Chloroquine resistance molecular markers in *Falciparum* malaria in Edo state

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**Abstract:** The epidemiology survey of *Pfcrt* and *Pfmdr 1* mutant genes in malaria infection in Edo State was carried out between June 2005 and May 2006. Five hundred and sixty one (561) subjects with a history of fever, joint pains, and bitterness of the mouth were enlisted for this study. With regard to place of residence, 229 subjects were from Edo South, 177 from Edo Central and 155 from Edo North. Genotyping of resistance markers “*Plasmodium falciparum* Chloroquine Resistance Transporter” (*Pfcrt* K76T) and *Plasmodium falciparum* Multi-Drug resistance (*Pfmdr 1*) was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). There was no statistical difference (P>0.05) in the prevalence of malaria infection among the three senatorial districts (90.8%, 94.2% and 96.1% respectively). The general prevalence of mutant *Pfcrt K76T* and *Pfmdr 1* mutant genes in the state were 21.9% and 15.1% respectively. No statistical difference existed when the prevalence of these genes was compared within the senatorial districts (P>0.05). The treatment of malaria has reached a crisis point in the sub-Sahara regions. The present findings also revealed a relatively high prevalence of these resistance genes in Edo State, underlining the need for urgent policy intervention before the situation escalates beyond control.

**Key words:** Prevalence, *Pfcrt K76T*, *Pfmdr 1*, mutant, *falciparum* malaria

**INTRODUCTION**

Malaria parasite resistance to the drug chloroquine (CQ) poses a severe and increasing public health threat. This inexpensive and widely consumed drug has been the main line of attack against the parasite, and its increasing failure accompanies a return of malaria-related morbidity and mortality levels not seen for decades [1]. The problem is most acute with regard to *Plasmodium falciparum* malaria, the species responsible for the most severe form of the disease.

In human erythrocytes, *P. falciparum* supports its growth by taking up host cell cytoplasm in an acidic digestive food vacuole [2]. Toxic heme, in its hematin (u-oxodimer) form, is released in the vacuole by hemoglobin digestion and crystallized into innocuous hemozoin, or malaria pigment. CQ is supposed to interfere with this process by complexing with hematin [3], thereby creating toxic complexes that cause parasite death. [4] identified the *Pfcr* (P. falciparum chloroquine resistance transporter) gene. CQ resistance is associated with a T76 mutation of the *P. falciparum* chloroquine resistance transporter gene (*pfcr*) [4], while a multidrug resistance analogue (*Pfmdr 1*) Y86 variation may modulate its degree [5]. Although the association of Pfcr alleles with CQ resistance in vitro and in vivo is evident, the role of other genes, such as the multidrug resistance gene *Pfmdr 1* [6], is less clear. The *Pfmdr 1* gene encodes an ATP-dependent trans-membrane protein, Pgh-1, that has also been localized to the parasite’s digestive vacuole [7]. Evidence from different studies has sometimes shown associations between CQ resistance and *Pfmdr 1* copy number or mutations [8].

This study aimed at establishing the prevalence of *Pfcr* K76T and *Pfmdr 1* 86Y mutation in the three senatorial districts of Edo State.

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MATERIALS AND METHODS

Area of the study

This study was carried out in the three senatorial zones of Edo State, namely Edo South, Edo Central and Edo North. Lying between longitude 05°4’E and 06°43’E and latitude 05°44’N and 07°34’N, Edo State has a typical climate characterized by two distinct seasons - the wet and dry seasons. The wet season occurs between April and October with a break in August and an average rainfall ranging from 150cm in the extreme north to 250cm in the south. The dry season lasts from November to April with a cold harmattan between December and January. Temperature averages about 25°C in the wet and 28°C in the dry season. The relatively poor hygienic and climatic conditions prevailing in the state enhance the development of anopheles mosquitoes, making malaria infection one of the most common causes of hospital attendance and admission.

Sample collection/analysis

DNA extraction

Fresh capillary blood samples were blotted onto filter paper. DNA was later extracted from the dried blood blots by a modified Saponin/Chelox (Sigma-Aldrich Corp., St. Louis, MO) method [9]. A total volume of 100 µl was obtained, of which 4 µl was used for the PCR.

TPCR-restriction fragment length polymorphism (RFLP) of the Pfmdr 1 and Pfcr t genes

Genotyping of the resistance markers Pfcr tK76T and Pfmdr 1 was carried out by PCR-RFLP. A fragment of the Pfcr t gene containing codon 76 was first obtained by PCR amplification using a novel “nested” approach conceived to overcome spurious PCR bands on agarose gels, thus increasing the specificity and sensitivity of amplification. Consequently, several primer pairs were designed and tested, of which only those consistently producing single PCR products were chosen for downstream analyses. The lower primer was 5’-AATAAGTTGATTTCTTCCAGAA-3’, hybridizing from positions 280 to 300 [10]. The upper primer was 5’-TGACCTCATGTTTTAACCTT-3’, hybridizing from positions 130 to 150 in the Pfcr t sequence (Genbank accession number AF495378) [9]. The PCR components, in a final volume of 25 µL, were 1.6 mM MgCl2, 640 µM deoxyribonucleotide triphosphate, 1X phosphate buffer saline, 0.3 µM of each primer, 0.5 U of AmpliTaq polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA, USA) and 4 µL of DNA samples.

For the Pfmdr 1, the PCR-RFLP assay used to distinguish between the asn-86 and ty-86 mutant was developed from that previously described by Frean et al. (1992). A semi-nested PCR was used to increase the sensitivity of the assay. In the first Nest I, primers A’-(5’-TTACCTAAGA-3’) and A/-(5’-ATTACTATg-3’) were used to amplify the region flanking codon 86. A nested primer A/-(5’TATCCATCTg-3’) was then used with A/ to amplify the PCR product in a Nest II reaction from the previous reaction mixture.

After amplification, a volume of 20 µL of PCR product was incubated overnight at 55°C with the mutation-specific restriction enzyme ApoI to detect the Pfcr t K76T mutation. In the PCR products, the DNA sequence was cleaved at the wild-type codon site (if present) into two fragments (100 and 30 bp), while the mutant allele was not cut (130 bp). An aliquot from the secondary amplification of Pfmdr 1 was also subjected to restriction digestion with the enzymes Alf III (New England Biolabs) in parallel. Alf III cuts the PCR product if a mutation exists at codon 86 tyr (TA T) to generate fragments of 190 and 110 base pairs. The digested products were separated by electrophoresis in a 2% agarose gel containing ethidium bromide, and DNA was visualized by ultraviolet transillumination.

Statistical analysis

The data generated from this study were analyzed using SPSS statistical software. The statistical formulae used were chi-square, analysis of variance (ANOVA).
and student’s t-test.

RESULTS

The findings from this study revealed that, of the 561 subjects screened for malaria parasites in Edo State using the polymerase chain reaction technique, 520 (92.6%) were infected with malaria parasites. Malaria parasitaemia was distributed among the Edo State senatorial districts as follows: 208 (90.6%) of the 229 subjects from Edo South, 166 (94.2%) of the 177 subjects from Edo Central and 149 (96.1%) of the 155 subjects from Edo North. This prevalence was not statistically different among the districts (P > 0.05) (Table 1).

The overall prevalence of $P_{fcr}$ and $P_{fmdr1}$ mutant genes (Fig 2 and 3) in the state was 21.9% and 15.1% respectively, no statistical difference existing when the prevalence was compared within the senatorial districts (P > 0.05) (Table 2).

DISCUSSION

This study revealed that most of the subjects had malaria and that a higher prevalence of malaria was evident among subjects screened with PCR than with the conventional thick film technique, although the difference was not statistically significant (P > 0.05). The high prevalence of malaria may be due to the fact that malaria is endemic in this area, individuals being regularly exposed to mosquitoes bites.

Several reports of chloroquine resistant $P. falciparum$ treatment failure rate of up to 53.6% in southeastern Nigeria

<table>
<thead>
<tr>
<th>Senatorial district</th>
<th>No. examined</th>
<th>No. infected</th>
</tr>
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<tbody>
<tr>
<td>Edo South</td>
<td>229</td>
<td>208 (90.8%)</td>
</tr>
<tr>
<td>Edo Central</td>
<td>177</td>
<td>166 (94.2%)</td>
</tr>
<tr>
<td>Edo North</td>
<td>155</td>
<td>149 (96.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>561</td>
<td>520 (92.6%)</td>
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<table>
<thead>
<tr>
<th>No. examined</th>
<th>$P_{fcr}$</th>
<th>$P_{fmdr1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>Mutant</td>
</tr>
<tr>
<td>Edo South</td>
<td>208</td>
<td>168 (77.9%)</td>
</tr>
<tr>
<td>Edo Central</td>
<td>166</td>
<td>131 (77.5%)</td>
</tr>
<tr>
<td>Edo North</td>
<td>149</td>
<td>120 (79.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>520</td>
<td>419 (78.0%)</td>
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</tbody>
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Fig. 2: Agarose gel depicting the K76 and 76T after RFLP digestion with Apo I: Lanes 2, 4, 6-8, b, d showing the K76 (Wild type) bands, Lanes 3, c, e-g showing the 76T (mutant) bands, Lanes 5 and h failed the RLFP digestion.

Fig. 3: Agarose gel depicting the Y86 and mutant type after RFLP digestion with AFLP1: Lanes 2’, 4’ and 5’, b’d and h’ showing the mutant type bands, Lanes 3, 6-8, c’, e’ f’ g’ showing the Y86 (Wild type) bands.
and up to 37\% in the southwestern part of Nigeria are available.

In line with reports on high clinical failure rates of CQ, the obtained data show that, in an area of seasonal transmission in Nigeria, \textit{P. falciparum} mutations conferring resistance to these drugs are moderate. In particular, \textit{pfcr} T76T and \textit{pfmdr} 1 mutation occurred in 21.9\% and 15.6\% respectively. [12] reported a prevalence of \textit{Pfcr} mutation as high as 100\% in Ethiopia. \textit{Pfcr} T76T, and to a lesser extent \textit{pfmdr1} Y86, are useful markers of CQ resistance in areas where prevalence is low to moderate [9, 13]. In the present study, the moderate prevalence of \textit{pfcr} and \textit{pfmdr1} mutations is consistent with the poor efficacy of CQ experienced in Benin City. The emergence of these mutant genes in the three senatorial districts of the state, namely Edo South, Edo Central and Edo North, indicates the possibility that these genes are widely distributed across the federation because Nigerian population is a mixture of people from different parts of the country. Inter-state migration may be the way in which these genes are transferred from one state or area to another, because people from different areas travel to other regions for social or business activities. Happi \textit{et al.} reported the occurrence of these genes in children treated with amodiaquine in Ibadan [14].

Considering the retraction of the drug at least from official first-line treatment, however, the perpetuation of resistant strains in the parasite population also seems possible. Unfortunately, pre-treatment drug levels could not be assessed in the present study. Nevertheless, the obtained results on CQ resistance markers may have important implications for subsequent monitoring: In areas where CQ was abandoned as a first-line antimalarial treatment, a recovery of CQ sensitivity and decrease in \textit{pfcr} mutations have been demonstrated [15, 16].

In conclusion, the emergence of \textit{P. falciparum} mutations in Benin City conferring resistance to CQ is worrisome. Our findings strongly underlines the need for vigilance and repeated surveillance of the parasites’ molecular markers as a way to monitor the development of resistance to CQ in the state.

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REFERENCES