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Original papers

Detection of *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) mutant gene amongst malaria-infected pregnant women in Calabar, Nigeria

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ABSTRACT. Chemotherapy is the mainstay in malaria control and management. For some time, chloroquine (CQ) was a drug of choice for the treatment of malaria. It was effective against all forms of malaria, cheap and readily available. The increased resistance of malaria parasites to CQ led to widespread abandonment of the drug in African and Asian countries on the prompting of the World Health Organization. Currently, artemisinin-based combination therapy is the gold standard for the treatment of malaria. This study investigates the presence of the *Plasmodium falciparum* Chloroquine Resistance Transporter (*Pf*CRT) mutant gene, a molecular marker responsible for CQ resistance in malaria parasites. A total of 369 pregnant women were microscopically screened for malaria infection using thin and thick blood films stained with Giemsa. Subsequently, malaria parasite DNA was extracted from the blood of malaria positive participants. The *Pf*CRT gene was amplified using Polymerase Chain Reaction (PCR). A Restriction Fragment Length Polymorphism analysis of the gene was performed to confirm mutant forms. The results showed that 251 (68.0%) of the participants had *Plasmodium falciparum* in their blood. Molecular examination revealed the presence of *Pf*CRT mutant genes in 28% of the study population. Notwithstanding the decline in the prevalence of *Pf*CRT T76 mutation since the antimalarial policy change in Nigeria, the 28% prevalence recorded in this study is considered high after ten years of the withdrawal of CQ in the treatment of uncomplicated malaria.

Key words: malaria, chloroquine, artemisinin-based combination therapy, microscopy, PCR, *Pf*CRT

Introduction

Malaria is one of the most lethal diseases on the planet today. A large population of individuals in developing and tropical countries throughout Africa, Latin America and across large parts of Asia, harbour malaria. About 1.5–2.7 million people die annually from malaria infection, with children below the age of five years accounting for the highest mortality rate [1,2]. The most dangerous form of the disease, *falciparum* malaria, is endemic in Nigeria and sub-Saharan Africa [3]. Twenty-five percent of all malaria cases in Africa occur in Nigeria [4]. In Calabar, Cross River State, one of the coastal States in Nigeria, approximately 60% of all out-patient attendances and 30% of hospital admissions are due to malaria infection [5]. Malaria is hyper-endemic in the city due to heavy rains, which occur almost all year round with peak periods between July and September, and the area is covered by typical rainforest vegetation and an abundance of female *Anopheles* mosquitoes [5].

To control malaria, an integrated approach which emphasizes chemotherapy, anti-vector activities and personal protection (particularly the use of insecticide treated nets) by the secondary host is employed [6]. Of prime interest is the management of cases using chemotherapy. It is known to reduce the parasite reservoir in man, thereby preventing the transmission of the parasites to the definitive host, the mosquito [7].

However, the effectiveness of chemotherapy is hampered by the development of drug resistance by the malaria parasite [8]. Chloroquine, which was

first discovered by Hans Andersag in 1934 [9] was the mainstay in the treatment and prevention of malaria for 50 years since the inception of its use in 1947. This was due to its efficacy against all four known malaria species, low cost and toxicity, relative ease to manufacture, chemical stability (even under extreme weather conditions) and its ease of storage and handling [10]. However, within a decade of its introduction, CQ failure was first reported in Thailand. Thereafter, there have been widespread reports of CQ resistance globally, particularly in *P. falciparum* infections [11].

Resistance to CQ by *P. falciparum* strains is associated with a parasite protein named *P. falciparum* Chloroquine Resistance Transporter (*Pf*CRT), located in the digestive vacuole of the parasite. The mutated form of this gene, a point mutation which occurs at position 76, is able to reduce chloroquine accumulation in this organelle. Thus, the mutated form of this gene is considered a molecular marker for chloroquine resistance [12].

A drug therapeutic efficacy trial (DTET) conducted on chloroquine in the six geopolitical zones of Nigeria showed a widespread clinical failure rate of CQ (17.4%) [13]. Later findings from another DTET carried out in Nigeria confirmed an increase in malaria parasite resistance to CQ from a 100% success rate in 1980 to a 60% failure rate in 2002. These findings necessitated the formulation of a new National Antimalarial Drug Policy in 2005 which emphasized the withdrawal of CQ in the treatment of malaria [5]. The National Health Policy, which allowed presumptive treatment of fever in children with CQ by caregivers (mothers, guardians etc.) at home, provided fertile ground for CQ abuse [13]. This was compounded by ignorance, poverty, poor quality or readily available counterfeited CQ on the market.

Currently, artemisinin-based combination therapy (ACT) is the approved drug for the treatment of malaria in Nigeria. ACT offers high efficacy and protection against the development of resistance. However, its usage is limited by its relatively high cost, dosing complexity and toxicity [14]. In addition, there are reports of the emergence of resistance by *P. falciparum* to some ACTs, as reported by Mwai et al. [15] in Kenya.

It is critical to know when sensitivity to CQ is restored in Nigeria, because it is a cost-effective antimalarial medication compared to ACTs. Also, over-the-counter purchase of most ACTs is common practice in Nigeria without prescription. It is therefore important to explore the possibility of re-introducing previously failed antimalarial medications to avoid running out of available drugs to tackle the malaria scourge.

The aim of this study is to determine the prevalence of the *Pf*CRT mutant gene amongst pregnant women in Calabar infected with malaria, more than ten years following the withdrawal of CQ in the treatment of malaria.

Materials and Methods

This study was conducted at the University of Calabar Teaching Hospital (UCTH), located in Calabar, Cross River State, Nigeria. Calabar, the capital of Cross River state, is located at 4°34’27"N, 6°58’32"E, in the South-South geopolitical zone within the coastal rainforest region of Nigeria, and has a population of 372,848 people [16]. The majority of the population speak Efik and Ejagham dialects. Calabar has a relatively good drainage system with tarred roads and gutters in some parts of the metropolis. The vegetation is typical of the tropical rain forest which makes malaria transmission stable throughout the year but more intense between April and early October (wet season) with a peak period in June/July due to relatively heavy rain during this period which usually helps to propagate the malaria vector, resulting in malaria hyper-endemicity [5].

Sample size. Three hundred and sixty nine (369) subjects were enrolled in the study: A value derived using the formula described by Daniel [17]. A malaria prevalence rate of 43.7% earlier reported for Calabar was used to calculate the sample size [5].

Study population. Pregnant women attending the Antenatal Clinic (ANC) at the University of Calabar Teaching Hospital (UCTH) were screened for *P. falciparum* malaria infection by microscopy.

Inclusion criteria. All pregnant women attending the ANC at UCTH who gave their consent were recruited for the study. Only participants who tested positive for malaria parasite were subjected to DNA analysis.

Exclusion criteria. Those who did not give consent or were receiving antimalarial chemotherapy or prophylaxis were excluded from the study.

Administration of questionnaire. A questionnaire entitled “An assessment of the demographics of subjects used in the study” was administered to all the participants. It included a

range of questions including participant age and current usage of any antimalarial prophylaxis.

Collection of blood samples. After disinfecting the collection site and applying a tourniquet, about 2mL of venous blood was collected from each participant aseptically into EDTA tubes using a syringe and needle. The blood was then stored in a refrigerator at 2–8°C for the study.

Diagnosis of malaria infection. Participants were screened for malaria by microscopy. Using an automatic pipette, 6μL and 2μL of blood from the same participant was placed at different sites on the same slide for the thick and thin films respectively. The slide was left on a flat surface to air dry, after which the thin smear was fixed for five seconds using absolute methanol. The entire slide was then flooded with freshly prepared 3% Giemsa solution for 30 minutes, after which the stain was washed off using buffered water at pH 7.2. The films were air dried and examined under the microscope using ×100 oil immersion objectives [18]. The stained thick smear was used for detection of the presence of malaria parasite, whereas the thin film was used for species identification.

Polymerase Chain Reaction technique. Only blood samples whose malaria screening tests were positive were transported to the Molecular Biology Laboratory at the Niger Delta University, located at Wilberforce Island, Bayelsa state, for extraction of malaria parasite DNA, and the amplification and detection of the *Pf*CRT point mutations gene by PCR. The following were purchased from BEI Resources, United States of America: primers, master mix, endonuclease enzyme (APO1), 100 and 200 base pair ladder, commercially-prepared Chloroquine-Sensitive (CQS) and Chloroquine-Resistant (CQR) *P. falciparum* strains (to serve as the negative and positive controls respectively). Malaria parasite-negative blood samples were also used as negative controls for *P. falciparum*.

Malaria parasite DNA extraction. Malaria parasite DNA were extracted from blood obtained from malaria-infected individuals using Quick-gDNA MiniPrep according to the manufacturer's instructions (Zymo Research Corporation). The eluted DNA was stored at -20°C for onward amplification.

DNA quantification and quality. Following extraction, a Nanodrop-1000 spectrophotometer was used to measure the concentration of the total DNA and estimate its quality.

***Pf*CRT gene amplification.** The extracted DNA was then added to a mixture of the master mix, buffer solution, Magnesium chloride, forward and reverse primers. The DNA sequence of the forward primer used for the primary amplification process was 5–CCGTTAATAATAAATACACGCAG3– while the reverse primer was 3–GCATGTTACAA AACTATAGTTACC5–. The forward primer used for the secondary amplification process was 5–TGTGCTCATGTGTTTAAACTT3– while the sequence for the reverse primer was 3–CAAAACTATAGTTACCAATTTTG5–. Other components were Taq polymerase (obtained from the Bacillus, *Thermos aquaticus*) and polymerase water on an ice rack. The cocktail was then placed on a PCR system for gene amplification via the following steps: denaturation, annealing and extension. A nested DNA amplification was performed i.e. both primary and secondary DNA amplification.

Primary amplification. The amplification took place as follows: A single cycle of the initial denaturation process was performed at a temperature of 95°C for five minutes. This was followed by 40 cycles of the following: 94°C for 30s, 56°C for 45s and 65°C for 45s. After the denaturation process, a drop in temperature to 56°C caused the exposed single strands to serve as a template for the attachment of primers (annealing process) and the cycle continued. DNA extension was performed at 65°C for seven minutes by the thermostable polymerase (Taq polymerase), thereby resulting in the creation of several copies of the *P. falciparum* DNA.

Secondary amplification. Amplified DNA from the primary amplification process was used as a template for the secondary amplification process (Nested PCR). After an initial denaturation step at 95°C for three minutes, secondary amplification was carried out on 35 cycles (94°C for 30s, 48°C for 30s, 65°C for one minute), followed by an extension process for seven minutes at 65°C. Polymerase water (water free of DNA) was used as a template to check for possible contamination of reagents.

Restriction Fragment Length Polymorphism analysis of the *Pf*CRT gene. The *Pf*CRT gene was detected using Restriction Fragment Length Polymorphism Polymerase Chain Reaction (PCR-RFLP). APO-1 was then added to the amplified DNA fragment from the secondary amplification process and incubated at 50°C for five hours in an incubator (endonuclease activity). Using an electrophoretic apparatus, the digested and

Table 1. Prevalence rate of wild and mutant *Pf*CRT genes by age

Age group (years)	No. of malaria positive samples examined	No.(%) of wild *Pf*CRT genes	No.(%) of mutant *Pf*CRT genes
18–22	12	10 (83.3)	2 (16.7)
23–27	10	8 (80.0)	2 (20.0)
28–32	28	21 (75.0)	7 (25.0)
33–37	30	20 (66.7)	10 (33.3)
38–42	18	11 (61.1)	7 (38.9)
>42	2	2 (100.0)	0 (0.0)
Total	100	72 (72.0) χ^2= 8.54 p<0.05	28 (28.0) χ^2= 52.53 p>0.05

0 denotes that no mutant *Pf*CRT gene was detected

undigested (secondary amplification) products, 100 and 200 base pairs (bp) DNA ladder and CQS (3D7), CQR (Dd2) DNA fragments and the system check (amplification done on polymerase water used as template) were run on 2% agarose gel tinged with ethidium bromide at 110 volts for 35 minutes.

The sizes of the DNA were determined by comparison against the 100 and 200bp DNA ladder under ultraviolet transillumination. CQS strains (wild type) have a protein sequence that is receptive (restriction site) to the enzyme APO-1. This enzyme digests the 145bp nested PCR product and cuts the nucleotide sequence to give two bands (111 and 34bp). APO-1 does not recognise the nucleotide sequence on CQR strains due to the point mutation (absence of restriction site), and so does not have any effect on the nucleotide sequence, resulting in only one band being produced at 145bp.

Data analysis. The data generated in this study were analysed for level of significance using Chi square test analysis. Differences were shown to be statistically significant where $p<0.05$.

Ethical approval and consent. The study protocol was reviewed and approved by the Health Research Ethics Committee of UCTH, while written informed consent was obtained from the participants before the study began.

Results

Of the 369 subjects screened for malaria, 251 (68.0%) were positive for malaria while 70 (18.9%) were negative (Fig. 1). *P. falciparum* was responsible for all cases of parasitemia.

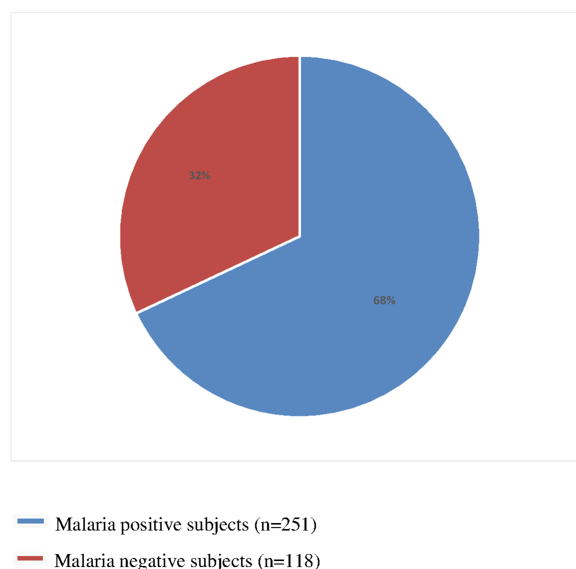

Fig. 1. Prevalence of malaria parasitaemia among pregnant women in Calabar

Figure 2 is a representative photomicrograph of some nested *Pf*CRT genes prior to digestion. N, L, 3D7 and Dd2 represent the negative control, 100bp DNA ladder, CQS and CQR genes respectively; 1, 3, 8, 10 and 11 are negative amplifications, while 2, 4, 5, 6, 7 and 9 are positive amplifications. The *Pf*CRT gene has a band size of 145 when compared against the 100bp mass ruler DNA ladder.

Figure 3 shows the band sizes of the *Pf*CRT genes after endonuclease activity. The digested amplicons and controls were run electrophoretically on 2% agarose gel treated with ethidium bromide. The band sizes of *Pf*CRT 76T (CQR) and *Pf*CRT K76 (CQS) genes were examined. As they are not

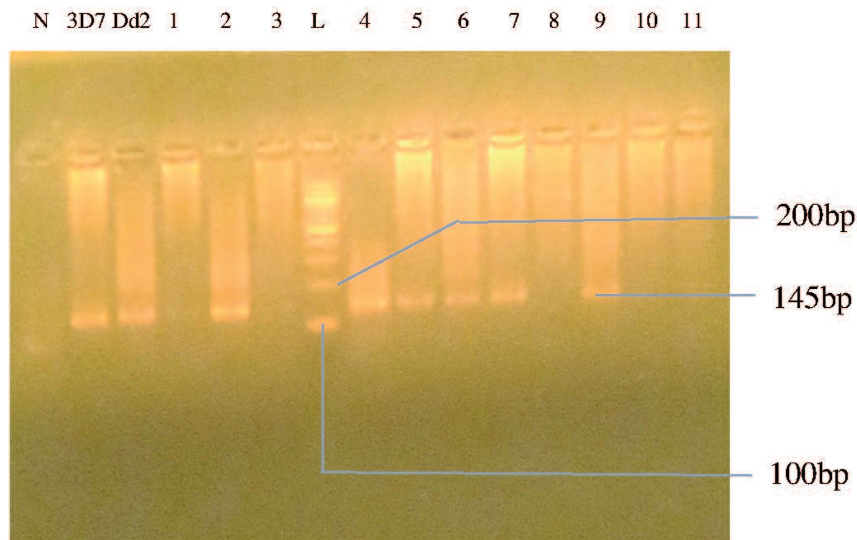

Fig. 2. Representative photomicrograph of some nested *Pf*CRT genes

digested by the APO-1 enzyme, CQR strains remain unchanged with a band size of 145bp, while CQS strains are cut by the enzyme into 111bp and 34bp.

Figure 4 shows the prevalence of the wild and mutant *Pf*crt genes in the study group. The wild *Pf*CRT K76 gene had a prevalence of 72% while the mutant *Pf*CRT 76T gene accounted for 28%.

Table 1 shows the distribution of the wild and mutant *Pf*CRT genes amongst the study population by age. There was a statistically insignificant difference, implying that the prevalence of the mutant *Pf*CRT genes was independent of age (χ^2=52.53; p>0.05). Overall, the presence of mutant *Pf*CRT genes increased with increasing age of subjects (16.7%, 20.0%, 25.0%, 33.3% and 38.9% for age groups 18–22, 23–27, 28–32, 33–37 and 38–42 years respectively). There was a significant difference in the distribution of wild *Pf*CRT genes (χ^2=8.54; p<0.05) which implied that age had an effect on the presence of the wild *Pf*CRT genes amongst the study subjects. Although no consistent pattern of change was found in the wild *Pf*CRT gene by age (83.3%, 80.0%, 75.0%, 66.7% and 61.1% for age groups 18–22, 23–27, 28–32, 33–37 and 38-42 years, respectively), the younger subjects tended to have a higher prevalence of the wild gene. However, the low number of participants aged 42 years and above may have been responsible for the 100% and 0% prevalence of wild and mutant *Pf*CRT gene recorded in the study, respectively.

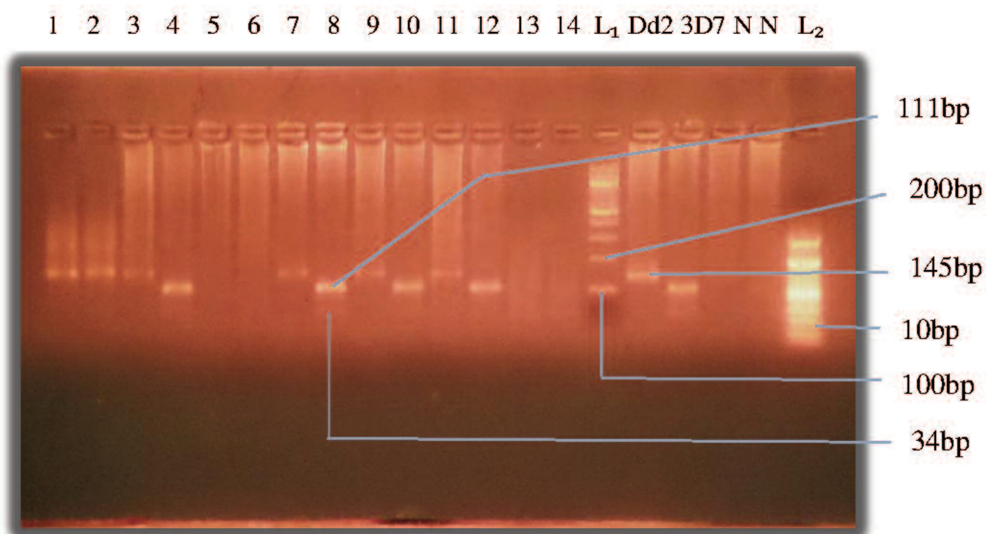

Fig. 3. Representative photomicrograph showing the results of some RFLP after digestion

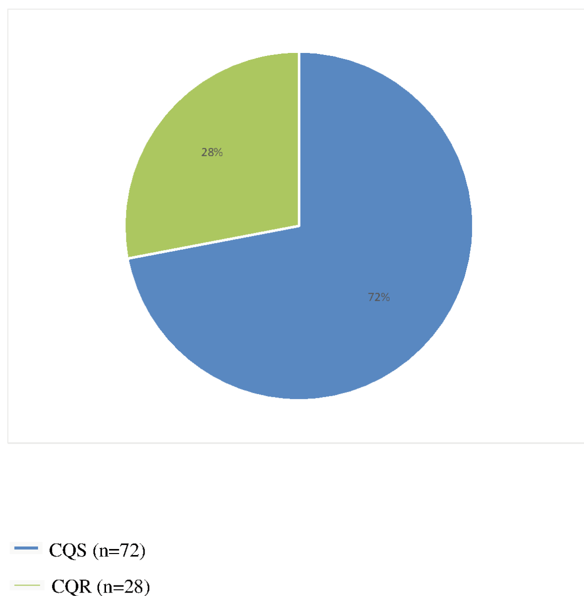

Fig. 4. Prevalence of CQS and CQR strains

Discussion

This study is the first in the city of Calabar that has assessed the prevalence of the *Pf*CRT mutant gene among *falciparum* malaria infected subjects. In this study, 68.0% of pregnant women investigated had malaria caused by *P. falciparum*. This is slightly lower than the 70.1% reported by Okafor et al. [19] amongst pregnant women who attended antenatal clinic in this same locality. Ojiezeh et al. [20] reported a prevalence of 71.2% of malaria among pregnant women in South West, Nigeria. The high prevalence of malaria infection amongst pregnant women in this study may be attributed to the stable transmission of malaria in the study area coupled with the poor drainage found in some areas of the study locality, the constant rainfall throughout the year, thick vegetation and poor observance and acceptance of malaria control activities [5]. Additionally, depressed immunity which is usually associated with pregnancy may also account for the high endemicity of malaria among the pregnant women enlisted in the study [21].

The findings in this study indicate that CQ resistance (*Pf*CRT 76T gene) was present in 28% of the study population, which is lower than the 48% recorded by Olasehinde et al. [22] in a study conducted in Ogun state, Nigeria ten years after CQ withdrawal. Efunshile et al. [23] recorded a 96.9% prevalence rate for the mutant *Pf*CRT gene five years after withdrawal of CQ in Ogun state, Nigeria. The findings in this study are similar to those in a study conducted in Southern Senegal by Magatte et al. [24] where they recorded a 25.2% prevalence of mutant *Pf*CRT gene. Elsewhere in Africa, particularly the countries of Southern Africa, complete or near complete sensitivity was reported.

A recovery of the susceptible *Pf*CRT gene from <15% to 100% was observed 13 years after a CQ policy change in Malawi, leading to a re-introduction of CQ for the treatment of uncomplicated malaria. In Tanzania, the prevalence of the mutant *Pf*CRT gene was reported to be just 8.3% [25]. The continuing high prevalence of CQ resistance reported in this study indicates that compliance with antimalarial withdrawal may still be incomplete. Another factor which may be responsible for the perpetuation of this mutant gene is the fact that both wild and mutant forms of the *Pf*CRT gene perform generally the same biological function, i.e. waste removal, amino acid metabolism and hydrogen ion homeostasis, although the mutant form allows increased efflux of CQ. Therefore, the malaria parasite has need of mutant *Pf*CRT genes as it does for the wild types. Also, the endemicity of malaria may have contributed to the continuous existence of the mutant gene. Gene transfer through conjugation and/or transformation from CQR malaria parasite strains to CQS strains from generation to generation either in the mosquito vector or human host may have ensured the continued survival of the mutant *Pf*CRT gene.

Though the prevalence rate of the mutant gene in this study falls short of WHO recommendations that any medication with a failure rate of greater than 10% should be withdrawn from use [26], this report offers the promise of a possible re-introduction of CQ: Our findings indicate a gradual fall from the 60% failure rate reported in 1997 [5]; this led to the implementation of the 2005 National antimalarial drug policy which required the withdrawal of CQ in the treatment of malaria.

Our findings reveal a strong association between the presence of the mutant *Pf*CRT gene and age. The older subjects may have successfully relied on CQ for malaria treatment in the past, prior to its removal, i.e. parasite clearance from blood and cure, and still hold the belief that the medication is still effective in the treatment of malaria. Thus, it is highly probable that self-medication with CQ may still be prevalent amongst older subjects. Younger subjects were found to have a higher prevalence of the wild PfCRT gene than their older counterparts, suggesting that there may be a higher level of

compliance on the non-usage of CQ by the younger people than the older ones. Also, it is likely that younger subjects may have never taken CQ, hence the lower prevalence of mutant genes and higher prevalence of wild genes.

The re-introduction of CQ in the treatment of malaria, a disease predominant among the poor, will be of immense public health benefit as it is less expensive than ACTs. More so, CQ is a drug of choice in rural areas, where malaria is more prevalent and the electricity supply tends to be poorer, because of its better ability to withstand adverse weather conditions and retain its potency: ACTs are easily affected by poor storage conditions. Also, CQ is safer for the treatment of malaria than ACTs amongst vulnerable groups, i.e. pregnant women and children under five years. Thus, the return to sensitivity of CQ will ensure the usage of this medication amongst this group of individuals.

The prevalence of malaria infection in pregnancy is still high amongst pregnant women living in the study area despite increased awareness and more thorough enforcement of malaria control intervention. There is still a high prevalence of tCQR malaria parasite strains in Calabar, although a decline has been observed in earlier studies conducted elsewhere in Nigeria.

In order to ensure the complete return of sensitivity to CQ, as seen in other African countries, relevant government agencies in Nigeria should adopt a more aggressive approach on compliance with the directive on the non-usage of CQ. Also, further studies on the prevalence of the mutant *Pf*CRT gene should be extended to cover a wider geographical spread in Nigeria with different epidemiological contexts. As CQS malaria strains have out-competed the CQR strains, the possibility of using chloroquine-based combination therapy is suggested.

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