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Plasmodium falciparum Multidrug Resistance-1 (pfmdr1) Gene Mutation in Adults Malaria Patients Attending Murtala Muhammad Specialist Hospital Kano, Northwest Nigeria

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ABSTRACT

Suspicion of failure in the effectiveness of artemisinin-based combination therapies (currently the first-line treatment of malaria, worldwide) is leading to the unofficial use of alternative antimalarials, including chloroquine and sulfadoxine/pyrimethamine in Kano state northwestern Nigeria. To facilitate evidence-based resistance management, antimalarial resistance mutations were investigated in *Plasmodium falciparum* multidrug resistance-1 (pfmdr1) gene. Hundred adult patients comprising 43 males and 57 females were recruited for the study. The mean age of participants is 36.4 years, minimum and maximum ages were 16 and 60 years respectively, while 41% of them fall within the range of 16 to 30 years. Blood isolates were then analyzed for the presence of malaria parasite using microscopy, the results show a high prevalence of *P. falciparum* infection in the subject (30%). Pfmdr1 gene, a molecular marker of artemisinin resistance, was successfully sequenced in 21 out of 100 *P. falciparum* isolates collected from recruited participants. Pfmdr1 mutations were found in 19.5% (4/33) of the samples isolated. The prevalence of the Pfmdr1 N86Y allele was found in 4 samples whilst Y184F and D1246Y were not detected. A total of 4 non-synonymous mutations at codon N86Y were detected. The presence of these mutations highlights the challenges for malaria treatment in Kano state, northwestern Nigeria using antimalarials such as artemether lumefantrine, mefloquine, amodiaquine quinine and lumefantrine.

INTRODUCTION

Malaria is a major global public health dilemma that exerts a high mortality and morbidity in many countries, particularly in resource-poor countries across Sub-Saharan Africa, Southeast Asia and Latin America, and probably contributes to keeping these countries in poverty [1]. Malaria is currently affecting more people in the world than any other disease. It is currently endemic in over a hundred countries and is one of the ten most prevalent and deadliest diseases in the world [2]. The World Health Organization estimates that 3.4 billion people globally are at risk of malaria and 0.6 million deaths occur due to malaria (90% in Africa) most being children and pregnant women [2]. The estimated number of malaria deaths stood at 435, 000 in 2017, a similar number to the previous year. In 2017, five countries accounted for nearly half of all malaria cases worldwide; Nigeria (25%), the Democratic Republic of Congo; (11%), Mozambique

(5%), India (4%) and Uganda (4%) [2]. Nigeria suffers from the world's greatest malaria burden with approximately 51 million cases and 207 000 deaths reported annually while 97% of the total population (approximately 173 million), is at risk of infection [3].

Various drugs are used for malaria prevention and cure. These include quinine (QN), chloroquine (CQ), amodiaquine, piperaquine (PPQ), mefloquine (MQ), lumefantrine (LUM), pyrimethamine (PYR), proguanil, sulfadoxine, atovaquone, primaquine, and artemisinin and its derivatives (ART) [4]. Artemisinin-based combination therapies (ACTs) are now recommended worldwide for treatment of *P. falciparum* infections [5]. Primaquine and the recently approved tafenoquine are the only antimalarials available against liver-stage parasites and hypnozoites [6]. Artemisinin-based combination therapies (ACTs) are the first-line therapy in almost all countries where

malaria is endemic due to the high efficacy, tolerability, and ability of ACTs to reduce ongoing transmission of the parasite. ACTs are comprised of two components: an artemisinin derivative and a partner drug. The artemisinin derivative has a high antimalarial potency, killing a large proportion of parasites; however, these compounds are rapidly eliminated, leaving a residual parasite population that, if left untreated, will likely recrudesce. A slowly eliminated partner drug is required to provide a sustained antimalarial activity that is capable of killing the remaining parasites [7]. ACT combinations available include artemether and lumefantrine (AL), artesunate and amodiaquine (AS/AQ), artesunate and mefoquine (AS/MQ), artesunate and chlorproguanildapsone (AS/CD), artesunate and sulphadoxine and pyrimethamine (AS/SP), dihydroartemisinin and piperaquine (DA/PQ), artesunate and piperazine (AS/PZ), artesunate and atovaquone and proguanil (A/AP). Out of these ACT combinations WHO recommended AL, AS/MQ, AS/AQ, and AS/SP [2].

Unfortunately, resistance (or treatment failure) has been reported from nearly all malarial regions. Efforts are being made to discover new antimalarial drugs and to understand the molecular mechanisms of drug resistance [8]. Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject". Emerging resistance was initially identified as delayed parasite clearance rates following treatment with artemisinin-based therapies. Confirmed partial artemisinin resistance is now defined by the WHO as ≥5% of patients carrying K13 resistance-associated mutations, all of whom have been found, after treatment with ACT or artesunate monotherapy, to have either persistent parasitemia by microscopy on day three, or a parasite clearance half-life of ≥5 hours [9].

Several factors influence the likelihood of resistance occurring and the speed with which it spreads. The mechanism by which the drug works against the parasite is important; simple modes of action such as enzyme inhibition are likely to lead to rapid evolution of resistance, as the number of genetic mutations required to alter enzyme structure is low. Understanding the mechanisms underlying drug resistance is of paramount importance. Knowledge of the gene(s) controlling resistance to a specific anti-malarial drug enables the monitoring of the spread of resistance, as well as potentially increasing the effectiveness of decisions concerning drug policy. Knowing which genes are involved in resistance to a drug can also help to understand the molecular basis of drug resistance and aid in the design of new versions of drugs that are unaffected by the mutations causing parasite drug resistance [10].

Some genes have been identified and proven to be involved in *P. falciparum* antimalarial drug resistance which includes: the genes encoding the *Plasmodium falciparum* Na+/H+ exchanger (Pfnhe-1), *Plasmodium falciparum* chloroquine resistance transporter (Pfcrt), *Plasmodium falciparum* dihydropteroate synthase (Pfdhps), *Plasmodium falciparum* dihydrofolate reductase (Pfdhfr), *Plasmodium falciparum* multidrug resistance 1 gene (Pfmdr1), *Plasmodium falciparum* multidrug resistance-associated protein 1 (Pfmrp1), *Plasmodium falciparum* cg2 (Pfcg2), *Plasmodium falciparum* Ca^{2+} ATPase (PfATP6) and kelch 13 gene. Single Nucleotide Polymorphisms (SNPs) in these markers are very important in monitoring or determining new drug-resistant patterns [11].

There is growing concern among healthcare providers about the loss of efficacy of antimalaria medications including QN, AQ, CQ, MQ, LU, and artemisinin-based combination therapies (ACTs) in Kano state Northern Nigeria. Part of the problem with malaria control is antimalarial resistance. Currently, there is limited or no research on the molecular basis of ACT resistance by the populace of Kano state, Nigeria. It is therefore imperative to establish if the molecular markers associated with antimalarial resistance elsewhere are present in Kano state, Nigeria.

MATERIALS AND METHODS

Study Area
The study was conducted Murtala Muhammad Specialist Hospital (MMSH), Kano State Nigeria. The hospital is usually attended by very low and moderate socio-economic groups and therefore it's affordable and accessible to most dwellers of the ancient city of Kano being the largest state-owned hospital. It has a coordinate of latitude 11.96ºN and longitude 8.55ºE.

Administration of Consent Forms and Structured Questionnaire
Consent forms and a structured questionnaire were administered to consenting individuals who met the inclusion criteria. This was used to obtain biodata and information relevant to this research.

Inclusion and Exclusion Criteria
All febrile patients presenting symptoms of malaria who were directed to the laboratory for a malaria parasite test and gave consent were included. All patients directed to the laboratory for laboratory tests other than malaria tests were and those who did not give consent were excluded. Patients with other ailments like diabetes, hyperglycemia and HIV were excluded.

Study population and Sample size
A total of hundred patients presenting with uncomplicated malaria were recruited for the study.

Sample Collection
Two millilitres (2 mL) of blood were drawn from the patient and dispensed into appropriately labelled EDTA containers.

Confirmation of the presence of *Plasmodium falciparum* using Microscopy
A thick blood film was made by smearing a drop of blood on a clean glass slide. The smear was dried at room temperature, fixed in methanol for a minute and left to dry. The fixed smear was dipped in field stain B for 5 seconds, washed in running tap water, dipped in field stain A for 15 seconds, washed in running water and left to air dry at room temperature. The stained slides were viewed under oil immersion at ×100 magnification. The number of parasites was counted and recorded [12].

DNA extraction from whole-blood
Samples were brought out from the refrigerator and allowed to thaw at room temperature before the commencement of extraction. Micropipettes to be used were cleaned by using 70% ethanol, allowed to soak for some minutes and blotted with a dry paper towel to avoid contamination during extraction. The work surface area was also cleaned. DNA extraction was carried out on the whole blood of 30 malaria-positive patients using QIAamp® DNA Mini Kit (QIAGEN, Germany), according to the manufacturer's instructions. 20 µL of proteinase K (a serine protease) was pipette into the bottom of labelled 1.5 mL micro centrifuge tubes followed by the addition of 200 µL of the sample and 200µL of buffer AL and mixed by pulse vortexing for 15

seconds. The mixture was incubated for 20 minutes at 56 °C and briefly centrifuged to remove drops from the inside of the lid after which 200 µL of 100% ethanol was added, mixed by pulse vortexing for 15 seconds and briefly centrifuged to remove drops from the inside of the lid. The content was carefully transferred into the QIAamp mini spin column which is placed in a clean 2 mL collection tube. The cap of the spin column was closed, and the tubes were centrifuged at 8000 revolutions per minute (rpm) for 1 minute. Thereafter, the spin columns were placed in a new clean 2mL collection tube while the old one was discarded.

The cap of the mini spin column was opened, and 500 µL of wash buffer AW1 was added after which the tubes were centrifuged for 1 minute at 8000 rpm. After centrifugation, the mini spin columns were placed in a new clean collection tube while discarding the old one. The caps of the tubes were opened and 200 µL of buffer AW2 was added. The caps were closed, and the tubes were centrifuged for 3 minutes at 13000 rpm. The filtrate from the 2 mL collection tubes was discarded, and the QIAamp mini spin columns were replaced in a new 2 mL collection tube and centrifuged at full speed for 3 minutes to eliminate the chances of possible buffer AW2 carryover.

The QIAamp mini spin columns were placed in clean 1.5 mL microcentrifuge tubes while the 2 mL collection tubes containing the filtrate were discarded. The mini spin columns were carefully opened, 100 µL of nuclease-free water was added and incubated at room temperature (15 - 25 °C) for 5 minutes and then centrifuged at 10,000 rpm for 1 minute. The extracted DNA was stored at 4 °C to be used for polymerase chain reaction (PCR) in subsequent days.

Amplification of fragment of *Pfmdr1* encompassing the N86Y, Y184F and D1246Y codons
The fragment of the *pfmdr1* gene encompassing the N86Y, Y18F and D1246Y was amplified to identify possible mutations in the DNA extracted in these regions. The forward and reverse primers used for the first fragment were *Pfmdr1*- F (5'- AGA GAA AAA AGA TGG TAA CCT CAG -3'), *Pfmdr1*- R (5' ACC ACA AAC, ATA AAT TAA CGG -3'). Forward and reverse primers used for amplification of D1246Y were (5'- GTGGAAAATCAACTTTTATGA 3') and (5' TTAGGTTCTCTTAATAATGCT 3') respectively [13].

The reaction mixture consists of 1.6 µL of genomic DNA, 0.8 µL of the forward and reverse primers, 10 µL of master mix (Promega, USA), and 6.8 µL of double distilled water in a final volume of 20 µL. The thermal cycler was programmed with an initial denaturation at 94 °C for 3 minutes, 35 cycles each of 94 °C for 30 seconds (denaturation), 60 °C for 1 minute (primer annealing), 72^0C for 1 minute (extension) and a final 5 minute extension step at 72 °C.

Agarose Gel Preparation and Confirmation of PCR product
2 g of agarose powder was dissolved in 100 mL of Tris Acetate EDTA (TAE) buffer and mixed thoroughly. The mixture was boiled for 5 minutes and allowed to cool at room temperature. 4 µL of ethidium bromide was added and mixed with the gel. The mix was poured into a cast with combs and allowed to dry.

The PCR products were separated using the above gel at 120V for 40 minutes. Positive bands were identified using Ingenius gel documentation unit (Syngene, UK). The size of the amplified gene compared to the DNA ladder (Thermo Fisher, USA), was around 610 base sequences.

Purification of PCR products and Sequencing
Purification of the PCR products was carried out using the QIAquick® PCR purification kit (QIAGEN, Germany). Into labelled 1.5 mL, 5 volumes of Buffer PB and 1 volume of the PCR product were added. This was followed by the addition of 10 µL of 3 M sodium acetate (pH 5.2) which helps in precipitating the DNA and enhancing yield. The mixture was vortexed for 30 to 60 seconds.

The mixture is then applied into a QIAquick spin column containing silica gel to which DNA selectively binds while the contaminants pass through upon centrifugation at 13,000 rpm for 1 minute. The supernatant was discarded and 750 µL of buffer PE was added to clean the column. The spin columns were centrifuged twice at 13,000 rpm for 1 minute to remove the remaining contaminants. DNA was eluted in 30 µL of nuclease-free water after centrifuging for 1 minute at 13,000 rpm. After purification, samples were sent for sequencing in the UK, using Sanger sequencing, by GeneWIZ (https://www.genewiz.com/en-GB/), and the primers provided above.
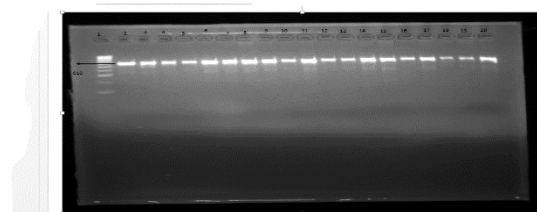
RESULTS AND DISCUSSIONS

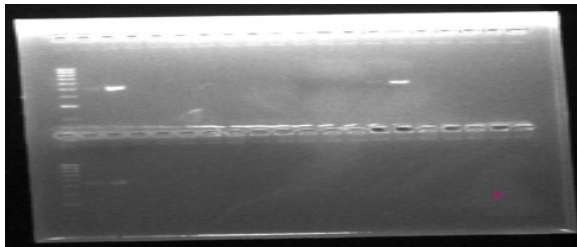**Socio-demographic characteristics of the study population**
The mean age of the participants is 36.4 years while the minimum age and maximum age are 16 years and 60 years respectively. Out of 100 participants, 80% of them had a malarial infection episode in less than three months and 20% in more than three months. 83% of participants of the participants have completed the dosage of prescribed ACT during the previous malarial infection, while 17% did not take the complete dose of ACT.

Amplification of fragment of *Pfmdr1* gene
DNA from 30 samples was used to amplify a 610bp partial fragment of the *pfmdr1* gene encompassing the 86th, 184th, and 1246th codons. Nineteen (19) samples were successfully amplified for codons 86 and 184. Two (2) samples were successfully amplified for codon 1246. **Fig. 1a** and **1b** shows the gel pictures of products amplified.
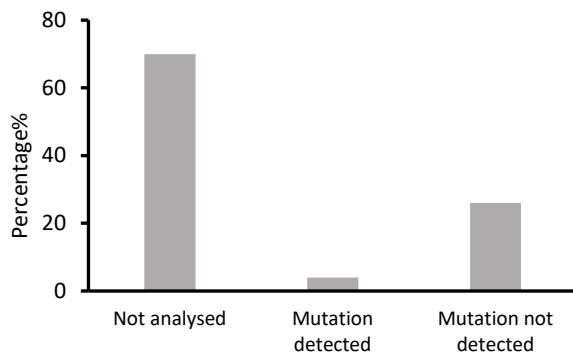

Fig. 1a. Agarose gel electrophoresis of a partial fragment of the *Pfmdr1* gene codon 86 and 184 from blood samples of *Plasmodium falciparum* isolates. Lane 1; Molecular ladder (610bp); lanes 2 – 20 which include samples 3, 7, 9, 15, 19, 24, 25, 29, 33, 38, 41, 47, 52, 59, 61, 76, 84, 93, 98 indicate amplified pfmdr1 gene.

Fig. 1b. Agarose gel electrophoresis of a partial fragment of the *Pfmdr1* gene codon 1246 from blood samples of *Plasmodium falciparum* isolates. Lane 1; Molecular ladder (610bp); lanes 2 and 14 show amplified mdr1 gene while lane 1, 3, 4, 5, 6, 7, 8, 9, 10, 11,12,13,15,16,17,18,19 shows no amplification.

Pfmdr1 gene single-nucleotide polymorphism-SNP

Fig. 2 below shows the percentage of samples that were sequenced and the percentage of those with mutations. A total of 21 out of 100 (21%) DNA samples were successfully sequenced and analyzed. Pfmdr1 gene mutations were found in 19.5% (4/21) of the samples obtained from different local government areas of Kano state.

Fig. 2. Polymorphism of Pfmdr1 gene.

were analyzed for polymorphism using BioEdit and multiple sequence alignment constructed in CLC viewer (**Fig. 3a** and **3b**). **Table 1** shows the changes that occur in amino acid sequence as a result of mutation. A total of 4 mutations were detected. All the four mutations were non-synonymous.

Table 1. Mutations observed in the study and the amino acid substitutions.

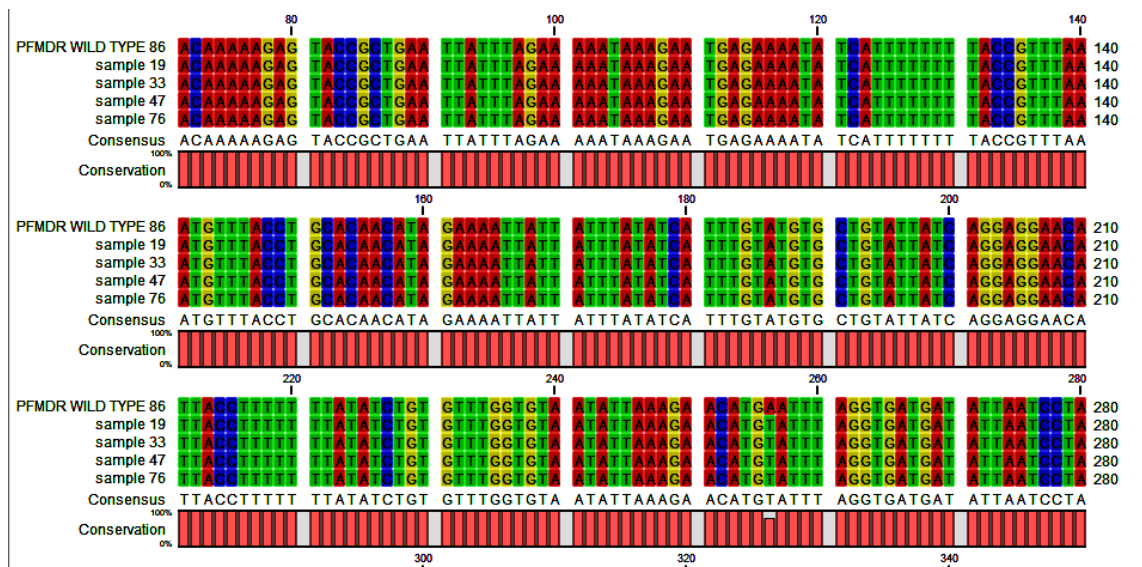| Samples	Mutation type	Codon position	Reference Nucleotide	Mutant Nucleotide	Reference amino acid	Amino acid substituted
19	NS	256	AAT	TAT	Asparagine	Tyrosine
33	NS	256	AAT	TAT	Asparagine	Tyrosine
47	NS	256	AAT	TAT	Asparagine	Tyrosine
76	NS	256	AAT	TAT	Asparagine	Tyrosine

Key: NS= Non-synonymous.

DISCUSSION

Screening of the participants reveals significant (up to 80%) presence of malarial infection in less than three months which is probably due to either recrudescence of the same parasite that is not completely cleared in their blood or as a result of a new infection [14]. Previous history of ACT drug compliance during the last malarial infection in this research indicated that 82% of participants have completed their prescribed ACT dosage as opposed to the previous national survey conducted in Lagos which reported less than 60% compliance [15].

Fig. 3a. Comparison of amino acid sequences of some *Plasmodium falciparum* isolates with the sequence of reference PFMDR Wild type.

Fig. 3b. Alignment of samples with the reference gene PFMDR wild type.

Sequencing and Polymorphism Analysis

Twenty-one (21) of the purified PCR products were sent for sequencing in the UK, using Sanger sequencing, with the appropriate primers. Fourteen (14) out of the 21 were successfully sequenced while the remaining failed. Sequences

In the microscopic screening of blood isolates of the participants using the approximation quantification method 68% of them were found to have a parasite density of 1+, 23% were found to have a parasite density of 2+, and 9% had a parasite density of 3+. Samples with 2+ and 3+ parasitemia were selected

for the study because high parasite density was considered as one of the important inclusion criteria for the research [16]. It is also considered to be an important parameter to assess the baseline information in evaluating parasite clearance after the commencement of ACT in order to establish resistant development in *Plasmodium falciparum* [17].

Single Nucleotide Polymorphisms (SNP) in PFMDR1 gene were assessed in patients with high parasite density. Only 21% of the isolates were successfully analyzed up to the level of sequencing, 4 non-synonymous SNP mutations were detected at codon N86Y which is responsible for the subsequent introduction of new amino acid in the protein sequence of the Pfmdr1 gene. The amino acid position affected was Asp86Tyr. This change in amino acid has resulted in a point mutation leading to a Synonymous mutation which altered the physiochemical properties as the substituted amino acid is more polar as compared to its substituent [18]. This result is similar to that obtained by Ayogu [19] who identified N86Y mutation and recorded a high prevalence of the mutation following post-treatment with artemether-lumefantrine (AL) in Enugu state, southern Nigeria. This is also in agreement with another work done in Uganda which reported a significant increase in the prevalence of *Pfmdr1* N86Y alleles from pretreatment samples to post-treatment. Another report from Zanzibar showed a significant accumulation of *Pfmdr1* N86Y among patients who had parasites after AL treatment [20]. However, no mutations were identified at codons 184 and 1246 from the study samples. This is contrary to the work of Auwal [21].

Mutations on the *Pfmdr1* gene play a pivotal role in variable parasite response to artemisinin, ACT and non-ACT, such as chloroquine, lumefantrine, primaquine, tafenoquine, piperaquine, and mefloquine [22-24] due to conformational changes in the transporter protein causing a decrease in intracellular drug accumulation and effect on the malaria parasite. Although this study did not assess *Pfcrt* mutation, there is a known synergy between mutations on *Pfmdr1* and *Pfcrt* genes. Studies in Nigeria and other areas have reported reduced *in vitro* and *in vivo* response in chloroquine-resistant isolates bearing the 76T mutation correlating strongly with *Pfmdr1* 86Y mutation [25-27].

CONCLUSION

There is a high malaria transmission rate in Nigeria. These parasites harbour mutations on *Pfmdr1*, which contribute to artemisinin partner drug resistance; surveillance strategies to reduce the spread of drug resistance in endemic areas are needed to eliminate the reservoir of malaria parasites that can mitigate the eradication of malaria in Nigeria.

AUTHOR'S CONTRIBUTION

This work was carried out in collaboration between all authors. Authors A.A. Imam Khadijah S and I.U. Muhammad designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors I.U. Muhammad and A. A Imam managed the analysis of the study and literature searches. All authors read and approved the final manuscript.

ETHICS APPROVAL

The study protocol for patient participation and collection of blood samples for laboratory testing was approved by the Kano State Ministry of Health through the board of ethical committee.

All methods were performed per the relevant guidelines and regulations.

CONSENT TO PARTICIPATE

All study participants provided written informed consent prior to their participation.

CONSENT FOR PUBLICATION

All study participants provided written informed consent for publication of the study.

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