Published in final edited form as:

Acta Trop. 2011 December ; 120(3): 224–230. doi:10.1016/j.actatropica.2011.08.013.

***In vitro* Amodiaquine Resistance and its Association with Mutations in *pfprt* and *pfmdr1* genes of *Plasmodium falciparum* isolates from Nigeria**

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Abstract

Amodiaquine (AQ) is currently being used as a partner drug in combination with artesunate for treatment of uncomplicated malaria in most endemic countries of Africa. In the absence of molecular markers of artemisinin resistance, molecular markers of resistance to AQ may be useful for monitoring the development and spread of parasites resistance to Artesunate-Amodiaquine combination. This study was designed to assess the potential role of polymorphisms on *pfprt* and *pfmdr1* genes and parasite *in vitro* susceptibility for epidemiological surveillance of amodiaquine resistance in *Plasmodium falciparum*. The modified schizont inhibition assay was used to determine *in vitro* susceptibility profiles of 98 patients' isolates of *Plasmodium falciparum* to amodiaquine. Polymorphisms on parasites *pfprt* and *pfmdr1* genes were determined with nested PCR followed by sequencing.

The geometric mean (GM) of AQ 50% inhibitory concentration (IC-50) in the 97 *P. falciparum* isolates was 20.48nM (95% CI 16.53–25.36nM). Based on the cut-off value for AQ *in vitro* susceptibility, 87% (84) of the *P. falciparum* isolates were sensitive to AQ (GM IC-50= 16.32nM; 95%CI 13.3–20.04nM) while 13% were resistant to AQ *in vitro* (GM IC-50= 88.73nM; 95%CI 69.67–113.0nM). Molecular analysis showed presence of mutant *CVIET pfprt* haplotype, mutant *pfmdr1Tyr86* allele and the double mutant *CVIET pfprt* haplotype+*pfmdr1Tyr86* in 72%, 49% and 35% respectively. The GM IC-50 of isolates harboring the wild-type *pfprt* CVMNK haplotype+*pfmdr1Asn86* allele (3.93nM; 95%CI 1.82–8.46) was significantly lower ($p=0.001$) than those isolates harboring the double mutant *pfprtCVIET* haplotype+*pfmdr1Tyr86* allele (50.40nM; 95%CI 40.17–63.24).

Results from this study suggest that polymorphisms in *pfprt* and *pfmdr1* genes are important for AQ resistance and therefore may be useful for epidemiological surveillance of *P. falciparum* resistance to AQ.

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Keywords

Plasmodium falciparum; Amodiaquine; Resistance; mutation; *pfcr*; *pfmdr1*

Introduction

The widespread of parasite resistance to chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) has been associated with increasing childhood morbidity and mortality in sub Saharan Africa (Trape, 2001). Artemisinin based combination therapies (ACTs) are currently recommended for the treatment of uncomplicated *falciparum* malaria (WHO, 2001). Nearly all malaria endemic countries of the world have already changed their first line treatment to ACTs (WHO, 2010a). In Nigeria, one of the ACTs in use as first line treatment of uncomplicated *falciparum* is Artesunate-Amodiaquine (AS-AQ). The rationale behind the use of AS-AQ is the rapid reduction of parasite biomass and fever in patients by AS and as well reduces chances of development of amodiaquine (AQ) resistance in the parasites (WHO, 2001). However there are concerns that parasites from this area might have quickly develop resistance to AQ due to the high level of CQ resistance (Happi et al., 2006a).

Very little is known about the mechanism or epidemiology of AQ resistance since its use as a therapeutic antimalarial drug (Meshnick and Alker, 2005). *In vitro* studies have found correlation between resistance to CQ and AQ, since the two drugs are chemically related (Ochong et al., 2003). Several *in vitro* (Childs et al., 1989; Basco and Le Bras, 1993) and clinical (Bloland and Ruebush, 1996; White, 1996; Sowunmi et al., 2001; Schellenberg et al., 2002) reports have shown cross-resistance between CQ and AQ or its active metabolite, desethylamodiaquine. A tool for monitoring emergence and spread of AQ resistance is required, as its increasing use will likely increase reduced susceptibility to this drug. This tool would be useful for monitoring the development and spread of parasites resistance to AS-AQ since molecular markers of artemisinin resistance are yet to be discovered.

The molecular mechanisms of cross-resistance between CQ and AQ are yet to be addressed. However, it has been argued that because CQ and AQ are structural analogs with likely common mode of action, they are also likely to have a common mechanism of resistance (Bray et al., 1998; Ginsburg et al., 1998). Some apparent cross-resistance suggest that molecular markers linked to CQ resistance might be useful for monitoring AQ resistance (Ochong et al., 2003). Two *P. falciparum* genes namely *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*) gene which encodes the Pgh1 protein and *Plasmodium falciparum* chloroquine resistance transporter (*pfcr*) gene which encodes parasite trans-membrane protein *Pfcr*, have been reported to play important roles in CQ resistance. Point mutations in these two genes have been associated with *in vitro* CQ resistance in laboratory parasite clones or field isolates (Duraisingh et al., 1997; Fidock et al., 2000; Reed et al., 2000; Durand et al., 2001; Thomas et al., 2002; Lim et al., 2003; Folarin et al., 2008) and *in vivo* in most malaria endemic countries (Adagu et al., 1996; Djimde et al., 2001; Dorsey et al., 2001; Maguire et al., 2001; Tinto et al., 2005; Happi et al., 2006b).

In vitro tests of parasite drug sensitivity can serve as an early warning system for the emergence of drug resistance because of its relative simplicity and low cost when compared with *in vivo* assessment (WHO 2005). This study investigates the association between *in vitro* AQ resistance in *P. falciparum* and polymorphisms on CQ resistance markers (*pfcr* and *pfmdr1* genes) in isolates obtained from children with *falciparum* malaria in Nigeria.

1. Material and methods

2.1. Study site

The study was carried out at the Malaria Research Laboratory clinic, College of Medicine, University of Ibadan, Nigeria. Malaria in Ibadan is hyperendemic, where transmission occurs all year round but is more intense during the rainy season from April to October (Happi et al., 2006b).

2.2. Patients Selection, and Sample Collection

Children aged 6 months to 12 years with microscopic confirmation of *P. falciparum* malaria infections were enrolled after clinical examination 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.

Five milliliter (5ml) of venous blood was obtained from each child enrolled into the study for *in vitro* sensitivity to antimalarial drugs and cryopreservation. Finger pricked blood sample was obtained for thin and thick blood film to determine the parasite density. In addition, finger pricked blood sample was also spotted on 3MM Whatman filter paper for the molecular analysis. Thick and thin blood films prepared from finger prick were Giemsa-stained and examined by light microscopy under an oil immersion objective at X1000 magnification. Parasitemia in thick film was estimated by counting asexual parasites relative to 200 leukocytes. The parasite density was calculated assuming leukocyte count of 6000/ μ l of blood (Sowunmi et al, 1995; WHO, 2010b).

2.3. Determination of in-vitro Sensitivity of Patient Isolates to Amodiaquine

In vitro sensitivity of each patient isolate of *P. falciparum* to amodiaquine (AQ) was determined using a modification of the schizont inhibition assay (Folarin et al., 2008). Briefly, a template containing three-fold serial dilutions of the working solution of AQ (10750nM) 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 25 μ l of the drug dilutions to each plate. Two hundred microliters (200 μ l) 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 different concentrations of AQ were incubated at 37°C for 24 to 36 hours in a plexiglass chambers containing a gas mixture (5% O₂, 5% CO₂, 90% N₂). The final concentration of AQ in wells A – G ranged from 1075nM to 1.5nM.

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 microtiter 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) of AQ on *P. falciparum* isolates were calculated and scatter plots were performed using GraphPad® Prism version 4.0 for windows software (GraphPad® software, San Diego, LA, USA, www.graphpad.com).

P. falciparum isolates with AQ IC-50 $\leq$ 60nM was considered sensitive to AQ while IC-50 $>$ 60nM was considered AQ resistant isolates (WHO 1997; Basco et al., 2002; Ferreira et al., 2007).

2.4. DNA extraction

Parasite genomic DNA was extracted from filter paper blood samples using saponin lysis with phenol-chloroform extraction method (Snounou et al., 1993). Briefly, 1X PBS with 0.05% saponin was added to small pieces of blood impregnated filter paper and incubated at 37°C for two hours. The Supernatant was discarded and lysis buffer (40mM of Tris-HCl pH 8.0, 80mM of EDTA, pH 8.0, 0.2% SDS) with 150µL Proteinase K was added to the pellet. The pellet in the lysis buffer was incubated at 37°C for two hours. After digestion, phenol-chloroform v/v was added to the sample and centrifuged for DNA purification. The supernatant was discarded and the pellet re-suspended in 50µL of milliQ-distilled water.

2.5. PCR detection of mutations on codons 72–76 and 86 of *pfprt* and *pfmdr1* genes in *Plasmodium falciparum*

Polymorphisms around codons 72–76 *pfprt* haplotype and 86 of *pfmdr1* genes were analyzed. Other loci on *pfmdr1* gene were not analyzed because previous reports (Happi et al., 2006a; Happi et al., 2009) in the same study site have shown the rarity/absence of other *pfmdr1* polymorphisms in isolates from Ibadan, Nigeria. Thus, region of the parasite genomic DNA spanning codons 76 and 86 of *pfprt* and *pfmdr1* genes respectively were amplified by nested PCR according to the method of Djimde and others (Djimde et al., 2001) in SN5333 Master cycler (Eppendorf, Germany). The PCR reaction consisting of 1X PCR buffer (10mM Tris-HCl PH 9.0, 50mM KCl), 250uM dNTPs, 1.5mM MgCl₂, 50pM each of forward and reverse primers and 2U of Taq polymerase was performed in final volume of 25µl. Two microliters (2µl) of the genomic DNA was used as template for the primary amplification while 1µl of the primary PCR product was used for the nest PCR. DNA from two laboratory adapted *P. falciparum* clones, 3D7 (Wild-type) and Dd2 (mutant) were used as negative and positive controls respectively for K76T polymorphism on *pfprt* gene. The K1 and 7G8 laboratory strains of *P. falciparum* were used for negative and positive controls respectively for N86Y polymorphism on *pfmdr1* gene.

2.6. Direct DNA sequence analysis

The nested PCR amplification products for each gene were purified using Wizard SV Gel and PCR Clean-Up System kit (Promega, UK) and sequenced directly on an ABI PRISM® 3100-Avant Genetic Analyzer with BigDye Terminator v3.1 Cycle Sequencing kit according to the manufacturer's instructions. The sequence products were analyzed for polymorphisms using 3D7 sequence obtained from PlasmoDB (www.Plasmodb.org) as control in BLAST analysis.

2.7. Data Analysis

For each isolate, the number of schizonts with three or more nuclei over a total of 200 WBC was counted. The IC-50 is defined as the drug concentration able to inhibit 50% of the parasite growth compared with drug free control wells. Data were expressed as the geometric mean IC-50 (GM IC-50) and the 95% confidence intervals were computed. Data were entered in SPSS 16.0. The proportions of isolates displaying AQ resistance were compared by the χ^2 test for trend. The GM IC-50 for each of the genotype was compared for significant difference using the Epi Info 6.0. A value of $p < 0.05$ was considered statistically significant.

The sequence analysis was performed using FinchTV (Geospiza) for sequence chromatogram visualization and the mutations were localized using Mutation Surveyor (Softgenetics). The sequence of 3D7 was used as reference and was obtained from PlasmoDB (<http://plasmodb.org/plasmo/>).

3. Results

3.1. In vitro susceptibility of *P. falciparum* isolates to Amodiaquine

In vitro susceptibility testing for AQ was successful in 97 of 107 (91%) of *P. falciparum* isolates analyzed. The geometric mean of parasite density at presentation was 38650 parasites/ul blood (95%CI 32333–46204).

In vitro susceptibility testing of *P. falciparum* isolates showed a wide range of response to AQ with IC-50 ranging from 1.25nM to 183.20nM (Fig 1).

Based on the IC-50 threshold for AQ *in vitro* resistance (WHO 1997; Basco et al., 2002; Ferreira et al., 2007), 87% (84 of 97) of the *P. falciparum* isolates had IC-50 < 60nM (GM IC-50=16.32nM; 95%CI 13.3–20.03nM) and were therefore considered AQ *in vitro* sensitive. Thirteen percent (13%) of the isolates analyzed showed IC-50 > 60nM (GM IC-50= 88.73nM; 95%CI 69.67–113nM) and were classified as AQ resistant.

3.2. Prevalence of *pfprt* and *pfmdr1* polymorphisms in *P. falciparum* isolates

Nested PCR followed by direct DNA sequencing was used to detect polymorphisms around codons 72–76 and 86 of *pfprt* and *pfmdr1* genes respectively. Of the 107 parasites genomic DNA sequenced for polymorphisms, 69 and 95 were successful for *pfprt* and *pfmdr1* genes respectively. Sequence analysis of *pfprt* haplotype (codons 72–76) showed the presence of wild-type CVMNK *pfprt* haplotype in 23% of the *P. falciparum* isolates analyzed. Mutant CVIET and CVMNT *pfprt* haplotypes were present in 72% and 5% of the isolates respectively. Wild-type *pfmdr1*Asn86 allele was present in 51% (49/95) of the isolates, while the mutant *pfmdr1*Tyr86 allele was present in 49% of parasites analyzed.

The wild type CVMNK *pfprt* haplotype+*pfmdr1*Asn86 allele was present in 12% (8/69) of the *P. falciparum* isolates while 35% harbored the mutant CVIET *pfprt* haplotype +*pfmdr1*Tyr86. Four percent (4%) of the parasite isolates analyzed harbored the CVMNT *pfprt* haplotype+*pfmdr1*Asn86 allele while only one (1) isolates harbored the CVMNT *pfprt* haplotype+*pfmdr1*Tyr86

3.3. Association between the presence of *pfprt* and *pfmdr1* polymorphisms and reduced AQ *in vitro* susceptibility

The AQ GM IC-50 in 48 *P. falciparum* isolates harboring wild-type *pfmdr1*Asn86 allele was 11.97nM (95%CI 9.69–17.0nM). All of these isolates were sensitive to AQ *in vitro*. Mutant *pfmdr1*Tyr86 allele was present in 47 *P. falciparum* isolates and showed increased IC-50. Of these 47 isolates, 72% (34) were sensitive to AQ *in vitro* (GM IC-50 26.18nM; 95%CI 19.91–34.42nM), while 28% (13) were resistant to the drug (GM IC-50=88.73nM; 95%CI 69.67–113.0nM) [Fig 2a]. AQ resistant parasites harbouring the *pfmdr1*Tyr86 allele showed significantly higher IC-50 (p=0.0001) compared to sensitive ones (Table 1).

Fifteen (15) isolates harbored the wild-type *pfprt* CVMNK haplotype and were all sensitive to AQ *in vitro* (GM IC-50= 5.65nM; 95%CI 3.37–9.48nM). Mutant *pfprt* CVMNT (GM IC-50=11.20nM; 95%CI 2.30–27.30nM) and CVIET (GM IC50=30.21nM; 95%CI 24.39–37.42nM) haplotypes were found in 4 and 50 isolates respectively. All the four isolates with the mutant *pfprt* CVMNT were sensitive to AQ *in vitro*, while 16% (8/50) with CVIET haplotype were resistant to AQ (GM IC-50=81.19nM; 60.06–109.8nM) [Fig2b].

Correlation analysis carried out between the presence of double mutant CVIET *pfprt* haplotype+*pfmdr1*Tyr86 allele and parasite susceptibility to AQ showed increased IC-50 (GM IC-50=50.40nM; 95%CI 40.17–63.24nM) in 24 isolates harboring these *pfprt* and *pfmdr1* mutations. Sixty six percent (16/24) of these double mutants (CVIET *pfprt* +

*pfmdr1*Tyr86) were sensitive to AQ *in vitro* (GM IC-50=39.55nM; 95% CI 31.98–48.92nM) while 34% (8/24) were resistant to AQ (GM IC-50=81.19nM; 95% CI 60.06–109.8nM) [Fig 2c]. Eight (8) *P. falciparum* isolates harbored the double wild-type CVMNK *pfcr* haplotype + *pfmdr1*Asn86 allele and were highly sensitive to AQ *in vitro* (GM IC-50=3.93nM; 95% CI 1.82–8.46nM) [Fig 2c]. All isolates harboring either mutant CVIET *pfcr* haplotype + wild-type *pfmdr1*Asn86 (26) or wild type CVMNK *pfcr* haplotype + mutant *pfmdr1*Tyr86 (7) allele were sensitive to AQ (GM IC-50=18.88nM; 95% CI 14.68–24.30nM and GM IC-50=8.56nM; 95% CI 4.00–18.31nM respectively) [Fig 2c]. However, there was a significant difference ($p < 0.005$) between the GM IC-50 of isolates harboring the *pfcr* wild-type CVMNK haplotype + *pfmdr1*Asn86 allele and those with mutant *pfcr* CVIET haplotype + *pfmdr1*Tyr86 allele (Fig 2c). Three of the four isolates harboring the CVMNT *pfcr* haplotype also harbored the wild-type *pfmdr1*Asn86 (GM IC-50=9.63nM), and one had the CVMNT *pfcr* haplotype + mutant *pfmdr1*Tyr86 allele (IC-50= 17.60nM) [Fig2c].

4. Discussion

This study demonstrates increasing trend of *in vitro* resistance of *Plasmodium falciparum* to AQ and the association between parasites' *in vitro* reduced sensitivity to AQ and polymorphisms on *pfcr* and *pfmdr1* genes in a clinical setting of Southwest Nigeria.

As many countries have adopted the use of ACTs to increase treatment efficacy and to delay the emergence of drug resistant parasites, monitoring parasites' susceptibility to the individual components of the combinations is therefore necessary in order to provide evidence based signs of resistance to components of ACTs. This monitoring can be done either by *in vitro* susceptibility testing or molecular markers. In this study, we demonstrated that 13% of *P. falciparum* isolates collected from children were resistant to AQ with an IC-50 >60nM according to the threshold suggested by the WHO guideline (WHO, 1997). Although *in vitro* response cannot be directly extrapolated to predict *in vivo* response, a similar level of *in vivo* resistance (13%) to AQ was previously reported in an AQ clinical efficacy study from the same area (Happi et al., 2006a). Similar reports have been made in other malaria endemic areas of Africa (Holmgren et al., 2006). The high level of *in vitro* parasites resistance to AQ as shown in this study could be due to the extensive use of CQ and the selection of chloroquine resistant parasites in the study area over the past 50 years. Cross-resistance between CQ and AQ have been observed in other malaria endemic areas (Olliaro and Mussano, 2003) of Africa. Cross-resistance between chemically similar drugs may be a source of concern for the long-term viability of AQ as a component of ACTs, especially in areas where CQ has been used extensively and CQ resistance has been reported.

Assessing polymorphisms in potential candidate genes of drug resistance is of utmost relevance to establish their role in modulating drug responses and to predict epidemiological dynamics of resistance. A high prevalence of the mutant *pfmdr1*Tyr86 alleles and CVIET *pfcr* haplotype were observed in the *P. falciparum* isolates analyzed. This is consistent with rates (60–100%) observed in previous study from the same area (Happi et al., 2006b) and other malaria endemic areas (Djimde et al., 2001; Johnson et al., 2004; Anderson et al., 2005; Holmgren et al., 2006; Nsobia et al., 2007). This high baseline prevalence of CQ resistance associated alleles are also in line with report from East and west Africa, where there has been long use of CQ (Sidhu et al., 2002) leading to selection of these alleles in the parasite population.

The GM IC-50 of *P. falciparum* isolates with mutant CVIET *pfcr* haplotype was observed to be 5 fold higher than that observed in isolates with wild-type CVMNK *pfcr* haplotype (Fig 2b). The significant difference ($p < 0.05$) in the GM IC-50 suggests that mutations at

condons 72–76 of *pfcr* may be primarily important in reducing the susceptibility of *Plasmodium falciparum* to AQ. This is supported by the fact that all the 15 *P. falciparum* isolates with the CVMNK wild-type *pfcr* haplotype are sensitive to AQ *in vitro* (Table 1). The presence of mutant CVIET *pfcr* haplotype allele in *P. falciparum* isolates with significantly high IC-50 further confirmed its primary involvement parasites reduced susceptibility to AQ, while other SNPs on the same gene or other *P. falciparum* genes may be important in modulating the resistance of the parasite to AQ. The mutant CVIET haplotype has been reported to have emerged from Southeast Asia and spread to Africa (Anderson et al., 2005), and has also been suggested to confer some level of cross resistance to AQ/DEAQ *in vitro* (Warhurst, 2003). Molecular studies have shown that SVMNT haplotype is sufficient to confer resistance to AQ/DEAQ (Sidhu et al., 2002), however this allele is only detected in parasite population from South America, Papua New Guinea and only recently in Tanzania where AQ monotherapy has been widely used due to high sulphadoxine-pyrimethamine failure (Alifrangis et al., 2006). It can therefore be suggested that this haplotype might be selected by artesunate-amodiaquine combination in malaria endemic areas where this ACT is been used for treatment of acute uncomplicated malaria.

Our study showed the presence of the mutant CVMNT *pfcr* haplotype in Nigerian isolates of *Plasmodium falciparum*. Although all the isolates harboring this haplotype are sensitive to AQ *in vitro*, their GM IC-50 is 2 fold higher than those with CVMNK haplotype. It is likely that reduced susceptibility to AQ is a stepwise process whereby polymorphisms accumulate first on codon 72–76 stretch of the *pfcr* gene, and followed by additional mutations in the same gene or in other parasite genes including *pfmdr1*. A proposed AQ/DEAQ resistance pathway suggest a drive from the wild-type CVMNK *pfcr* haplotype via CQ resistance associated *pfcr* haplotype CVIET (Ariey et al., 2006) and then via further selection or import to the putatively DEAQ resistance associated *pfcr* haplotype SVMNT (Alifrangis et al., 2006; Holmgren et al., 2007). It is also possible that the CVMNT haplotype is first selected from the CVMNK haplotype and followed by CVIET. It is however not clear if SVMNT will be selected in *P. falciparum* isolates from Africa as AQ pressure increases.

In addition to *pfcr* polymorphisms, observation from this study showed that the N86Y polymorphism on *pfmdr1* gene might play a modulating role in reducing the susceptibility of parasites to AQ. *P. falciparum* isolates harboring the mutant *pfmdr1*Tyr86 allele showed a 3-fold increase and a significantly high GM IC-50 compared to isolates with wild-type *pfmdr1*Asn86 allele. Previous studies from the same study site have shown not only the rarity of other *pfmdr1* polymorphisms (at codons 184 and 1246) in *Plasmodium falciparum* isolates (Happi et al., 2009), but also the selection and modulatory role of the *pfmdr1* N86Y mutation in AQ resistance (Happi et al., 2006b). Other studies in other part of Africa have reported the selection of Y86-Y184-Y1246 *pfmdr1* haplotype in patients who failed treatment with AQ (Nsobya et al., 2007; Humphreys et al., 2007). Interestingly, a study from Madagascar (Rason et al., 2007) has reported highly CQ and AQ sensitive *Plasmodium falciparum* isolates with mutant *pfmdr1*Tyr86, without the mutant *pfcr*Thr76 allele. Overall, these reports from malaria endemic areas of Africa confirm the primary and modulatory roles of mutation(s) in *pfcr* and *pfmdr1* genes respectively in AQ resistance.

It has been suggested that since AQ and CQ have similar chemical structures and likely common mode of action (Bray et al., 1998; Ginsburg et al., 1998), these two compounds may have similar molecular mechanisms of resistance (Happi et al., 2006b). Our data show that the double mutant CVIET *pfcr* haplotype+*pfmdr1*Tyr86 allele that has been severally reported to be good CQ resistance marker (Bray et al., 1998; Fidock et al., 2000; Djimde et al., 2001; Sidhu et al., 2002; Happi et al., 2006a) is also associated with reduced susceptibility/resistance to AQ in *P. falciparum*. These observations are also similar to

results from Colombia (Echeverry et al., 2007). Although, it is also obvious from this study and other previous reports (Holmgren et al., 2007) that additional polymorphisms in these two genes or other genes may be involved in reduced parasite sensitivity to AQ. There is an urgent need for the identification and evaluation of these *Plasmodium falciparum* polymorphisms.

A regular monitoring of the *in vitro* drug response and/or molecular markers is warranted for surveillance of drug resistance especially in this current era of ACTs. *In vitro* drug susceptibility testing is a laboratory tool that can determine the parasite's response to individual drugs without interference of host factors. A major technical limitation of the *in vitro* testing is that the parasite phenotype is largely determined by the predominant parasite population as infections in this area is mostly polyclonal as reported in previous studies from the same area (Happi et al., 2003, 2004). If equal proportions of drug susceptible parasites and drug resistant parasites are tested, the resulting IC-50 may tend to reflect the response of the resistant parasites. Despite this limitation, *in vitro* drug susceptibility still remains an important component of research on surveillance of drug resistance.

Molecular markers have proven to be very useful and effective in predicting drug resistance in many settings of Africa and even in situation of epidemics (Djimde et al., 2004). Although, there are still variations in the predictive value of the molecular markers of resistance to antimalarial drugs in some peculiar areas of Africa like Madagascar (Rason et al., 2007).

In conclusion our study shows increase in parasites with reduced susceptibility/resistance to AQ *in vitro* in Southwestern Nigeria. The association observed between AQ *in vitro* resistance and double mutant *CVIET pfert* haplotype+*pfmdr1*Tyr86 allele may contribute to understanding the long known cross-resistance observed between AQ and CQ and could be useful for monitoring the spread of AQ resistance even as it is used as a partner drug with artemisinin derivatives.

Acknowledgments

The authors thank all the patients, their parents or guardians for volunteering to participate in the study and our laboratory staff Mr M.O. Olatunde for assistance with the study.

This study was supported by the NIH/Fogarty International Centre, The European Union Developing Countries Clinical Trial Partnership (EDCTP), the UNICEF/UNDP/World Bank/WHO/TDR, and the Multilateral Initiative for Malaria in Africa (MIM)/TDR. Christian T. Happi is the 2011 recipient of the Exxon-Mobil Corporation Malaria Research Leadership Award and is supported by the EDCTP Grant Award no. TA2007/40200016 for Senior Research Fellowship; the Fogarty International Research Collaboration Award (FIRCA) no. NIH RO3TW007757-03 and the UNICEF/UNDP/World Bank/WHO/TDR Grant ID A50337. Grace O Gbotosho is supported by the MIM/TDR project ID A20239.

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Research Highlights

- There is increasing trend in reduced susceptibility of *P. falciparum* to Amodiaquine.
- Double mutant CVIET *pfcr1* haplotype+*pfmdr1*Tyr86 allele is associated with AQ resistance *in vitro*.
- These may serve as epidemiological tool for monitoring the spread of AQ resistance as it is a partner drug in the ACTs.
- There is a need to evaluate the role of other SNPs on these genes or other genes in AQ resistance

Fig 1.
Scatter plot of Amodiaquine IC-50 of *Plasmodium falciparum* isolates obtained children with uncomplicated malaria infection

Fig 2.
Distribution of Amodiaquine IC-50 of *P. falciparum* isolates and the *pfmdr1N86Y* genotype (a), *pfcr1* haplotype (b) and *pfcr1* haplotype+*pfmdr1N86Y* genotype (c)

Table 1

Geometric mean IC-50 of Amodiaquine sensitive and resistant *P. falciparum* isolates and *pfprt* and *pfmdr1* polymorphisms

Gene/Allele/Haplotype	<i>In vitro</i> amodiaquine sensitive isolates (n)	<i>In-vitro</i> Amodiaquine resistant isolates (n)	p-value
<i>Pfmdr1</i> Asn86	11.97nM (48)	-	-
<i>Pfmdr1</i> Tyr86	26.18nM (34)	88.73nM (13)	0.0001
C72-V73-M74-N75-K76 (CVMNK)	5.65nM (15)	-	-
C72-V73-M74-N75-T76 (CVMNT)	11.20nM (4)	-	-
C72-V73-I74-E75-T76 (CVIET)	25.03nM (42)	81.19nM (8)	0.0001
CVMNK+ <i>pfmdr1</i> Asn86	3.93nM (8)	-	-
CVMNK+ <i>pfmdr1</i> Tyr86	8.56nM (7)	-	-
CVMNT+ <i>pfmdr1</i> Asn86	9.63nM (3)	-	-
CVMNT+ <i>pfmdr1</i> Tyr86	17.6nM (1)	-	-
CVIET+ <i>pfmdr1</i> Asn86	18.88nM (26)	-	-
CVIET+ <i>pfmdr1</i> Tyr86	39.55nM (16)	81.19nM (8)	0.0001

Lys–Lysine; Asn–Asparagine; 72–76–Aminoacid positions on *pfprt*; CVMNK–Cysteine, Valine, Methionine, Asparagine, Lysine; CVIET – Cysteine, Valine, Isoleucine, Glutamate, Threonine.