistant to CQ *in-vitro*, while 10 (31%) had parasites that were sensitive to the drug. *P. falciparum* isolates obtained from 23 (44%) of the 52 patients who were cured with CQ, showed a resistant profile to CQ *in-vitro*. A significant association (p=0.029; RR=2.774; 95%CI= 1.098-7.005) was observed between *in-vivo* treatment outcome and *in-vitro* sensitivity of *P. falciparum* isolates to CQ.

DISCUSSION

This study has demonstrated high level *P.falciparum* resistance to CQ *in-vitro* and *in-vivo* in Ibadan, Southwest Nigeria. *In-vitro* and *in-vivo* CQ resistances were 54% and 38% respectively. The current level of *in-vivo* resistance (38%) is apparently lower compared to 51% [24-26] reported previously from the same study site. This sharp reduction in the level of *in vivo* CQ resistance may be explained by the 2005 antimalarial treatment policy change from CQ to artemether-lumefantrine or artesunate-amodiaquine as first lines treatments for acute uncomplicated malaria in Nigeria, although, chloroquine has not been completely withdrawn, despite the change in drug policy. It has been argued that the withdrawal of CQ or SP as first line treatment for uncomplicated malaria in other disease endemic settings of Africa has led to the reemergence of drug sensitive *Plasmodium falciparum* [8-10]. The fact that *in-vitro* CQ resistance (54%) is higher than *in-vivo* resistance (38%), may be attributed to immunopotentiation of antimalarial drugs by patients immunity, which would have helped older patients (>5years of age) clear drug CQ resistant parasites.

Table 5. Association between *pfcrt*/*pfmdr1* Mutations in *P. falciparum* Isolates Obtained from Children at Enrollment and CQ *In-Vitro* Susceptibility Profile

***In-Vitro* Susceptibility Profile**				
Alleles of Genes/Haplotypes	**Sensitive**	**Resistant**	**OR (95% CI)**	**P Value**
Pfcrt				
K76	15	8	3.553 (1.278-9.875)	**0.013***
T76	19	36		
Pfmdr1				
N86	16	10	2.590 (0.993-6.76)	0.05
Y86	21	34		
Y184	23	34	0.203 (0.050-0.818)	**0.029***
F184	10	3		
***Pfmdr1*Y86-Y184-S1034-N1042 haplotype**				
Present	15	31	4.133 (1.555-10.99)	**0.004***
Absent	20	10		
Mutants *pfcrt*T76 + *pfmdr1* Y86				
Present	14	28	2.706 (1.103-6.635)	**0.028***
Absent	23	17		
***Pfcrt*T76+*pfmdr1*Y86-Y184-S1034-N1042 haplotype**				
Present	14	27	2.464 (1.009-6.002)	**0.046***
Absent	23	18		
***Pfcrt*K76+ Any *pfmdr1* haplotype**				
Present	15	7	0.27 (0.096-0.764)	**0.011***
Absent	22	38		

*P value statistically significant; Alleles of *pfcrt* and *pfmdr1* genes are the same as described in Table **2**.

Previous reports from malaria endemic areas have shown that children over the age of 5 years who have acquired some level of immunity to *Plasmodium falciparum* can clear drug resistant parasites [26, 32-36]. The value of *in-vitro* susceptibility testing of *P. falciparum* for elucidating the epidemiology of drug resistant malaria cannot be overemphasized. This study showed a strong association (p=0.029) between resistance to CQ *in-vitro* and *in-vivo*, confirming the fact that despite the technical challenges posed by the *in-vitro* technique in disease endemic countries of Africa, it is still a very useful tool for monitoring drug resistance. To our knowledge, this study is one of the very few studies in West Africa that shows a correlation between *Plasmodium falciparum* resistance to CQ *in-vitro* and *in-vivo* resistance to the drug.

This study showed similarities in the parasite msp-2 genotype from pre and post treatment infections in 72% (23 of 32) of the children who failed CQ treatment. The rest of the isolates (9) obtained from children that failed CQ treatment showed the presence of new infections in the post treatment isolates in addition to similarities in the pre and post treatment infections. These new infections observed in the post treatment isolates are confirming the polyclonality of infections observed in this study and in previous study in the same area [24, 26, 32]. It is possible that these new genotypes represent minor parasite populations in pre-treatment isolates amplified by PCR below the threshold of ethidium bromide detection. Polyclonality of infections may also have implications in epidemiology of antimalarial drug resistance. Ten patients who failed CQ treatment in spite of the parasite sensitivity to CQ *in-vitro* is one of the discrepancies between *in-vitro* and clinical outcome. One possible reason to this may be the complexity of infection as shown by msp-2 genotyping. It is possible that pre-treatment isolates consisted of parasites with different drug susceptibilities. In this case, a major population sensitive to CQ would have been cleared by the drug leaving a minor population which is resistant to CQ.

This may be supported by the fact that 6 of these isolates have borderline *in-vitro* sensitivity to CQ and, from the msp-2 genotyping, a significant (p= 0.03) reduction was observed

Table 6. Association between *pfcrt*/*pfmdr1* Mutations in *P. falciparum* Isolates Collected from Children at Enrollment and CQ Treatment Outcome

Alleles of Genes/Haplotypes	*In-Vivo* CQ Outcome		OR (95%CI)	P Value
	Cured	Failed		
Pfcrt				
K76	16	7	1.77 (0.628-4.983)	0.277
T76	31	24		
Pfmdr1				
N86	20	6	2.778 (0.967-7.981)	0.053
Y86	30	25		
Y184	33	24	0.41 (0.102-1.661)	0.344
F184	10	3		
***Pfmdr1* Y86-Y184-S1034-N1042**				
Present	25	21	3.36 (1.157-9.759)	**0.022***
Absent	24	6		
***PfcrtT76*+*pfmdr1* Y86**				
Present	22	20	2.121 (0.856-5.258	0.102
Absent	28	12		
***PfcrtT76*+*pfmdr1* Y86-Y184-S1034-N1042**				
Present	20	21	2.864 (1.138-7.209)	**0.024***
Absent	30	12		
***Pfcrt* K76+any *pfmdr1* haplotype**				
Present	16	6	0.49 (0.169-1.427)	0.187
Absent	34	26		

*P value statistically significant.
Alleles of *pfcrt* and *pfmdr1* genes are the same as described in Table **2**.

in the average number of clones (3.8) in the post treatment isolates compared to pre treatment isolates (5.3).

Although point mutations in *pfcrt* and *pfmdr1* genes as molecular markers of CQ resistance represent valuable tools for surveillance and monitoring changes in CQ efficacy, the interplay between polymorphisms in these two genes is not fully understood, as they may account for the differences observed in different malaria epidemiological settings [37]. This study analyzed the association of mutations at codon 76 of *pfcrt* gene and codons 86, 184, 1034 and 1042 of *pfmdr1* gene with *in-vitro* and *in-vivo* CQ resistant or sensitive phenotypes. The *pfmdr1*Y184F mutation was identified in 14% (10 of 70) of isolates analyzed in this study. This point mutation has been reported to be associated with higher *in-vitro* resistance in laboratory strains [14] and field isolates from South America [38, 39]. No isolate analyzed in this study showed mutants *Pfmdr1*C1034 or *pfmdr1*D1042. This is in contrast with previous observations made in South America where these two alleles have been associated with high grade CQ resistance in isolates of *P. falciparum* [39, 40].

Correlation analysis between mutations in *pfcrt* and *pfmdr1* genes and *in-vitro* CQ susceptibility in 82 isolates for which both *in-vitro* and molecular data were available, showed a very interesting feature. Some isolates of *P. falciparum* harboring the mutant *pfcrtT76* and *pfmdr1* Y86 alleles were sensitive to CQ *in-vitro*. These finding are similar to earlier reports from Senegal [41], the Philippines [42] and South East Asia [24, 43].

The occurrence of molecular markers of CQ resistance (mutant *pfcrtT76* or *pfmdr1* Y86 alleles) in some isolates of *P. falciparum* that are CQ sensitive *in-vitro* can be attributed to either the involvement of compensating mutations in either these two genes or other *P. falciparum* genes not previously associated with modification of drug response.

In addition, a recent report from Johnson and colleagues [43] showed that the presence of a mutation at codon 163 of the *pfcrt* (S163R) gene, where a serine (S) is replaced by an arginine (R) is associated with CQ sensitivity in some isolates of *Plasmodium falciparum*, despite the presence of the

pfcrtT76 mutation. However, the role of parasites population dynamics on the detection of either the mutant or wild-type alleles of both *pfcrt* and *pfmdr1* depends on the predominant parasites populations in patients' isolates and cannot be ignored in an area of intense transmission like Ibadan, Nigeria. Further studies are needed for a better understanding of the association between *in-vitro* susceptibility of parasites to CQ, markers of CQ resistance and clinical outcome in this area of high malaria transmission.

Although there was no association between *in-vitro* CQ resistance and mutant *pfmdr1*Y86 alone, association between the double mutant *pfcrt*T76+*pfmdr1*Y86 or *pfmdr1*Y86-Y184 haplotype confirms the involvement of *pfmdr1* in CQ resistance *in-vitro*. This is in agreement with previous observations in South East Asia where *pfmdr1* Y86-Y184-S1034-N1042 haplotype is associated with CQ resistance in cultured isolate [44]. Previous reports [21, 24, 26, 45] have shown the primary role of the mutant *pfcrt*T76 as the major determinant of CQ resistance while mutation in *pfmdr1* gene plays a modulatory role in the mechanism of resistance. It is also possible that the presence of wild type *pfmdrY184* in isolates harboring mutant *pfmdr1*Y86 also confer more fitness on the parasite against CQ.

The association observed between mutant *pfcrt*T76 allele+*pfmdr1* Y86-Y184 haplotype and *in-vitro* CQ resistance in our study further confirms the role of polymorphisms in these two genes in the mechanism of CQ resistance in *P. falciparum*. This is further strengthened by one interesting observation in our study, as we show an association (p=0.011) between *in-vitro* CQ sensitivity and the presence of wild type *pfcrt*K76 allele+ any *pfmdr1* haplotype (Table **5**). This observation demonstrates and confirms the primary and important role of *pfcrt* polymorphisms, irrespective of *pfmdr1* polymorphisms and perhaps other genes in *P. falciparum* CQ resistance.

It has been difficult to establish an association between the *Plasmodium falciparum* mutations and patients' treatment outcomes in Ibadan, Nigeria [24] and in many other malaria endemic countries of Africa and Madagascar [37]. The high prevalence (60%) of the mutant *pfcrtT76* allele in pre-treatment isolates of all patients treated with CQ in this study makes it difficult for this allele to be predictive of treatment failures. This high prevalence of the *pfcrtT76* allele is consistent with rates ranging from 60% to 100% reported in other malaria endemic regions [13, 22, 23, 33, 41, 46-48].

Unlike the observations made *in-vitro*, a significant association was observed between CQ treatment failure and *pfmdr1* Y86-Y184 haplotype (p= 0.022) or mutant *pfcrt*T76+*pfmdr1* Y86-Y184 haplotype (p=0.024) (Table **6**). The significant association between *in-vivo* CQ failure and *pfmdr1* Y86-Y184 haplotype or mutant *pfcrt*T76+*pfmdr1*Y86-Y184 confirms that polymorphisms on these two genes are involved in *in-vivo* CQ resistance. The mutant *pfmdr1* Y86-F184 haplotype was observed to significantly (p=0.007) increase in post treatment isolates compared to pre treatment samples indicating a selection of the haplotype. The reasons for this selection remain unclear.

Some patients treated with standard doses of CQ cleared infections containing mutant allele of *pfcrt* and/or *pfmdr1* that have been associated with both *in-vitro* and *in-vivo* CQ resistance. The ability of such patients to clear parasites with mutant genotype may be due to acquired immunity, as previously shown in other studies [26, 32, 35].

CONCLUSIONS

Overall, this study showed that the mutant *pfcrt*T76+*pfmdr1*Y86-Y184 haplotype is associated with *in-vitro* and *in-vivo* CQ resistance and can be used to identify *Plasmodium falciprum* resistant phenotypes in isolates from Nigeria. To our knowledge, this is one of the few studies in West Africa that clearly demonstrates the role of parasites mutations in phenotypic CQ resistance *in-vitro* and *in-vivo*. Further studies in other malaria endemic countries are needed in order to validate these findings, especially, in areas where ACTs have been introduced, and there is a need to monitor the return of CQ sensitivity.

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