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Detection of antimalarial drug resistance polymorphisms in *Plasmodium falciparum* chloroquine resistance transporter and *Plasmodium falciparum* multidrug resistance 1 genes of *Plasmodium falciparum* found in Kano State, Nigeria

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ABSTRACT

Objectives: In 2018, malaria claimed an estimated 380,000 lives in African region, with Nigeria accounting for 24.0% (91,368) of malaria deaths from the region. Mutations in *Plasmodium falciparum* chloroquine resistance transporter (Pfcrt) and *P. falciparum* multidrug resistance 1 (Pfmdr-1) genes had reduced the effective use of artemisinin combination therapy through the development of resistance to these antimalarial agents. Our study set out to determine the antimalarial drug resistance polymorphisms in Pfcrt and Pfmdr-1 genes of *P. falciparum* isolates among patients in Kano State, Nigeria.

Material and Methods: Malaria positive samples were collected across the three senatorial districts of Kano State. The samples were amplified using nested polymerase chain reaction to detect the Pfcrt and Pfmdr-1 genes. The amplicons were sequenced and bioinformatic analysis was done using CLC Sequence viewer 8.0 and BioEdit sequence alignment editor to detect the single-nucleotide polymorphisms.

Results: In the Pfcrt gene, CVIET haplotype was seen in 26.2% of the samples while only two samples showed the 86Y mutation in the Pfmdr-1 gene. All the 86Y mutations and majority of the CVIET haplotypes were detected in the patients from rural settings where some of them noted that they consumed modern and traditional (herbs) antimalarial agents. One sample was observed to have the CVIET haplotype and N86Y mutation while the other five CVIET haplotypes were seen in five separate samples. A new mutation V62A was found in the Pfmdr-1 gene as observed in one of the sample.

Conclusion: It is imperative to ensure the rational use of the right antimalarial agents and employ continuous resistance surveillance/mapping to ensure synergy in malaria containment and elimination strategies.

Keywords: *Plasmodium falciparum* chloroquine resistance transporter, *Plasmodium falciparum* multidrug resistance 1, Antimalarials, Resistance, Polymorphisms

INTRODUCTION

Malaria is a potentially life-threatening protozoan disease that is a major public health problem in developing countries causing considerable morbidity and mortality, especially in sub-Saharan Africa. Nigeria is the most malaria burdened country globally, followed by Democratic Republic

of Congo and Uganda.[1] An estimated 228 million cases occurred in 2018, 93.8% of which was seen in the African region.[1] Similarly, 93.8% of estimated global malaria mortality was recorded in African region accounting for 380,000 deaths.[1]

Antimalarial agents inhibit growth of *Plasmodium* species by inhibiting hemoglobin utilization and parasite metabolism.[2] Polymorphisms in two genes of *Plasmodium falciparum* genome are the molecular basis of many antimalarial drugs; *P. falciparum* chloroquine resistance transporter (Pfcrt) gene and *P. falciparum* multidrug resistance 1 (Pfmdr-1) gene. Pfcrt polymorphisms at codons 74, 75, and 76 were found to confer resistance to chloroquine. Pfmdr-1 polymorphisms, notably at codons 86, 184, 1034, 1042, and 1246, have been implicated to influence susceptibilities to lumefantrine, artemisinin, quinine, mefloquine, halofantrine, and chloroquine.[3] The mutant CVIET haplotype of Pfcrt gene had been reported largely from Africa with varying alleles,[4,5] while the SVMNT haplotype was found mostly in South America and Southeast Asia. However, some studies indicated that SVMNT had been detected in parasite strains from Angola[6] and Tanzania.[7] A study from Africa identified 11 Pfmdr-1 haplotypes based on variations at codons N86Y, F184Y, and 1246, and noted that the NFD, YFD, and N Y/F D were the most prevalent mutant haplotypes in Africa.[5] The occurrence of resistant malaria parasites in Northwest Nigeria had been reported, with mutant Pfcrt (76T) prevalence of 28.3% and 12.4% for Pfmdr-1 86Y haplotype.[8] Considering the paucity of documented data on antimalarial drug resistance polymorphisms in Kano State coupled with the wide availability of antimalarial drugs, and often self-prescription attitudes of individuals,[9] researches on antimalarial drug resistance are useful for identifying regions at risk, thereby alerting malaria elimination programs. This study is determined to detect the common polymorphisms found in Pfcrt and Pfmdr-1 genes in *P. falciparum* among patients in Kano State, Nigeria.

MATERIAL AND METHODS

Ethical approval and informed consent

Ethical approval was obtained from Kano State Research Ethics Committee, Ministry of Health (Reference No. M84/70FT/797/T.I/130). Informed consent was obtained from individual patient or their guardian with respect to children.

Study area and population

The study was conducted in selected hospitals from the three senatorial districts of Kano State, Nigeria. All patients (1–70 years, $n = 200$) who presented with signs or symptoms of uncomplicated malaria and consented were recruited using systematic random sampling for the study. All patients that have signs or symptoms of severe malaria, are pregnant, or refused consent were excluded from the study.

Collection of samples, parasites identification, and estimation

Whole blood was collected by venepuncture from each participant.[10] Thick and thin blood films were made on clean, grease-free, frosted end slides, and stained using 3% Giemsa stain solution. The malaria parasites were identified by microscopy using morphological characteristics on the thick and thin films by method earlier described[11] and parasites density was determined according to method described by the WHO.[11] A dry blood spot was prepared from each sample on Whatman No. 1 filter paper, packed in separate polythene bags containing desiccant and stored at controlled room temperature for the molecular studies.[12]

Parasite DNA extraction and amplification

The parasite DNA was extracted from the dried blood spot using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).[13] The extracted DNA was amplified using the nested polymerase chain reaction (PCR) technique using primers (Eurofins Genomics, Germany) earlier described.[14] The primers were used to target amino acid positions 57–146 and 29–188 for Pfcrt and Pfmdr-1 genes, respectively. Amplification was carried out at the following conditions for the primary and nested PCR: Pfcrt; initial denaturation at 94°C for 3 min, then 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 30 s for 35 cycles, and final extension at 72°C for 10 min and Pfmdr-1; initial denaturation at 95°C for 1 min, then 95°C for 20 s, annealing at 52°C for 20 s, extension at 60°C for 30 s for 35 cycles, and final extension at 60°C for 3 min. All products were kept at −20°C until required.

Sequencing and results analysis

Some selected amplicons were sent to MYTACG Bioscience Enterprise, Selangor, Malaysia, for sequencing analysis. Sequence results were analyzed using CLC sequence viewer v8.0 (http: www.clcbio.com) and BioEdit software version 7.2.3.0 for alignment and manual comparison with an antimalarial susceptible 3D7 control (PF3D7_1343700). Statistical analysis was done using SPSS Version 20.

RESULTS

The predominant *Plasmodium* parasite seen was *P. falciparum* where a total of 197 samples showed *P. falciparum* infection (98.5%) while three samples had mixed infection of *P. falciparum* and *Plasmodium ovale* (1.5%). A parasite

density range of 93–132,761 parasites/μL and mean of 11,496 parasites/μL (SD = 22,237) was observed in this study. A total of 63 (31.5%) samples that have parasites density of ≥5000 parasites/μL were used for DNA extraction. Out of the 63 samples subjected to nested PCR, the Pfcrt gene was detected in 36 (57.1%) samples after the primary and nested PCRs, respectively. The Pfmdr-1 gene was detected in 45 (71.4%) samples by both the primary and nested PCRs. The Pfcrt gene yielded an amplicon size of 270 bp, as shown in Figure 1, which targeted the amino acid positions including 72, 73, 74, 75, and 76. For the Pfmdr-1 gene, the amplicon size was 520 bp, as shown in Figure 2, which covered amino acid positions including 86 and 184.

Twenty amplified samples and the 3D7 control were selected for sequencing the Pfcrt and Pfmdr-1 genes each. The results from the sequence analysis of Pfcrt gene showed that 73.7% of the samples have sequence similar to the wild type, CVMNK, and control 3D7 while 26.3% (5 samples) have the mutant sequence at codons 74, 75, and 76 [Figure 3] giving rise to the haplotype, CVIET. There were no other mutations seen on the sequenced Pfcrt gene for all the samples. Only 2 (11.1%) samples showed mutations at codon 86 [Figure 4] out of the 20 Pfmdr-1 samples sent for sequencing. No mutation was observed at amino acid position 184. However, another mutation was seen at codon 62 [Figure 5]. Only one sample that had CVIET haplotype for the Pfcrt gene possessed N86Y mutation on the Pfmdr-1 gene. The other six samples have either the CVIET, N86Y, or V62A haplotypes.

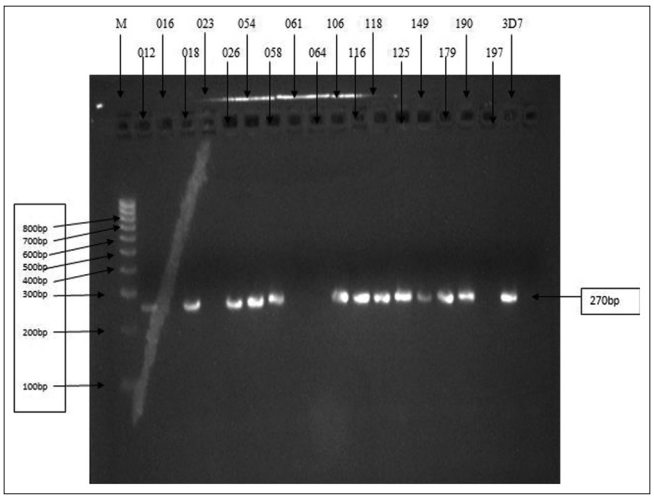

Figure 1: Gel electropherogram of Pfcrt gene of 270bp: Negative samples (016, 023, 061, 064, and 197); positive samples (012, 018, 026, 054, 058, 106, 116, 118, 149, 179, and 190) and control (3D7), Key: M – Marker (100 bp ladder).

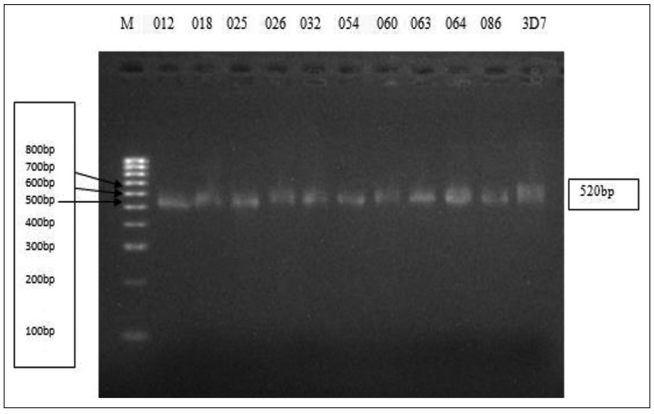

Figure 2: Gel electropherogram of Pfmdr1 gene at 520 bp showing all samples being positive and control (3D7), Key: M – Marker (100 bp ladder).

Table 1 shows the sociodemographic characteristics (sex, household type, type of medication used, and education status) of the patients, where no significant difference ($P > 0.05$) exists between those with mutant haplotype and those that have the wild type haplotype. Although the number of female with mutant Pfcrt haplotype (CVIET) exceeded that of the males, no statistical difference was observed. Likewise, only one CVIET haplotype was seen from samples collected from urban setting, while the other four CVIET haplotypes were all from samples collected from rural settings.

Only those that combined treatment (modern and traditional) or used modern drugs (only) possessed the mutant gene. All those that have the mutant 86Y were rural dwellers and used modern medication.

The CVIET haplotype was found in all the education status groups except in those that were not of school age. The 86Y haplotype was seen in the post-primary and tertiary school levels.

DISCUSSION

This was the first CVIET haplotype reported to be seen in Kano, Nigeria, though it had been reported elsewhere in Nigeria and West Africa. A higher 69.9% prevalence of the mutant allele was reported in Ogun State, Nigeria,[15] where the rural settings predominated having the mutant type. A lower prevalence of 5.4% in 76T was seen among malaria asymptomatic *Almajirai* in Northeast Nigeria.[16] In contrast, a higher prevalence of the mutant was observed in Khartoum, Sudan.[17] Other haplotypes, SVMNT, CVMNT, and mixed type which were predominant in South American countries, were not detected in our study. It can be said that the low prevalence observed in our study could be attributed to the withdrawal of chloroquine in Nigeria for over a decade now. A similar trend was seen in Ghana where a decreasing mutant (76T) profile was observed from 2005 to 2017.[18,19] However, despite the chloroquine withdrawal in Ethiopia, the mutant haplotype CVIET is still in full (100%) circulation.[20] This calls for the need to improve on the antimalarial agents resistance surveillance until it is proven that no mutant haplotype exists in the country and region.

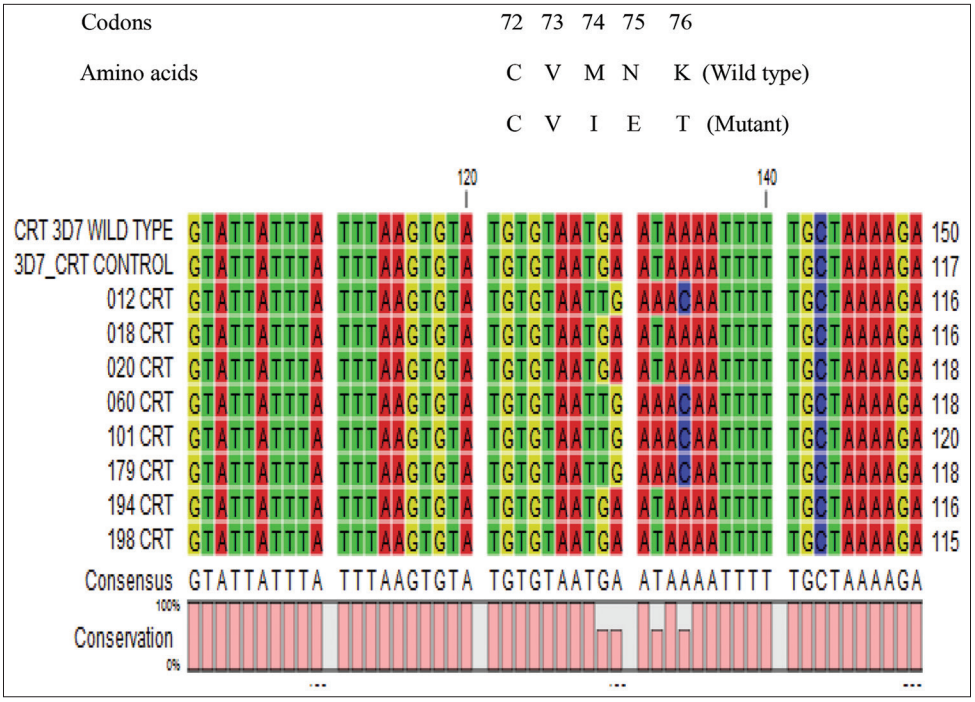

Figure 3: Multiple nucleotide sequences alignment of samples highlighting polymorphisms in the Pfcrt gene of samples 012, 060, 101, and 179, Courtesy: CRT 3D7 wild type (NCBI GenBank Accession number: AL844506.3).

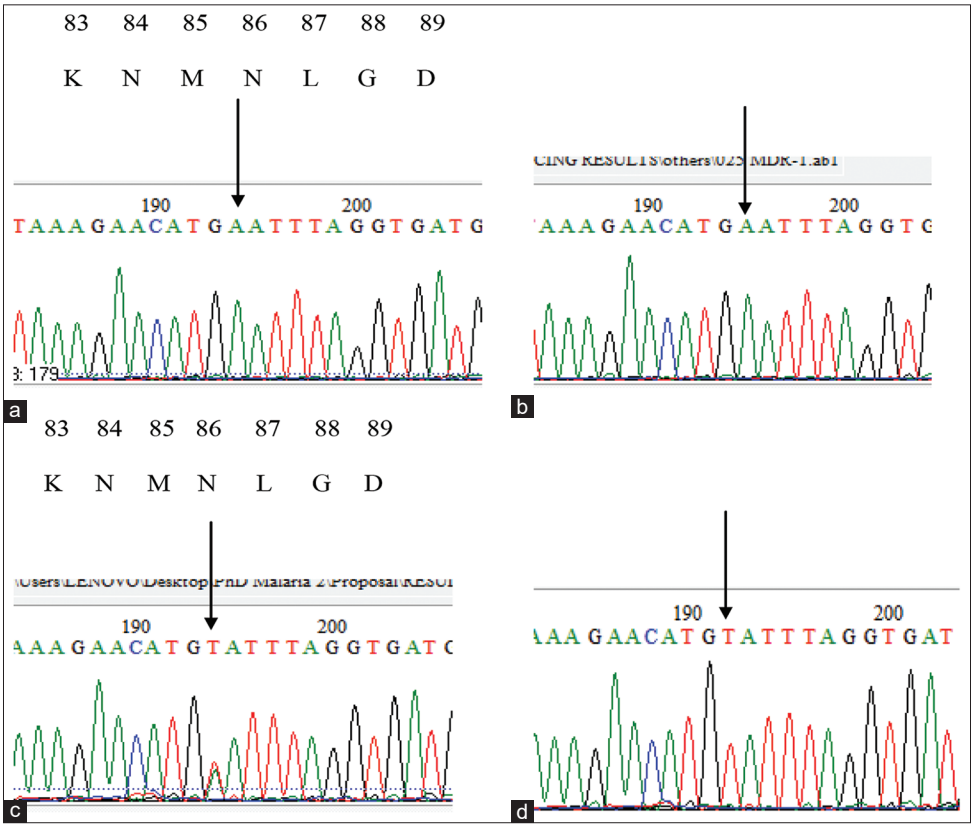

Figure 4: Chromographs showing nucleotide sequence analysis of the Pfmdr-1 gene at position 86. (a) Wild type (control) with N86 codon, (b) sample with wild type gene (N86), (c and d) mutant haplotypes with 86Y codons. Arrows show single-nucleotide polymorphisms in (c and d).

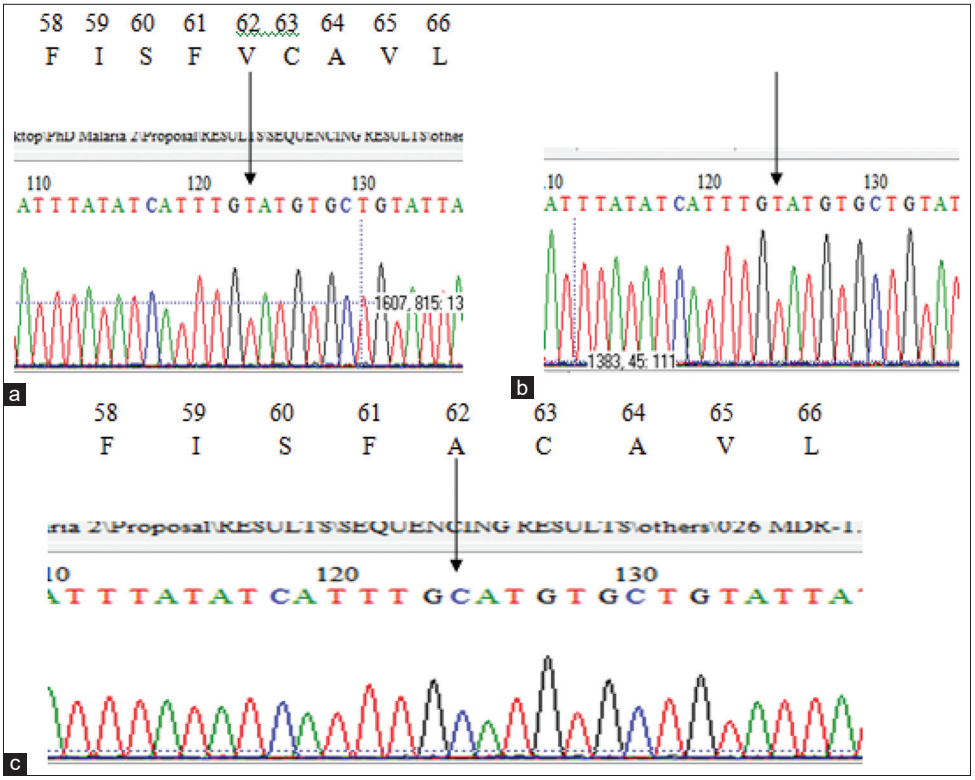

Figure 5: Chromographs of nucleotide sequences showing mutation depicted on the Pfmdr-1 gene at position 62. (a) Wild type 3D7 (Control), (b) haplotype with no mutation (V62), (c) mutant haplotype with mutation (62A).

Table 1: Sociodemographic characteristics of patients according to Plasmodium falciparum haplotypes seen.

Characteristics	Pfcrt haplotype (*n*=19)		χ2	*P*-value	Pfmdr-1 haplotype (*n*=18)		χ2	*P*-value
	IET	MNK			86Y	N86		
Sex								
Female	3	10			1	12		
Male	2	4	0.317	0.483	1	4	0.423	0.521
Household type								
Rural	4	11			2	12		
Urban	1	3	0.89	0.634	0	4	0.741	0.553
Type of medication used								
Modern	3	9			2	9		
Traditional	0	2			0	2		
Both	2	3	1.292	0.283	0	5	1.197	0.714
Education status								
None	0	2			0	2		
Informal	1	5			0	6		
Primary	1	7			0	8		
Post-primary	2	0			1	0		
Tertiary	1	0	4.356	0.36	1	0	2.222	0.695

A new mutation V62A was seen in the Pfmdr-1 gene where a non-synonymous missense mutation occurred. However, this mutation is probably a conservative replacement because valine has similar properties with alanine when compared using Grantham's chemical distance.[21] It is likely that the protein formed may be structurally functional and probably none of its major activities may be hindered. This mutation had not been reported elsewhere. Mutations in Pfmdr-1 were also associated with an increase in resistance to artesunate.

The presence of CVIET may probably be that there are places where chloroquine is still being use for treating malaria in the rural settings despite its withdrawal. It could also be due to ingestion of local herbs that have similar chemical properties/activities with quinolines. Some of the factors that could likely account for the presence of the mutants could be due to drug pressure accompanied by non-compliance in completing dosage coupled with administration of substandard drugs.[9]

The limitations in this study included the few number of samples sequenced. Other known mutation points of Pfcrt (codon 220) and Pfmdr-1 (codons 1024 and 1246) were not covered during the amplification and sequencing due to financial constraints.

CONCLUSION

The Pfcrt-resistant haplotype, CVIET, and Pfmdr-1 86Y mutation were found in the study population. A new mutation V62A was observed in the study which needs further research. It is imperative to ensure the rational use of the right antimalarial agents in treating *Plasmodium* infection and employ the use of continuous surveillance/resistance mapping to ensure synergy in malaria elimination strategies.

Contribution details

AAY conceived the study. AAY, OOS, IHI, and SAB designed the research protocol: AAY performed the experiments and analyzed the data. OOS, IHI, and SAB reviewed the paper. All authors read and approved the final manuscript.

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Declaration of patient consent

Institutional Review Board (IRB) permission obtained for the study.

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Conflicts of interest

There are no conflicts of interest.

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