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Detection of Multidrug Resistance Gene 1 (MDR1) Mutation from Plasmodium Falciparum Isolated from some Patients in Kano Metropolis

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Abstract

It has been suspected that the diminishing efficacy of artemisinin-based combination therapies, which are presently the primary therapy for malaria globally, is causing people most especially in northern Nigeria to resort to using alternative antimalarials. To bolster evidence-based strategies for managing resistance, Scientists studied mutations linked to antimalarial resistance in the pfmdr1 gene of Plasmodium falciparum. The study involved the recruitment of one hundred adult malaria patients. Blood samples were examined via microscopy to ascertain the presence of the malaria parasite. The P.

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falciparum infection was found in all the samples screened for parasite density. Only samples with high parasitemia were used for molecular analysis. DNA from positive malaria samples was extracted using a DNA extraction kit. The pfmdr1 gene from the extracted DNA was amplified and resolved using gel electrophoresis and was sequenced. The gene (Pfmdr1), an essential molecular indicator signaling resistance to artemisinin, was successfully sequenced in 14 out of 100 P. falciparum isolates obtained from participants residing in selected local government areas within Kano state. Notably, the mutations were detected in 19.5% (4/30) of the isolated samples. The study identified a significant mutation associated with antimalarial resistance within the pfmdr1 gene in P. falciparum isolates from Kano, Nigeria. The N86Y allele of Pfmdr1 was found in four samples., while the Y184F and D1246Y alleles were not observed. Specifically, four non-synonymous mutations at codon N86Y were identified, with three originating from Kano municipal and one from Fagge L.G.As. The study revealed that socio-demographic characteristics has no significant association with pfmdr1 mutation ($p<0.05$). The existence of these mutations underscores the difficulties faced in treating malaria in northern Nigeria with antimalarial drugs. The study indicates the presence of P. falciparum strains in Kano, Northern Nigeria, that show reduced sensitivity to the artemisinin component of ACT (artemisinin-based combination therapy).

Keywords: Antimalarial resistance, Artemisinin Combination Therapy, Malarial, Mutation, *Pfmdr1*

INTRODUCTION

Malaria poses a significant public health challenge worldwide, causing substantial mortality and morbidity, especially in resource-limited nations across Sub-Saharan Africa, South East Asia, and Latin America. It likely plays a role in perpetuating poverty within these regions (Roll back malaria, 2020). At present, malaria afflicts a larger global population more than any other sickness. It is prevalent in more than one hundred countries, and about 249,000 new cases were recorded in 2022 which makes it among the top ten most widespread and fatal diseases worldwide (WHO, 2023). Lately, advancements in malaria reduction have stalled. The 2023 World Malaria Report indicated that, after major setbacks in 2020 due to the COVID-19 pandemic, the rates of new malaria cases have somewhat stabilized. Despite this, malaria cases and deaths in 2022 were higher than in 2019 (WHO, 2023). The WHO African Region bears the greatest burden, responsible for 94% of malaria cases and 95% of related deaths (WHO, 2023). Current data on malaria in Nigeria indicates that the country bears the largest share of the global malaria burden. In 2022, Nigeria was responsible for approximately 55% of malaria cases in West Africa (WHO, 2023). Malaria stems from a protozoan parasite belonging to the Plasmodium genus. Five Plasmodium species—*Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae,* and *Plasmodium knowlesi* are responsible for human malaria infections. Malaria spreads through the bite of female anopheles mosquitoes that are infected. The introduction of sporozoite forms by mosquito bites initiates the development of blood stages, which can lead to various clinical outcomes, spanning from asymptomatic cases to severe malaria and potential fatality (WHO, 2023). Malaria is associated with varied hematological abnormalities leading to clinical complications (Prasad *et al*., 2018).

The *P. falciparum* multidrug resistance 1 (pfmdr1) gene, also referred to as P-glycoprotein homologue 1 (pgh1), is located on chromosome 5 and has been linked to the parasite's susceptibility to various currently available antimalarial drugs (Veiga *et al*., 2016). Multiple single nucleotide polymorphisms (SNPs) in the pfmdr1 gene have been identified, including D1246Y, N1042D, S1034C, Y184F, and N86Y (Witch *et al*., 2020). Research has demonstrated that these five SNPs are most commonly found in parasites from Asia and Africa. They

contribute to increased resistance to chloroquine (CQ) and amodiaquine (AQ) while simultaneously enhancing susceptibility to a variety of first-line artemisinin-based combination therapy (ACT) drug (Witch *et al*., 2020).

A variety of medications are employed for both the prevention and treatment of malaria (Gorobets et al., 2017). The ACT drugs serve as the primary treatment in nearly all malaria-endemic regions owing to their notable effectiveness, tolerability, and capacity to diminish continued parasite transmission. ACTs consist of two constituents: an artemisinin derivative paired with another drug (WHO, 2023).

MATERIALS AND METHODS

Study Area

The research took place at Murtala Muhammad Specialist Hospital (MMSH) in Kano State, Nigeria. The hospital is located on Muhammad Abdullahi Wase Road in Kano Municipal, Kano State, and primarily caters for individuals from low to moderate socio-economic backgrounds. As the largest state-owned hospital in the region, it is both affordable and easily accessible to the majority of residents in Kano city and neighboring local government areas.
The hospital is situated at latitude 11.96°N and longitude 8.55°E. Kano State shares borders with Kaduna State to the southwest, Bauchi State to the southeast, Jigawa State to the east, and Katsina State to the west. Covering a total area of 20,131 square kilometers (7,773 square miles), Kano State has an estimated population of around 11 million people. The region is predominantly characterized by Sudan savannah vegetation, with an average annual rainfall of 800-900 mm and temperatures ranging from 25–40 °C (with an average of approximately 26 °C), along with a mean relative humidity of 47.43%. The wet season spans from May to October, while the dry season lasts from November to April.

Ethical Clearance

The protocol for patient involvement and blood sample collection in the study received approval from the Board of Ethical Committee under the Kano State Ministry of Health. All procedures were conducted following applicable guidelines and regulations. Before participating, all study participants provided written informed consent. Prior to collecting data and samples, the patients or their guardians (in the case of children) were briefed on the objectives and methods of the study. Subsequently, written and signed informed consent was acquired from all adult participants, as well as from the parents or guardians of participants under 18 years of age.

Administration of questionnaire

Individuals who agreed to participate and met the inclusion criteria were given consent forms and structured questionnaires. These documents were utilized to gather demographic information and pertinent data for the research.

Criteria for inclusion and exclusion

Individuals with fever features referred to the laboratory for malaria parasite testing and provided consent were enrolled in the study. Those directed to the laboratory for tests unrelated to malaria or who declined consent were not included. Additionally, individuals with diabetes, hyperglycemia, or HIV were excluded from the study.

Population under study and size of the sample

The sample size was determined using the formulae

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

Where:

Z = selected critical value of desired level of confidence or risk = 95% confidence level (the value of (1-∞) in standard normal distribution Z- table which is 1.95 for 95%)

p = estimated proportion of an attribute (for prevalence of the attribute) that is present in the population or max variability of the population (prevalence of *pfmdr1* 86Y allele in North-West Nigeria is 7.49%) = 0.0749 (Adamu *et al*., 2020)

d = desired level of precision or margins of error (5%) = 0.05

$$n = \frac{\left((1.95 \times 1.95) \times\ 0.0749\ \times\ (1 - 0.0749)\right)}{(0.05\ \times 0.05)}$$

$$n = \frac{0.248}{0.0025}$$

n = 105.4

A grand total of one hundred participants were enlisted for the study.

Sample Collection

A simple random sampling method was used to select the patients for the study. Samples were gathered during the months of September and October 2019, coinciding with periods of elevated annual rainfall. A volume of two milliliters (2 mL) of blood sample was withdrawn from each participant and placed into properly labeled EDTA containers.

Identifying the existence of *P. falciparum* via microscopic examination.

Thin blood films were fixed using absolute methanol (BDH, England) for 10 seconds, followed by air drying at room temperature before the staining process. Thick blood films were stained with 3% Giemsa (BDH, England) in Gurr® buffered water at pH 7.2 (BDH, England) for 30 minutes (Cheesbrough *et al.,* 2020). All thick and thin blood films were analyzed under a microscope at ×100 magnification using immersion oil. A minimum of 200 fields were observed before labeling a sample as 'negative' or, more precisely, 'no malaria parasites seen.'. This is done to detect the presence of malarial parasite. Positive results were graded on the thick smear using the 'plus' system scale.

Molecular analysis

Extraction of Genomic DNA

The blood samples were taken out of the fridge and allowed to thaw at ambient temperature before the separation process commenced. Micropipettes designated for use were sanitized by moistening them with 70% ethanol, left to soak for a few minutes, and then dried with a disposable handkerchief to prevent contact with dirt during separation.

The extraction of DNA was performed on the complete blood samples of 30 patients who tested positive for malaria using the QIAamp® DNA Mini Kit (QIAGEN, Germany), following the manufacturer's guidelines.

Amplification of *Pfmdr1* fragments

The gene (pfmdr1) segment containing codons 86, 184, and 1246 mutations was amplified from 30 samples to detect potential mutations within the gene. For the initial fragment (N86Y and Y184F), the forward primer Pfmdr1-F (5'- AGA GAA AAA AGA TGG TAA CCT CAG -3') and the reverse primer Pfmdr1-R (5' ACC ACA AAC, ATA AAT TAA CGG -3') were

employed (Basco and Ringwald, 1998). For amplification of the D1246Y fragment, the forward primer (5′-GTGGAAAATCAACTTTTATGA 3′) and the reverse primer (5′ TTAGGTTCTCTTAATAATGCT 3′) were used. The reaction mixture, totaling 20 µL in volume, comprised genomic DNA, primers, master mix from Promega (USA), and double-distilled water. The thermal cycler was set for an initial denaturation at 94°C for 3 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, primer annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and a final extension step at 72°C for 5 minutes.

Agarose Gel Electrophoresis

Polymerase chain reaction (PCR) products were subjected to electrophoresis on a 2% agarose gel. To prepare the gel, size of the amplified DNA fragments was determined using a DNA ladder (Thermo Fisher, USA).

Purifying PCR products

The PCR products were purified utilizing the QIAquick® PCR purification kit from QIAGEN, based in Germany.

DNA Sequencing

DNA sequencing was performed using the Sanger sequencing method in both forward and reverse directions, employing the respective forward and reverse primers. The double-stranded DNA was first denatured into single-stranded DNA. Subsequently, a primer corresponding to one end of the sequence was attached. Four polymerase solutions containing four types of deoxyribonucleotide triphosphates (dNTPs) were utilized. The process of DNA synthesis was initiated, and the chain was elongated until the termination nucleotide was integrated. The resulting DNA fragments were then transformed into single-stranded DNA through denaturation. These denatured fragments were separated by gel electrophoresis, facilitating the sequencing process.

Sequences were examined for single nucleotide polymorphism using BioEdit software tool 7.6.2.1 and multiple sequence alignments constructed in CLC sequence viewer Version 8.0.

Statistical Analysis

The data were examined using an Excel spreadsheet. Statistical analyses were conducted, and variations were assessed utilizing a Chi-square test for categorical variables. A significance level of $p < 0.05$ was deemed indicative of non-significance.

RESULTS

Socioeconomic and demographic traits of the study population

Figure 2 present the frequency of different age group of the participants. The mean age was 36.4 years. Participants were in the range of 16 years to 60 years. 41% out of 100 are within the ages 16 years to 30 years, 29% are within the age of 31 years to 45 years and 30% out of 100 are greater than 45 years of age.

A total of 100 Patients were screened. Figure 3 illustrates the gender distribution of the study participants. 57% (n=57) of the participants were identified as females, whereas 43% (n=43) were males.

Figure 4 depicted the local governments of residence of all the participants involved in the research work. 40% were from Kano municipal, 14% from Gwale, 20% from Fagge, 8% from Nasarawa, 8% Dala and 10% from other local governments.

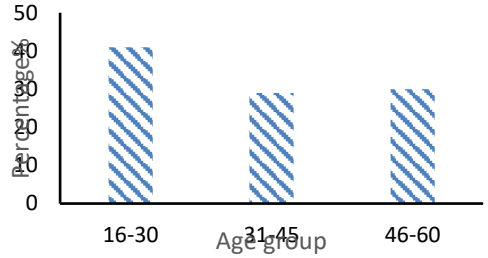

Figure 2: Age group distribution of participants (N=100)

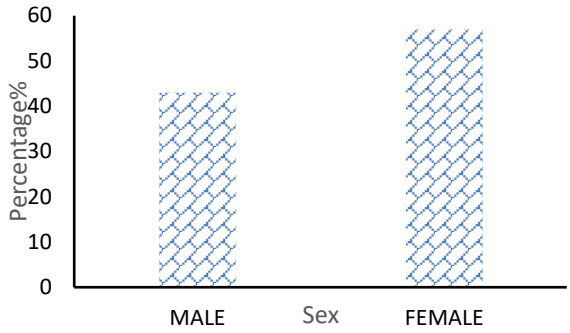

Figure 3: Population distribution by sex of participants (N=100)

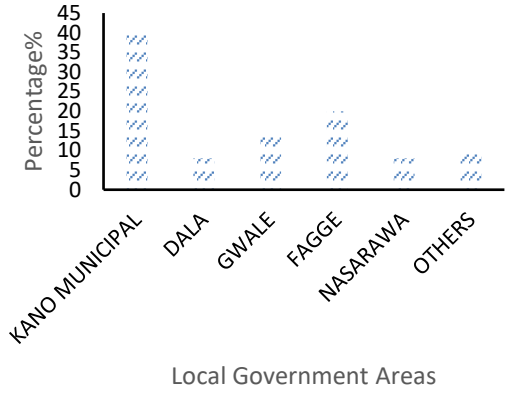

Figure 4: Local Government Areas of participants (N=100)

Educational level of study participants is illustrated in figure 5. Participants with primary education were found to be the majority (56%). Participants with tertiary education have the least number of participants (12%).

The employment status of the research participants is presented in figure 6. The employment status revealed that 62% of participants were self-employed, 17% were civil servants and 21% of study population were not employed.

Previous history of malarial and antimalarial drugs compliance

Figure 7 displays the past malarial infection history of the participants. A total of 80% of the participants reported having had a malarial infection within the preceding three months.

Figure 8 present the number of participants that took the complete dosage of prescribed ACT (83%) and those that did not complete their drug dosage during the previous malarial infection (17%).

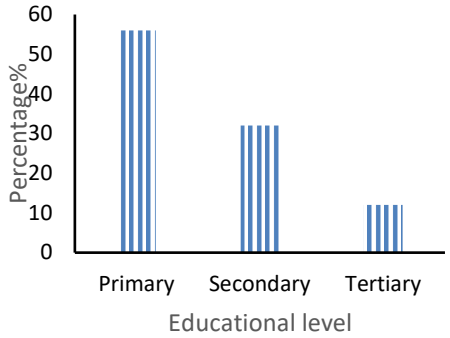

Figure 5: Level of education of study participants (N=100)

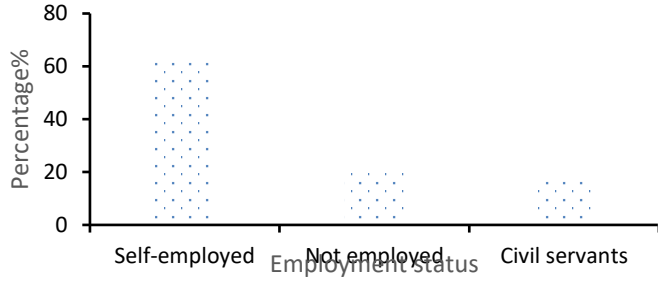

Figure 6: Employment status of study participants.

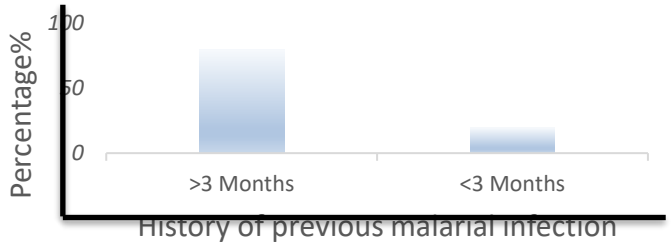

Figure 7: History of previous malarial infection in the study participants (N=100)

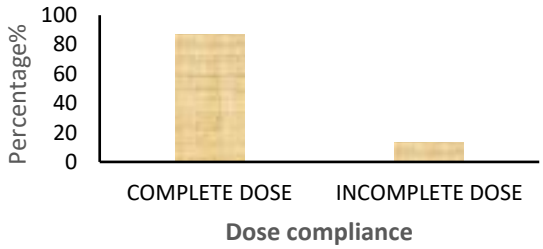

Figure 8: Past compliance with drug dosage during prior malaria infections among the participants in the study.

Parasite density

Figure 9 presents the parasite density of blood isolates obtained from the participants. 68% of the participants have 1+ parasitemia which is higher than those participants that have 2+ parasitemia. Participants with 3+ are found to have the lowest number 9%.

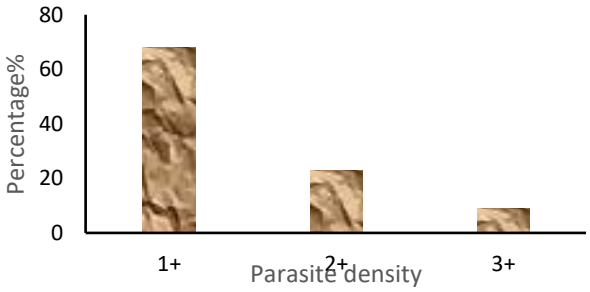

Figure 9: Parasite density of blood isolates found in the study participants.

Amplification of fragment of *Pfmdr1* gene

The outcomes of the PCR products derived from the pfmdr1 gene isolates collected from the participants are illustrated in Plate 1 and Plate 2. As illustrated in Plate 1, all samples underwent successful amplification for codons 86 and 184, yielding an amplicon size of 610bp.

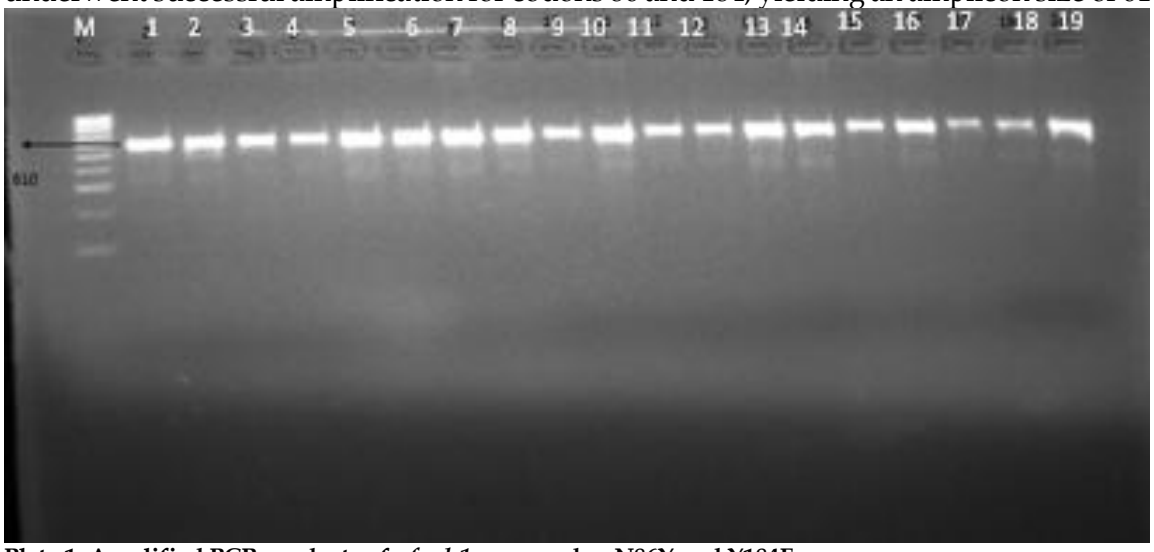

Plate 1: Amplified PCR products of *pfmdr1* gene codon N86Y and Y184F
Lane M: DNA ladder Lanes 1-19: Amplified *pfmdr1* gene

Plate 2 presents the electrophoregram of amplified PCR products of blood isolates for 1246th codon.

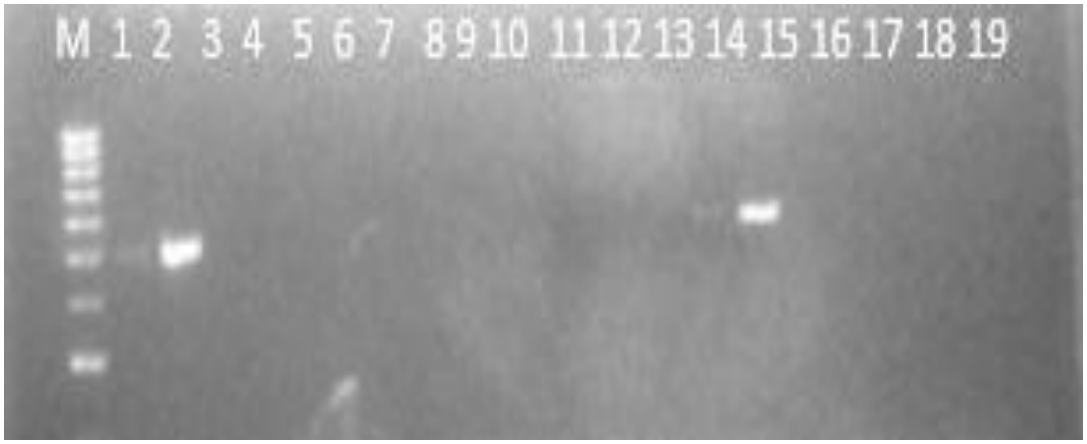

***Plate 2:* Amplified PCR products of *pfmdr1* gene codon D1246Y**
Lane M: DNA ladder Lanes 1-19: DNA samples

Sequencing and Polymorphism Analysis

Figure 10 presents the alignment of the sequences obtained with the reference gene pfmdr1. A total of fourteen (14) out of twenty-one (21) DNA samples sent for sequencing were successfully sequenced and analyzed; the remaining seven (7) failed. Pfmdr1 gene mutations were found in four (19.5%) of the samples studied. SNP occurred at position 256 where AAT was replaced with TAT.

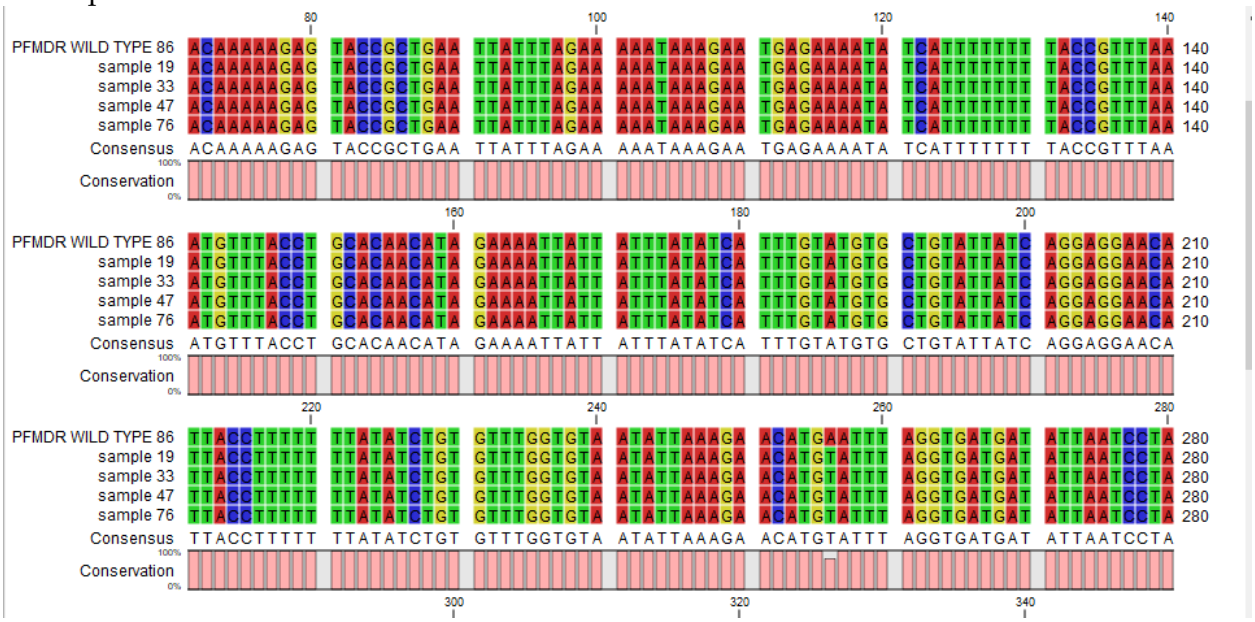

Figure 10: Sequencing Isolates with the reference gene

PFMDR WILD TYPE 86: Sequence of susceptible strain (reference gene)

The nucleotide sequence of the pfmdr1 gene was converted into the corresponding amino acid sequence of the gene's protein, as depicted in Figure 3.4.2. At codon 86, the amino acid Asparagine (N) was substituted with Tyrosine (Y).

Figure 11: **Analyzing the sequences of aminoacid against the reference pfmdr1 gene.**

PFMDR WILD TYPE 86: Sequence of susceptible strain (reference gene).

Single nucleotide polymorphism (SNPs) were detected in four (4) samples. Samples 19, 47 and 76 were from Kano municipal while sample 33 was from Fagge local government area of Kano State.

Table 1: Changes observed in the *pfmdr1* gene and the amino acids substitutions

Samples	Variation	L.G.A	Codon location	Reference Nucleotide	Mutant Nucleotide	Reference amino acid	Amino acid substituted
19	NS	Kano municipal	86	**A**AT	**T**AT	Asparagine	Tyrosine
33	NS	Fagge	86	**A**AT	**T**AT	Asparagine	Tyrosine
47	NS	Kano municipal	86	**A**AT	**T**AT	Asparagine	Tyrosine
76	NS	Kano municipal	86	**A**AT	**T**AT	Asparagine	Tyrosine

NS: Non-synonymous.

Table 2 presents the relationship between sex, previous malarial infection, dose compliance and age group as a possible predictor of *Pfmdr1* SNP mutations. Based on the analyses performed, the chi-square value suggests that sex, previous malarial infection, dose compliance and age group had no influence on the development of SNP mutations.

Table 2: Association between risk factors and *Pfmdr1* SNP mutations

Risk factor	χ^2	*df*	*P-value*	Level of significance
Sex	2.56	1	0.1096	Not-Significant
Previous malaria infection	3.24	1	0.0719	Not-Significant
Dose compliance	1.96	1	0.1615	Not-Significant
Age group	1.17	1	0.0813	Not-Significant

DISCUSSION

In Nigeria, there is significant concern regarding decreased efficacy of ACTs. Recent research has shown that mutations in drug targets and transporters are key drivers of drug resistance, emphasizing the evolving nature of resistance mechanisms in *Plasmodium falciparum* (Zhou *et al.,* 2023). Since its adoption as the primary treatment in 2005, ACTs have been extensively utilized for uncomplicated malaria treatment in Nigeria. Recent reports from Nigeria have indicated reduced efficacy or the emergence of partial resistance to artemisinin combination therapies (ACTs), consistent with earlier findings (Lucie *et al.,* 2022; Onwujekwe *et al.,* 2021). This study investigated the involvement of single nucleotide polymorphisms (SNPs) in the Pfmdr1 gene of malaria patients in Kano.

The study suggests that socio-demographic factors, such as age, sex, occupation, and geographical location, do not have a significant association with malaria prevalence. This finding aligns with the research by (Shalu *et al*., 2018), which indicated that sociodemographic and household characteristics may not necessarily impact malaria. However, a separate study by Joao et al (Joao *et al.,* 2022) on modeling socio-demographic factors found that age and a prior history of malaria treatment were significant predictors of the disease.

The comparison in the study highlights the consistency and divergence in findings between different research works. The observation of mutations in codon 86 (N86Y) of the *Pfmdr1* gene aligns with Ayogu *et a*l. (2016), who also identified this mutation following artemether-lumefantrine treatment in Nigeria, suggesting a potential association between the N86Y mutation and resistance to this antimalarial drug. This convergence in results supports the idea that the N86Y mutation may play a significant role in antimalarial resistance in *Plasmodium falciparum*.

However, the absence of mutations at codons Y184F and D1246Y in the current study contrasts with the findings of Dokunmu *et al.* (2019) and Auwal *et al.* (2017), who reported the presence of the D1246Y mutation and the N86Y mutation. These differences might be attributed to regional variations in the prevalence of specific mutations, differences in study populations, or methodological approaches used in the different studies. The discrepancies also suggest that while certain mutations like N86Y are common across various settings, other mutations (such as D1246Y) may exhibit more localized or less frequent patterns depending on the specific context of the study, including the geographic location and the treatment regimen employed.

The occurrence of the 184 mutation has also been documented in Nigeria at a high rate. For instance, studies have shown a prevalence of 29.27% in southeast Nigeria (Ikegbunam *et al*., 2019), 28.1% in the southwestern region of Nigeria, and as high as 69.0% in another investigation conducted in the same region (Oladipo et al., 2014). Although the 184F mutation is considered to have limited impact on its own, its coexistence with N86 (N86+184F double mutant) is associated with decreased susceptibility to piperaquine. The existence of the 184F mutation predominantly alongside 86N was observed in recurrent infections following treatment with artemether–lumefantrine in a prior investigation (Dokunmu et al., 2015). In this research, the D1246Y mutation was not found, this could be due to Geographic or Temporal Variation, Sample size, Different Strain Variants or Assay Variability.

Yet, some studies have recorded occurrences of 1246Y at rates of 3.66% in the southeast and 18.6% in the southwest regions of Nigeria, respectively. A study recently published in Lagos, located in the southwestern region of Nigeria, discovered uncommon mutations in Pfmdr1, namely N504K, N649D, F938Y, and S967N, which had not been reported previously (Idowu *et al*., 2019). This underscores the significance of proactive monitoring of this gene and verifying newly identified mutations to address the issue of resistance to multiple drugs. The high prevalence of chloroquine (CQ)-susceptible alleles, such as N86, observed in this study, may be linked to the resurgence of sensitive haplotypes or selective pressure from treatments like artemether-lumefantrine (Nadeem *et al*., 2023). Indeed, the withdrawal of chloroquine in regions with established resistance has been shown to result in the resurgence of chloroquine-susceptible parasites, as seen in several studies, including in Malawi (Mwai *et al*., 2019) which could also promote the selection of alternative resistance associated with the N86 allele, as mentioned earlier.

A prevalence investigation conducted on the spread of drug resistance alleles of Pfmdr1 and Pfcrt in Northwestern Nigeria. (Muhammad *et al*., 2017; Adam *et al*., 2021) showed that in Kebbi state, the prevalence of drug-susceptible alleles N86 of pfmdr1 was higher at 57.8%, followed by Kano state at 52.8%, Katsina at 45.1%, Jigawa state at 39.5%, and Kaduna state at 33.96%. Conversely, Pfmdr1 drug-resistant alleles 86Y exhibited a decline across the states, Declining from 33.0% in Katsina, 32.56% in Jigawa, 31.1% in Kebbi, 18.9% in Kaduna, and 11.32% in Kano. This has indicated a revival of the wild-type alleles. Similar patterns were observed for pfmdr1 N86Y, with a decline in the prevalence of the mutant variant from 46% to 28% between 2005 and 2010, alongside an increase in the wild-type N86 strain from 77% to 86% in Ghana (Akinbo and Mufutau, 2021).

Mutations occurring in the Pfmdr1 gene play a crucial role in determining the varying responses of parasites to artemisinin, as well as to both ACT and non-ACT treatments (Wurtz *et al*., 2014; Kaewpruk *et al*., 2016; Gil and Krishna, 2017). These mutations induce

conformational alterations in the carrier protein, resulting in decreased drug accumulation within the cell and subsequent impacts on the parasite that cause malaria. While this research did not specifically examine Pfcrt mutations, it is well-established that mutations in both the Pfmdr1 and Pfcrt genes often exhibit a synergistic relationship, contributing to chloroquine resistance (Agnandji et al., 2023) Research conducted in Nigeria and other regions has documented diminished responses observed in laboratory settings and within living organisms in isolates resistant to chloroquine containing the 76T mutation, which is closely associated with the Pfmdr1 86Y mutation (Atroosh *et al.,* 2012; Olasehinde *et al.,* 2014; Shrivastava *et al.,* 2014).

CONCLUSION

The study population's *Plasmodium falciparum* parasites possess the drug-resistant gene pfmdr1. The expression of this gene induces resistance to commonly employed antimalarial medications, which could consequently poses a notable public health concern in the treatment and containment of malaria in the study region. The results of this extensively characterized marker should be taken into account when formulating resistance management plans in the study area. Nonetheless, the socio-demographic attributes of the participants do not appear to impact the development of mutations in *P. falciparum* parasites.

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