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MOLECULAR ANALYSIS OF DRUG RESISTANCE GENES IN CLINICAL SAMPLES OF *PLASMODIUM FALCIPARUM* OBTAINED IN ZARIA, NIGERIA

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ABSTRACT

Malaria is a mosquito-borne disease caused by parasites that belong to the genus *Plasmodium*. It is a disease of public health concern in Nigeria, responsible for millions of deaths worldwide. This study aimed to carry out molecular analysis of drug resistance genes in clinical samples of *Plasmodium falciparum* in Zaria, Kaduna State, Nigeria. A total of 300 consenting participants were enrolled in this study from January to June 2019 at Hajiya Gambo Sawaba General Hospital, Zaria. Blood samples were collected from them and screened for *Plasmodium falciparum* by RDT and confirmed by microscopy. The polymerase chain reaction was used to amplify portions of *pfmdr1, pfcrt,* and *pfkelch13* genes carrying known mutations associated with antimalarial drug resistance. Afterward, the *pfcrt* amplicons were sequenced and the closest matches to the nucleotide sequences were determined using the BLAST tool on the National Centre for Biotechnology Information (NCBI), the relatedness between sequences was determined by creating a phylogenetic tree. The *pfmdr1* gene was detected in 20% (3/15) of the samples while the *pfcrt* gene was also amplified successfully in five of the samples analyzed, giving a prevalence of 33.3% (5/15). The *pfkelch13* was not amplified in all the 15 samples. The detection of segments of *pfmdr1* and *pfcrt* genes harboring single Nucleotide Polymorphisms associated with chloroquine resistance showed that the *Plasmodium falciparum* population in the study area may still be chloroquine-resistant. It is therefore recommended that Government should maintain the ban on the use of chloroquine for the treatment of *falciparum* malaria.

Keywords: Chloroquine, Resistance, *Plasmodium*, Microscopy, *pfcrt*, Zaria

INTRODUCTION

Malaria is a mosquito-borne disease caused by parasites that belong to the genus *Plasmodium*. People with malaria often experience fever, chills, and flu-like illnesses. Left untreated, they may develop severe complications and die (1). Of the five *Plasmodium* species that cause human infection, *Plasmodium falciparum* is the most virulent and is responsible for the large majority of infections in sub-Saharan Africa (2). Infection with *Plasmodium falciparum* results in one of three possible outcomes: asymptomatic parasitaemia, defined as the presence of asexual parasites in the blood without symptoms; uncomplicated malaria, which entails febrile illness not associated with signs of severe disease; and severe malaria, characterized by various syndromes of organ dysfunction, which if not treated promptly may result in death (3).

Antimalarial drug resistance has been associated with the presence of particular gene single nucleotide polymorphisms (SNPs) of *P. falciparum* (4). These SNPs serve as molecular markers which are believedshortlye to represent molecular epidemiological surveillance tools of antimalarial resistance, which may replace the more conventional and logistically complex *in vitro* or *in vivo* phenotyping approaches (4). This molecular surveillance can also help in slowing down drug resistance if supported by a careful drug usage policy (5).

The resistance to different anti-malarial drugs is due to single nucleotide polymorphisms (SNPs) in different *P. falciparum* genes, including *pfdhfr, pfdhps, pfcrt, pfatcase6, pfk13, and pfmdr1*. The accumulation of SNPs in these parasites can produce *in vivo* resistance (6)**.** This stresses the importance of understanding the molecular mechanisms of resistance to potentially prevent its emergence or spread (7).

Following the development and spread of resistance to antimalarials such as chloroquine and sulfadoxine-pyrimethamine, Artemisinin-based Combination Therapy (ACT) was adopted as the first-line treatment for uncomplicated *Plasmodium falciparum* malaria worldwide. However, artemisinin resistance, as measured by delayed parasite clearance, has now been confirmed in multiple countries in the Greater Mekong Sub-region (8,9). There is growing concern that artemisinin resistance may spread to Africa and other parts of Asia as was the case with chloroquine and sulfadoxine-pyrimethamine resistance (10,11). While *in vivo* therapeutic efficacy studies (TES) are considered the gold standard for determining anti-malarial

efficacy, the WHO recommends that data from these studies be complemented with molecular markers of drug resistance (12).

After a long search to identify a specific locus implicated in artemisinin resistance, the kelch propeller domain of the K13 gene (*PF3D7_1343700*) on chromosome 13 was identified as a molecular marker of artemisinin resistance (13). Several mutations in the kelch propeller domain have now been associated with *in vitro* ring-stage survival assays and delayed parasite clearance rates in patients treated with artemisinins (13,14). As a result, sequencing the kelch propeller domain of the *K13* gene is becoming an important tool in the global surveillance of antimalarial drug resistance in *P.falciparum* (14,15).

MATERIALS AND METHODS

Study Area

The study was conducted at Hajiya Gambo Sawaba General Hospital, Zaria, Kaduna State, Nigeria. Zaria is a major city in Kaduna state, Nigeria, as well as a Local Government Area. It is located at 11.11^{o} North latitude and 7.72^{o} East longitude and it is situated at an elevation of 644 meters above sea level. Zaria has a population of 975,153 making it the second-largest city in Kaduna (16). Zaria experiences extreme seasonal variation in monthly rainfall. The rainy period of the year lasts for 7 months; from March to October. The most rain falls around August, with an average total accumulation of 10 inches. The rainless period of the year lasts for 5 months, from October to March (17).

Ethical Approval, Study Design, and Sample Size

Full ethical approval was obtained from the Health Research Ethical Committee (HREC) of Kaduna State Ministry of Health and Human Services. The study was a cross-sectional study that lasted for 6 months (from January to June 2019). All febrile patients presenting symptoms of malaria, who were directed to the laboratory for the malaria parasite (MP) test, were included, while patients directed to the laboratory for laboratory tests other than the MP test were excluded. The sample size was determined using a previous prevalence of 22.4% (18) in Kaduna State, and the formula below described by Naing *et al.* (19).

$$n=\frac{Z^2 p(1-p)}{d^2}$$

The sample size calculated was 267, however, 300 blood samples were collected and used for the study.

Administration of Structured Questionnaire and Sample Collection

A structured questionnaire was administered to individuals who met the inclusion criteria after obtaining their consent to participate in

the study. This was used to obtain bio-data and other information relevant to the research. The venipuncture technique was employed for blood sample collection. A soft tubing tourniquet was fastened to the upper arm of the patient to enable the index finger to feel a suitable vein. The puncture site was then cleansed with methylated spirit (methanol) and venipuncture made with the aid of a 21 G needle attached to a syringe. When sufficient blood (2ml) was collected, the tourniquet was released and the needle was removed immediately, while the blood was transferred into an EDTA bottle.

Screening of Blood Samples for *Plasmodium falciparum*

Blood samples were screened by CareStart^{TM} Malaria HRP2 Rapid Diagnostic Test (RDT) Kit (Access Bio, Inc, Somerset, NJ), specific for the detection of *Plasmodium falciparum.* The RDT test was carried out according to the manufacturer's instructions and positive samples were confirmed by microscopy as described by Cheesbrough (20).

Extraction of DNA

Total deoxyribonucleic acid (DNA) was extracted from 15 of the 65 malaria-positive blood samples using Zymo Research Quick-DNA ^{TM} Miniprep Plus Kit (Irvine, California). The DNA samples were extracted according to the manufacturer's instructions. These DNA samples were used for the detection of *Plasmodium falciparum* genes by polymerase chain reaction (PCR).

The PCR Primers

The primers used in this study were designed by Zhang *et al*. (2008) to amplify the SNPs 391T/A, 392G/C, 399G/T, 400A/G,402T/A,404A/C at codons C72S, M74I, N75E, K76T of *pfcrt* and 256A/T,257A/T at codon N86Y/F of *pfmdr1* as shown in Table 1 below.

Table 1: Genes, primers, and single nucleotide polymorphisms amplified

S/N	Gene	Primer sequence	Size (bp)	SNPs (codons)
1	*Pfcrt*	F: GGAGGTTCTTGT CTTGGTAAAT R: ATATTGGTAGGT GGAATAGATTCT	315	C72S, M74I, N75E, K76T
2	*Pfmdr1*	F: TGTTGAAAGATG GGTAAAGAGCA GA R: TCGTACCAATTC CTGAACTCACTT	514	(Codon N86Y/F)
3	*Pfkelch13**	F: GCCAAGCTGCCA TTCATTTG R: GCCTTGTTGAAA GAAGCAGA	849	

Source: (21, 22*)

Amplification of *pfmdr1* by PCR

The polymerase chain reaction was used to amplify portions of *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*) gene spanning codons N86Y/F using the primers in Table 1. The PCR was carried out using the following cocktail mix: 2.5 µL of 10×PCR buffer, 1.5 µL 50mM $MgCl_2$, 1.0 µL each of 5pMol forward and reverse primers, 1.0 µL Dimethylsulfoxide (DMSO), 2.0 µL of 2.5 Mm dNTPs, 0.15 µL of Taq polymerase 5µ/µL, 2.0 µL of 100ng/ µL DNA and 13.85 H_2O to make a total volume of 25 µL. The following PCR programme was used: Nine cycles of initial denaturation at 94^o C for 5 minutes, 15 seconds of denaturation at 94^o C, 20 seconds of annealing at 60^o C, and 30 seconds of extension at 72^o C. Thirty-five cycles of denaturation at 94^o C for 15 seconds, 20 seconds of annealing at 50^o C, 30 seconds of extension at 72^o C, 7 minutes of final extension at 72^o C. The PCR products were resolved in 1.5% agarose gel, stained with ethidium bromide in Tris-acetate-EDTA. The molecular weight marker confirmed the expected product size, 514 base pairs.

Amplification of *pfcrt* by PCR

The polymerase chain reaction was used to amplify portions of *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene spanning codons C72S, M74I, N75E, K76T using the primers in Table 3.1 below. The PCR was carried out using the following cocktail mix: 2.5 µL of 10×PCR buffer, 1.5 µL 50mM $MgCl_2$, 1.0 µL each of 5pMol forward and reverse primers, 1.0 µL dimethylsulfoxide (DMSO), 2.0 µL of 2.5 Mm dNTPs, 0.15 µL of Taq polymerase 5µ/µL, 2.0 µL of 100ng/ µL DNA and 13.85 H_2O to make a total volume of 25 µL.

The PCR condition used was as follows: Nine cycles of initial denaturation at 94^o C for 5 minutes, 15 seconds of denaturation at 94^o C, 20 seconds of annealing at 60^o C, and 30 seconds of extension at 72^o C. Thirty-five cycles of denaturation at 94^o C for 15 seconds, 20 seconds of annealing at 50^o C, 30 seconds of extension at 72^o C, 7 minutes of final extension at 72^o C.

The PCR products were resolved in 1.5% agarose gel, stained with ethidium bromide in Tris-acetate-EDTA. The molecular weight marker confirmed the expected product size, 315 base pairs.

Sequencing of *pfcrt* and Phylogenetic Analysis

Polymerase Chain Reaction products were purified using Exo-SAP-IT (USB, Affymetrix, USA) and directly used as templates for DNA sequencing using the BigDye terminator v. 1.1 cycle sequencing

kit (Applied Biosystems, Foster City, USA) on an ABI 3130XL DNA sequencer.

The Genbank database was searched for similar sequences using BLAST (National Center for Biotechnology Information; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the output was then analyzed to find a significant homology. The *pfcrt* DNA multiple sequence alignments were performed with the *pfcrt* sequences obtained from GenBank and evolutionary analyses were conducted by creating a phylogenetic tree using Molecular Evolutionary Genetics Analysis (MEGA) X 10.0.5 software for in MEGA X. The analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 13743 positions in the final dataset.

PCR Amplification of *P. falciparum kelch13*

The researchers tried to amplify the *Plasmodium falciparum kelch13* gene using the primers in Table 1 as described by Huang *et al.* (2015), but could not replicate the nested PCR protocol after several optimization attempts. Hence, they resorted to using the primers for the secondary PCR reaction. The PCR was carried out using the following cocktail mix: 2.5 µL of 10×PCR buffer, 1.5 µL 50mM $MgCl_2$, 1.0 µL each of 5pMol forward and reverse primers, 1.0 µL dimethylsulfoxide (DMSO), 2.0 µL of 2.5 Mm dNTPs, 0.15 µL of Taq polymerase 5µ/µL, 2.0 µL of 100ng/ µL DNA and 13.85 H_2O to make a total volume of 25 µL. The PCR condition used was as follows: Nine cycles of initial denaturation at 94^o C for 5 minutes, 15 seconds of denaturation at 94^o C, 20 seconds of annealing at 60^o C, and 30 seconds of extension at 72^o C. Thirty-five cycles of denaturation at 94^o C for 15 seconds, 20 seconds of annealing at 50^o C, 30 seconds of extension at 72^o C, 7 minutes of final extension at 72^o C. The PCR products were resolved in 1.5% agarose gel, stained with ethidium bromide in Tris-acetate-EDTA.

RESULTS

The *pfmdr1* and *pfcrt* genes were amplified in the 15 *Plasmodium falciparum* DNA samples, but the *kelch13* gene could not be amplified. The *pfmdr1* gene was detected in 20% (3/15) of the samples (lanes 7, 12, and 15 in Plate I), while the *pfcrt* gene was also amplified successfully in five of the samples analyzed (lanes 4, 9, 12, 13 and 15 in Plate II), giving a prevalence of 33.3% (5/15). The relatedness between all the *pfcrt* sequences is shown in the molecular phylogenetic tree (Figure 1). The *kelch13* was not amplified in all the 15 samples; this is shown in Plate III.

The evolutionary history was inferred using the Neighbor-Joining method. The

evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

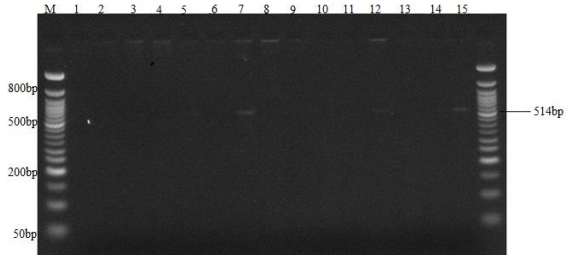

Plate I Agarose gel electrophoresis showing amplification of *pfmdr1* gene. (Lane M= Molecular ladder, Lanes 1-15= DNA samples)

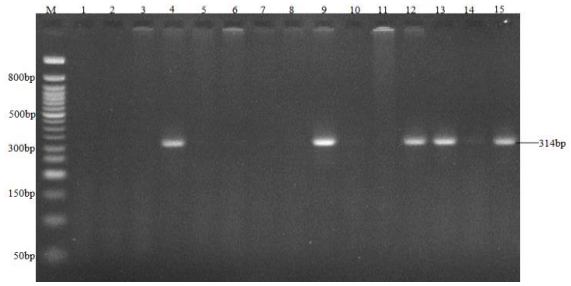

Plate II Agarose gel electrophoresis showing amplification of *pfcrt* gene

Lane M= Molecular ladder (50bp), Lanes 1-15= DNA samples)

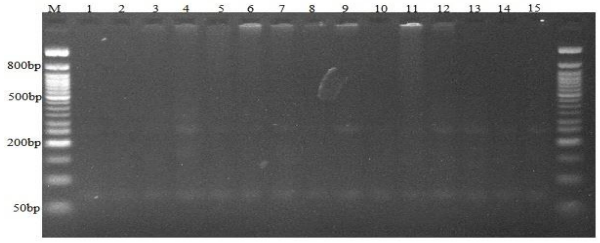

Plate III Agarose gel electrophoresis showing no amplification of *kelch13* gene at the expected size of 849bp (Lane M= Molecular ladder, Lanes 1-15= DNA samples)

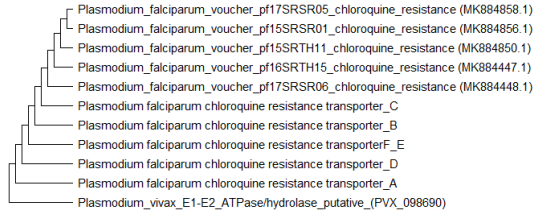

Figure 1 Phylogenetic tree showing the relatedness between the *pfcrt* gene sequences
Key: *Plasmodium falciparum* chloroquine resistance transporter A-E = *pfcrt* sequences from this study, all other sequences are Genbank sequences, *Plasmodium vivax* = outgroup

DISCUSSION

The widespread of chloroquine-resistant *Plasmodium* parasites prompted the WHO to recommend Artemisinin-based Combination Therapy for the management of malaria in endemic malaria regions. In Nigeria, Artemisinin-based Combination Therapy is currently used as the first-line treatment for uncomplicated *Plasmodium falciparum* malaria. The first report describing the prevalence of polymorphisms in *pfcrt* conferring chloroquine resistance showed that all the *Plasmodium falciparum* strains were carrying the *pfcrt* mutant alleles (23). In this study, we used *pfmdr1* and *pfcrt* primers designed by Zhang *et al*. (21) to amplify segments of *pfmdr1* containing the SNPs associated with chloroquine resistance. The *pfmdr1* and *pfcrt* genes were detected in only 20% (3/15) and 33% (5/15)

of the *Plasmodium falciparum* isolates used for PCR respectively. This finding highlights the fact that there are still some *Plasmodium falciparum* parasites in the study population carrying mutant *pfmdr1* genes, despite the withdrawal of chloroquine by the Nigerian government as the first-line malaria treatment drug years back. Chloroquine resistance may still be present in Zaria. This is in agreement with Folarin *et al*. (24) who reported an association between the presence of mutant *pfmdr1* with chloroquine resistance *in vitro* and *in vivo* in Nigeria. Many other studies have associated the presence of certain mutations (SNPs) in *pfmdr1* with resistance to antimalarials such as chloroquine (25, 26). Studies in other African countries such as Malawi (27), Kenya (28), and Tanzania (29) reported a rapid spread of chloroquine susceptible *pfcrt* populations. The detection of *pfcrt and pfmdr1* genes in this study indicated that chloroquine is still not a drug of choice for the treatment of falciparum malaria in Zaria, despite its withdrawal from the treatment of uncomplicated falciparum malaria over a decade ago by the Nigerian government. This may be due to non-compliance with the withdrawal of chloroquine for treatment of falciparum malaria in Nigeria (30), and it further underscores the need for wide-scale monitoring to withdraw chloroquine from circulation in Nigeria. In their study, Orimadegun *et al*. (30) reported a high prevalence of chloroquine treatment, despite the national drug policy change from chloroquine to ACTs. They also reported that pre-hospital administered chloroquine was associated with an increased risk of severe malaria, with a concurrent increase in mortality. Most of the early treatments for malaria occur through self-medication with antimalarial drugs bought from medicine sellers. These have led to increasing calls for interventions to improve treatment obtained in these outlets because they often increase the possibility of drug resistance.

The molecular phylogenetic analysis showed the relatedness between our *pfcrt* sequences and those obtained from the Genbank. All the *pfcrt* sequences shared a common ancestral lineage.

However, the sequences from GenBank were more closely related to each other than they were to sequences from this study except for the outgroup (*Plasmodium vivax*).

Clinical artemisinin resistance is defined as a reduced parasite clearance rate, expressed as an increased parasite clearance half-life or persistence of microscopically detectable parasites on the third day of ACT or high presence of *kelch13* mutants (31). We were not able to establish the presence of artemisinin resistance genes due to the non-

amplification of the gene in our samples. We used species-specific primers which were designed and used by Huang *et al*. (22) for the detection of K13 propeller domain mutations associated with drug resistance in *P. falciparum.* The inability to amplify the *kelch13* gene may be due to our failure to replicate the protocol of Huang *et al.* (22). Their protocol involved the use of primary and secondary (nested) PCR for the amplification of the *kelch13* gene. We used the secondary PCR primers after several attempts to use both primers (primary and nested) failed. We could not amplify the *kelch13* gene; as a result, we were unable to establish the presence or absence of the molecular markers of artemisinin resistance.

CONCLUSION

Even though *in vivo* or *in vitro* sensitivity test of antimalarial drugs on the *Plasmodium falciparum* isolates was not carried out, the results of this study suggest that the *Plasmodium falciparum* population in the study area may still be resistant to chloroquine due to the presence of mutant *pfmdr1* and *pfcrt* gene amplified in some of the *Plasmodium falciparum* strains. It is therefore recommended that Government should maintain the ban on the use of chloroquine for the treatment of *falciparum* malaria.

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