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Prevalence of the molecular marker of chloroquine resistance (*pfcrt* 76) in Nigeria 5 years after withdrawal of the drug as first-line antimalarial: A cross-sectional study

Michael Efunshile, *BSc, MB BS, MSc*

Lagos University Teaching Hospital, Idi-Araba, Lagos, Nigeria, Ebonyi State University, Abakaliki, Nigeria, and Otto-von-Guericke University, Magdeburg, Germany

Tamramat Runsewe-Abiodun, *MB BCh, FWACP (Paed)*

Olabisi Onabanjo University, Sagamu, Ogun state, Nigeria

Beniam Ghebremedhin, *MD, MSc, PhD*

Wolfgang König, *MD, MSc, PhD*

Brigitte König *BSc, MSc, PhD*

Otto-von-Guericke University, Magdeburg

Corresponding author: *T Runsewe-Abiodun (dr_abiodun@yahoo.com)*

Background. In line with the World Health Organization (WHO) guideline on chloroquine (CQ) resistance, CQ was withdrawn as the first-line antimalarial drug in Nigeria in 2005 as a result of widespread resistance. It was expected that its sensitivity and clinical usefulness would be restored with time. This study therefore aimed to determine the level of CQ resistance in Nigerian children aged less than 60 months.

Methods. We monitored the resistance pattern 5 years after withdrawal of CQ, using the *pfcrt* K76T mutation as a molecular marker for CQ resistance.

Results. Of 98 *Plasmodium falciparum*-positive blood samples, 95 (96.9%) showed the K76T mutation. Twenty-seven (27.6%) of the children had been treated with CQ at home before presentation at the clinic, while 50 (51.0%) had taken other antimalarials.

Conclusion. Our results indicate that there is an urgent need to re-evaluate antimalarial drug policy in Nigeria, especially when 27.6% of our study population still use CQ at home despite its withdrawal as first-line antimalarial. This may require effective legislation against the manufacture, importation and use of CQ in Nigeria, if the purpose behind its withdrawal is to be achieved.

With 3 billion people at risk of infection in 109 malarious countries and territories, and with around 250 million cases annually, leading to approximately 1 million deaths, malaria is one of the leading causes of death worldwide from a single infectious disease.[1]

Malaria remains a public health problem in Nigeria, causing about 60% of all outpatient attendances and 30% of all hospital admissions. It is responsible for nearly 110 million clinical cases and an estimated 300 000 deaths per year, including up to 11% of maternal mortality. Malaria's economic impact is also enormous, with about N132 billion lost to the disease every year in the form of treatment costs, prevention, loss of working hours, etc.[1]

The World Health Organization (WHO) currently advocates the use of integrated malaria management (IMM) as the most effective tool to check the menace of malaria.[2] IMM consists of three tools: effective case management (ECM), integrated vector control (IVC) and personal protection (PP). These tools have to be applied simultaneously to produce the desired impact. ECM includes the use of effective antimalarial drugs to remove parasites from the infected human host, thereby preventing transmission to mosquitoes.[3] IVC is the use of adult mosquito killing measures such as indoor insecticide sprays and environmental management to remove the mosquito breeding sites, thus lowering the population densities of malaria vectors.[4] PP includes measures that prevent contact between man and mosquitoes, such as insecticide-treated nets, indoor residual spraying, and window and door screens.[5]

To further malaria management, the monitoring of antimalarial drug resistance is of critical importance for the national Malaria Control Programme.[6,7] Chloroquine (CQ) was withdrawn as the first-line antimalarial drug in Nigeria in 2005 because of a widespread and high-level clinical failure rate across the country. Artemisin-based combination therapy (ACT) is now the recommended antimalarial drug in the treatment of uncomplicated malaria in Nigeria. It is expected that sensitivity to CQ will be restored about 10 years after withdrawal, as has happened in other countries.[8,9] There is a need to continue to monitor the resistance pattern of this drug in order to know when it can be reintroduced.[10]

The use of molecular markers as a surveillance tool helps to predict when a drug can safely be reintroduced. CQ resistance has been linked to a number of mutations in the *Plasmodium falciparum* genes. Current evidence from transfection studies strongly suggests that mutations in the *P. falciparum* CQ resistance transporter (*pfcrt*) gene confer resistance to CQ.[11] CQ resistance is said to involve a progressive accumulation of mutations in the *pfcrt* gene, the mutation at position 76 being the last in the long process leading to CQ clinical failure.[8] According to Umar *et al.*, CQ treatment failure in *P. falciparum* infection is significantly associated with the K76T mutation and this could therefore be used as a population marker for CQ resistance in Nigeria.[12]

Methods

Study area

The study was conducted in the Paediatric Department of Olabisi Onabanjo University Teaching Hospital, Sagamu, Ogun state, Nigeria, one of the 36 states of the Federation in Nigeria. Malaria in the region is hyper-endemic, its transmission being highest during the rainy season (May - October).

Ethical issues

Written informed consent was obtained from the participants, while ethical approval for the study was granted by the ethics and scientific committee of Olabisi Onabanjo University Teaching Hospital.

Study population

Children aged 0 - 60 months with clinical features of uncomplicated malaria were recruited for the study. Children with fever from other causes were excluded.

Study design

This cross-sectional descriptive study was a subsection of a larger study aimed at comparing malaria diagnostics. The study was conducted in June - July 2009. Whattman paper blots of blood samples confirmed by blood microscopy to be positive for malaria parasites were collected for the polymerase chain reaction (PCR) assay.

Research methodology

Venous blood from all children aged 0 - 60 months with fever or a history of fever 48 hours prior to presentation was collected into EDTA bottles for malaria microscopy, antigen detection using a rapid diagnostic test kit and PCR from samples on 3 MM Whattman filter paper blood spots.

Children aged 0 - 60 months with microscopically confirmed *P. falciparum* infection were recruited into the study.

Diagnosis of malaria

By antigen detection. SD BIOLINE Malaria Antigen *Plasmodium falciparum* (Korea), a malaria rapid diagnostic test kit that detects histidine-rich protein-2 (HRP-2) specific for *P. falciparum*, was used for initial screening of blood samples at the point of sample collection.

By microscopy. Thick and thin Giemsa-stained blood smears were examined for malaria parasites at the Nigeria Institute of Medical Research (NIMR) by accredited laboratory scientists, using standard methods. The parasite density was determined by counting the number of parasite against 200 leucocytes, assuming the total leucocyte count to be 8 000 leucocytes/µl blood. The haemoglobin concentrations of the children were also measured.

By species-specific PCR for *P. falciparum* small subunit rRNA. Drops of blood were collected on filter papers each time blood was obtained for blood smear. Filter paper blots were transported from Nigeria to Germany, where DNA was extracted using a tris-EDTA buffer-based extraction method, which yielded 100 µl of DNA in buffer. Nested PCR was carried out with primers as described by Snounou *et al.*[13] Two genus-specific primers for *Plasmodium* spp. (-rPLU5-, (CCTGTTGTTGCCTTAAACTTC) and -rPLU6-, (TTAAAATTGTTGCAGTTAAAACG)) were used for the first cycle of amplification. *P. falciparum* species were detected using species-specific primers (-rFAL1-, (TTAAACTGGTTTGGGAAAACCAAATATATT) and -rFAL2-,(ACACAATGAACTCAATCATGACTACCCGTC)) in the second cycle (nested) reaction. The PCR assays were performed using the Peltier Thermal Cycler (PTC-0225) DNA Engine Tetrad (MJ Research Inc., Waltham, Massachusetts, USA).

The thermal profile was 95°C for 1 minute (5 minutes at cycle 1), 60°C for 2 minutes, and 72°C for 2 minutes for 45 cycles. A volume of 1 µl of the primary PCR product was used in the nested PCR reaction for 30 cycles. In both the first and the nested PCR, reaction volume was 25 µl. The final concentration of each reagent was 1 × PCR reaction buffer (10 × PCR buffer – $MgCl_2$), 1.5 mM $MgCl_2$, 125 µM dNTP, 250 nM primers and 0.02 U/µl Taq Polymerase (QIAGEN, Hilden, Germany). PCR products (1 100 bp and 205 bp, respectively) were visualised by gel electrophoresis and ethidium bromide staining.

Detection of *pfcrt* K76T

Nested PCR followed by restriction endonuclease digestion was used to detect *pfcrt* K76T. The first-round primers were CRTP1 (5´-ccgttaataataaatacacgcag-3´) and CRTP2 (5´-cggatgttacaaaactagttacc-3´) and the second-round primers were CRTD1 (5´-TGTGCTCATGTGTTTAAACTT-3´) and CRTD2 (5´-CAAAACTATAGTTACCAATTTTG-3´). The first round and second round of amplification yielded a 537/145 bp PCR product. Aliquots were digested with 1 unit *Apo*I, which cleaves the wild type into 111 bp and 34 bp fragments. Dd-2 strain DNA, HB-3 strain DNA and water were used as mutant-type allele, wild-type allele and negative controls, respectively. Sequencing of the 537 bp PCR product was performed randomly to confirm the results obtained by digestion.

Results

The data are summarised in Tables I, II and III. A total of 98 blood samples from 98 children infected with *P. falciparum* were analysed. The children were aged between 2 months and 60 months, mean 24.97 (standard deviation 18.9) months. The male/female ratio was 1.28:1. Sixty-eight of the participants (69.4%) had taken some form of treatment for the fever before presenting to the clinic, while 27 (27.6%) had actually taken CQ.

The *pfcrt* T76 allele was present in 95 out of the 98 samples. The prevalence of the *pfcrt* K76T mutation was therefore 96.9%.

TABLE I. AGE AND GENDER DISTRIBUTION OF THE STUDY POPULATION

	Gender	
Age (mo.)	Male (N)	Female (N)
2 - 12	24	14
13 - 24	15	10
25 - 60	16	19
Total	55	43

TABLE II. FREQUENCY OF USE OF DRUGS BEFORE PRESENTATION

Drug	Frequency (N=98)	%
CQ	27	27.6
SP	21	21.4
ACT	11	11.2
Unknown	9	09.2
Herbs	10	10.2
None	20	20.4

CQ = chloroquine; SP = sulphadoxine-pyrimethamine; ACT = artemisinin-based combination therapy.

TABLE III. PREVALENCE OF *pfcrt* K76 MUTANT STRAINS

	Presence of *P. falciparum* (*N*=98)	Presence of *pfcrt* K76 mutant strains (*N*=98)
Yes	98 (100%)*	96 (98%)
No	0	2 (2%)

*Figures in brackets are percentages of the total.

Discussion

There are basically two methods for monitoring antimalarial drug resistance: *in vivo* and *in vitro* tests. *In vivo* tests are traditionally the 'gold standard' method for detecting drug resistance. However, *in vivo* tests involve administration of a standard dose of the respective drug to a clinically ill patient with follow-up to evaluate the clinical response. Because ACT is the first-line antimalarial in Nigeria, it would be unethical to conduct *in vivo* tests with CQ, which has been confirmed to be a failed antimalarial drug in the country. Meanwhile it is accepted that the prevalence of T76 genotypes may be used for population-based surveillance of CQ resistance, at least in Nigeria.[12,14,15] The use of *pfcrt* K76T as a molecular marker surveillance tool will help to predict when these drugs can be reintroduced safely.[8,6]

CQ was withdrawn as the first-line antimalarial in Nigeria in 2005 because of widespread and high-level clinical failure across the country. It is expected that sensitivity to CQ will be restored about 10 years after withdrawal, as has happened in other countries. In this regard in Malawi the prevalence of the *pfcrt* T76 allele, which is associated with CQ resistance, declined rapidly after the withdrawal of CQ from 85% in 1992 to undetectable levels by 2001. A clinical trial later confirmed that these changes in the prevalence of resistance-mediated *pfcrt* T76 allele have been accompanied by a dramatic increase in CQ efficacy for the treatment of malaria in Malawi.[9]

CQ has many desirable attributes as an antimalarial drug: it is inexpensive, rapid acting, long acting, and safe in all age groups including children. It is an excellent drug for preventing malaria in travellers and may be an ideal candidate for intermittent preventive treatment, which has shown promise in studies involving pregnant women, infants and children. The prevalence of the *pfcrt* K76T mutation before withdrawal of CQ in south-western Nigeria was between 73.8% and 88%.[14,15] Surprisingly, 96.6% of the isolates in our study exhibited the mutated allele at codon 76 (K76T) of the *pfcrt* gene 5 years after CQ had been withdrawn as first-line antimalarial. This may be a result of weak drug control policy in the country, which allows the drug still to be freely available for use outside government hospitals. Cross-resistance between CQ and amodiaquine (AQ) may be a contributory reason, owing to the similarities in their modes of action.[6] AQ 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 the active metabolite of AQ.[16] In this regard, Ochong *et al.* could show the impact of *pfcrt* markers on *in vivo* efficacy of AQ.[17] Their data have clearly demonstrated that the *pfcrt* allele at codon 76, the most common marker for CQ resistance, is also associated with AQ resistance *in vivo*.

Conclusion

This study has demonstrated that there is still a huge level of CQ resistance in south-western Nigeria, especially among children, 5 years after the withdrawal of the drug as the first-line treatment for uncomplicated malaria.

Arising out of the study, there is an urgent need to re-evaluate the malaria treatment policy in Nigeria, and ensure effective legislation against the manufacture, importation and use of CQ in Nigeria, especially for children under 5 years old, so that the purpose behind its withdrawal can to be achieved.

Competing interests. We jointly declare that we have no competing interests.

Authors' contributions. ME: conceived the study, was part of the team that collected the blood sample in Nigeria, performed the PCR assay in Germany and contributed to the writing of the manuscript. TR: conceived the larger study, led the team for the field work, transported the blood samples to Germany and contributed to the writing of the manuscript. BG: made the RDT kits and microscope available. WK: made the RDT kits and microscope available and reviewed the write-up for professional clarity. BK: performed the PCR assay and wrote the manuscript

Disclosure. The RDT kits and microscope were provided by the Medical Microbiology Department of Otto-von-Guericke University, Magdeburg. The PCR assay was done at Otto-von-Guericke University under the aegis of a DAAD fellowship to one of the authors.

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