Genetic Diversity and Molecular Surveillance of Antimalarial Drug Resistance of *Plasmodium falciparum* among Hospitals Patients in Benue State Nigeria

O. A. Adulugba [a*], O. Amali [a], M. M. Manyi [a], T. F. Ikpa [a] and V. U. Obisike [b]

[a] *Department of Zoology, Federal University of Agriculture, Makurdi, Benue State, Nigeria.*
[b] *Department of Animal and Environmental Biology, Abia State University, Uturu, Abia State, Nigeria.*

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Introduction: Malaria is a febrile illness caused by parasites of the genus *Plasmodium* and transmitted by female *Anopheles* mosquitoes. The genetic diversity and antimalarial drug resistance of *Plasmodium falciparum* are some of the major challenges of malaria control programme in Nigeria.
Aim: This study was aimed at determining the genetic diversity, and molecular surveillance of antimalarial drug resistance among patients attending Government hospitals in Benue State, Nigeria.
Methodology: *Plasmodium falciparum* deoxyribonucleic acid was extracted from dried blood spots of 60 positive malariacases among the patients. The diversity of *Plasmodium falciparum* was done by genotyping 3D7 and FC27 families of merozoite surface protein- 2 alleles. The *Plasmodium falciparum* multidrug resistance 1 and *Plasmodium falciparum kelch13* genes of *Plasmodium falciparum* were also amplified and assessed by restriction fragment length polymorphism (RFLP) to survey molecular resistance to antimalarial drugs.

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*Corresponding author: E-mail: adulugbaode@gmail.com;

Results: The results showed that the frequency of 3D7 allele 37(61.7%) was higher than FC27 allele 18(30.0%). The frequency of merozoite surface protein- 2 infections with both allelic types was 5(8.3%). There was a significant difference in the distribution of the merozoite surface protein two alleles (χ^2=25.9,df=2 P<.0.001). Both the *Plasmodium falciparum* multidrug resistance 1 Asparagine 86Tyrosine (N86Y) and Aspartic acid 1246Tyrosine (D1246Y), had 100 % mutant while the 100% while the *Plasmodium falciparum kelch13* G449A had 100% wild type allele.
Conclusion: The current study underscores the need for frequent monitoring of indicators of antimalaria drug resistance in Nigeria.

1. INTRODUCTION

Malaria is a febrile illness caused by parasites of the genus *Plasmodium* and transmitted by female *Anopheles* mosquitoes [1]. *Plasmodium falciparum* is the most virulent and prevalent malaria parasite in Nigeria, accounted for 99.7% of estimated malaria cases [2]. Despite the enormous efforts in control and elimination strategies which include the distribution of long-lasting insecticide-treated bed nets (LLINs), indoor residual spraying (IRS), larval control , improved diagnosis using malaria rapid diagnostic tests (RDTs) and the availability of artemisinin-based combination therapy (ACT); Nigeria had the greatest burden of global malaria cases (27%) and malaria deaths (23%) worldwide in 2019 [3].

The genetic diversity of *P. falciparum* and its innate ability to develop resistance to antimalarial drugs are some of the major obstacles in control and elimination of malaria. Genotypes of the merozoite surface protein two (MSP2) have been used to assessed genetic diversity in Nigeria [4-6]. While, *P.falciparum* multidrug resistance-1 (*Pfmdr-1*) and *Pf*kelch 13 propeller (*PfK-13*) genes have been used to assess antimalarial drug resistance elsewhere [7,8]. The Genetic diversity is one of the predominant features of *P. falciparum* infections [9]. It survives the host's immune responses due to its diversity, which results from several factors such as recombination, chromosome rearrangements, antigenic variation and allelic polymorphism [10]. Genetic diversity in the parasite regulates transmission dynamics, disease severity, anti-malarials drug resistance and impede the development of an effective vaccine against the malaria parasite, since antigenic diversity reduced the efficacy of acquired protective immunity to malaria [7,9,11,12].

Moreover, *P. falciparum* drug resistance is one of the most important problems in malaria control, due to the increasing resistance to almost all anti-malarial drugs, including amodiaquine, chloroquine, mefloquine, sulfadoxine /pyrimethamine and artemisinin-based combinations [13,14].

According to Federal Ministry of Health [15], chloroquine and sulphadoxine-pyrimethamine are no longer effective in treating malaria due to high treatment failures resulting from widespread resistance in Nigeria. Nigeria adopted Artemisinin-based combinations (ACTs) as a first-line treatment of uncomplicated *P. falciparum* in 2005. ACTs are made up of a rapid but short-acting artemisinin and a long-acting partner drug combined to reduce the emergence of resistance [16]. Artemether–Lumefantrine (AL) and artesunate-amodiaquine are the recommended artemisinin-based combinations for the treatment of uncomplicated malaria in Nigeria, and their suitability has been confirmed by results of therapeutic efficacy studies, which showed a cure rate of greater than 95% [17]. Some studies that investigated the prevalence of mutations in the kelch13 gene did not observe any of the major mutations associated with artemisinin resistance from Nigeria [14,18,].The present study was conducted in continuation of efforts to monitor the diversity of *P. falciparum* and molecular evidence aimed at determining resistance to antimalarial drugs among patients attending Government hospitals in Benue State, Nigeria.

2. MATERIALS AND METHODS

2.1 Study Area

The study was conducted in six selected General Hospitals in Benue State. The General Hospitals are located in Adikpo, Gboko, Naka, Okpoga, Otukpo and Sankera. Benue State is in the north central of Nigeria, with its capital at Makurdi. Its geographical coordinates lies between latitudes 6°30′ and 8°10′N of the Equator and between

longitudes 7°50′ and 10°E′ of the Greenwich Meridian. It is surrounded by six states, namely, Nassarawa to the north, Taraba to the northeast, Cross River and Ebonyi to the south, Enugu to the southwest and Kogi to the west. There is also a short international boundary between the state and the Republic of Cameroun along Nigeria's southeast border. Benue State is made up of 23 local government areas (LGAs); 14 and 9 of these LGAs correspond with the ethnic territory of the Tiv and Idoma people, respectively.

The State had a total population of 4,253,641 in 2006 census, made up of 2,144,043 males and 2,109,598 females. The state had a sex ratio of 1.02, a literacy rate of 44.7% among the population aged 6 years and above, and a population density of about 130 persons per square kilometer (Federal Republic of Nigeria, 2009; National Population Commission [20]. There are three major ethnic groups inhabiting the state. The Tiv form about two thirds of the population and are found in 14 of the 23 LGAs. The Idomas and the Igedes occupy the remaining 9 LGAs. Other ethnic groups include the Etulo, who are mainly found in Katsina Ala and Buruku LGAs, the Afia in Ado, the Nyifon in Buruku, and the Jukuns in Makurdi and Guma LGAs.

Agriculture is the mainstay of the economy, engaging over 75% of the state farming population. The State also boasts of one of the longest stretches of river systems in the country with great potential for a viable fishing industry, dry season farming through irrigation and for an inland water highway. The vegetation of the southern parts of the state is characterised by forests, which yield trees for timber and provide a suitable habitat for rare animal's types and species. The state thus possesses potential for the development of viable forest and wildlife reserves.

Malaria is transmitted through the bites of infected female *Anopheles* mosquitoes. These Anopheles species are widely distributed in Benue State. Malaria is present throughout the year with a marked increase during the raining season due to climatic factors most especially temperature as reported by Manyi et al., [21].

2.2 Study Design, Study Population and Sample Size

The study was conducted between September, 2018 and August, 2019; which was hospital based and it considered only outpatient subjects who were referred to Laboratory Department for medical diagnosis. The population consisted of 1200 subjects, including both children and adults who were screened for *P. falciparum* infections. Sixty (60) positive samples of dry blood spot were used for molecular analysis.

2.2.1 Sample collection

Falciparum malaria positive blood samples were detected from the subjects using CareStart[TM]*Pf*RDTs and microscopy. Thereafter, the positive blood samples were collected on 3 MM Whatman filter papers (Whatman International Ltd., Maidstone, England) and labeled accordingly. The blood-spotted filter papers were allowed to air dry completely, transferred into individual plastic bags, labeled and stored at room temperature in a desiccator containing silica gel and were then transported to Zoology Laboratory, Federal University of Agriculture, Makurdi for molecular analyses.

2.2.2 Extraction of parasite DNA and confirmation of *Plasmodium falciparum* infections by PCR

Parasite DNA was extracted from 60 of the malaria positive blood samples using a paper punch to remove a circular piece of the dry blood spot. The circular piece of the dry blood spot was divided into 4 pieces with a scalpel blade and transferred into a 1.5 ml microcentrifuge tube. The parasite DNA was then extracted using the tissue protocol of Zymo -DNA[TM] Miniprep Plus Kit (Zymo, Irvine, California, USA) based on the manufacture's recommendation. All samples were PCR tested to confirm the presence of *P. falciparum* as previously described by Snounou and Singh (2002). Confirmed *P. falciparum* positive samples were subsequently used for *MSP-2* genotyping, *PfMdr1* and *PfK-13* RFLP analysis.

2.2.3 Genotyping of *Plasmodium falciparum* MSP-2 alleles

Plasmodium falciparum DNA samples were analyzed by amplification of the highly polymorphic regions of merozoite surface protein-2 (MSP-2 Block 3),using a set of specific primers in nested PCR reactions as previously described by Snounou et al. [22]. The sequence of oligonucleotide primer sets used for detecting the two families of MSP-2 (FC27 and 3D7alleles) are given in (Table 1). All reaction conditions

were the same as previously described, except that the reaction volume for both primary and the secondary PCR in each case was 25.0µl.

2.2.4 Molecular surveillance of *Pfmdr1* mutations in *Plasmodium falciparum* isolates

The sequences of codons, Asparagine 86Tyrosine (N86Y) and Aspartic acid 1246Tyrosine (D1246Y) were amplified with primer pairs *PfMDR* 754-F/754-R and *PfMDR-5*/*MDR*-6 to have the fragment length of 355 bp. The total reaction mixture of 25 µl consisted of 1x PCR buffer, 1.5mM $MgCl_2$, 0.2 mM of each deoxynucleosidetriphosphate (dNTP), 0.2 µM of each oligonucleotide primers, 1.0 unit of *Taq* polymerase (New England Biolabs) and 2.0µl of DNA template. The target amplification of MDR1 was programmed for initial denaturation at 95°C for 3 min, 35 cycles of 94°C for 30sec, 55°C for 30sec, 72°C for 20 sec and final extension at 72°C for 5min. The PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized with ultraviolet (UV) transillumination [13].

2.2.5 Restriction enzymes digestion of *PfMdr1* PCR products

After amplification of the 355 bp fragments by each of the primer pairs (754-F/754-R and MDR5/MDR6) the polymorphisms in the *Pfmdr1* genes were assessed by restriction digestion with appropriate enzymes. For detection of N86Ymutant, the amplified fragment was digested with *Nsp1*, the D1246Y mutant was detected by digestion with *Eco*RV restriction endonucleases (New England BioLabs). All incubations were setup following the manufacturer's recommendation. Appropriate control DNA of samples with known *Pfmdr1*

sequences was used in parallel with field-collected parasite isolates in every PCR-RFLP protocol. The products resulting from restrictions of *pfmdr1* N86Y and *pfmdr1* D1246Ydigests were run on 2% agarose gels and stained with ethidium bromide and visualized under UV transillumination.

2.2.6 Amplification of *PfKelch13* and restriction digestion of G449A

Plasmodium falciparum kelch13 gene sequence with codon Glycine 449 Alanine (G449A) was amplified from the DNA using semi nested PCR following the protocol previously described by Khammaneeet al. [23], with some modifications. In the first PCR, the full-length *Pfkelch13* gene sequence (630bp) was amplified with a primer pair F0/R0 to produce amplicons of 630 bp followed by a semi nested amplification of 260bp fragment with primers F0/R1(Table 2). In each PCR reaction, 2.0 µl of DNA template was used in a total reaction volume of 25 µl. All other reaction conditions were as previously described by Lopes et al. [24] and Khammanee et al. [23]. The PCR products were separated on 2% agarose gel electrophoresis stained with 0.5µg/ml ethidium bromide and visualized under UV light.The amplified 260 bp fragment was subsequently digested, with *Alu* restriction enzyme as previously described by Lopes et al. [24] and separated on 2% agarose gel electrophoresis.

2.3 Statistical Analysis

Simple percentage and Chi-squared test were used for data presentation. Statistical analysis was performed using Statistical Product and service solution (SPSS) software version 18 (IBM., USA).The significance level was considered at P≤0.05.

Table 1. Sequences of the oligonucleotide primers used to Genotype *P. falciparum*

Gene	Primer	Sequence
	M2-OF	5'- ATGAAGGTAATTAAAACATTGTCTATTATA-3'
	M2-OR	5'–CTTTGTTACCATCGGTACATTCTT -3;
msp²	M2-FCF	5' – AATACTAAGAGTGTAGGTGCARATGCTCCA -3'
	M2-FCR	5' – TTTTATTTGGTGCATTGCCAGAACTTGAAC

Gene	Primer	Sequence
		-3'
	M2-ICF	5' – AGAAGTATGGCAGAAAGTAAKCCTYCTACT -3'
	M2-ICR	5' – GATTGTAATTCGGGGGGATTCAGTTTGTTCG -3'

Snounou et al. [25].

Table 2. The sequences of codons 86, 1246 and k13 were amplified with primer pairs

| Codons	Primer sequences	Restriction enzyme	Size
N86Y	MDR1F(5'-AGAGAAAAAAGATGGTAACCTCAG-3') MDR1R(5'-ACCACAAACATAAATTAACGG-3')	*Nsp1*	355bp.
D1246Y	MDR2F (5'-GCGGAGTTTTTGCATTTAGTTCAGATGATG-3') MDR2R (5'-AGCAGCAAACTTACTAACACGTTTAACATC-3')	*EcoRV*	355bp
PfKelch13 (G449A)	FO- (5' –CAAAAGATTTAAGTGAAAGTGAAGCCTTGTTG-3') RO-(TAAAAGCTGCTCCTGAACTTCTAGCTTC-3') R1(5'AAAGCCTTATAATCATAGTTATTACCACCAAACACGT-3')	*Alu1*	186bp 74bp

Lopes et al. [24]; Price et al. [26];Khammanee et al. [23]

3. RESULTS

The genetic diversity of the *P. falciparum* isolates using *MSP*-2 Block 3 as a genetic marker showed that the frequency of isolates possessing 3D7 family with, 37(61.7%) was higher than the FC27 allele with 18(30.0%). Infections with both allelic types were identified in 5(8.3%) of parasite isolates (Plate 1 and Fig. 1). There was a significant difference (χ^2=25.9, df=2, P< 0.001) between the frequency of FC27 and 3D7 alleles in the study area.

Prevalence of the single nucleotide polymorphism (SNPs) in the *pf*mdr-1 and Kelch13 gene.

Out of the 60 samples of 355 bp amplicons of 754-F/754-R primers, and MDR-5/MDR-6 primer pairs each digested with *Nsp1* and *EcoRV* to assess the mutations at codon 86 and 1246 of PfMdr-1 gene, all codons remained uncut, yielding 100% of mutant alleles at codons 86 and 1246 of the PfMdr-1 gene. In contrast, the Alu1 restriction digest of 260 bp fragment of F0/R1 primer pair of *Pf*Kelch-13 gene for mutation assessment at codon 449 yielded 100% digestion into 186 bp + 74 bp indication wild type alleles (Plate 2 and Table 3).
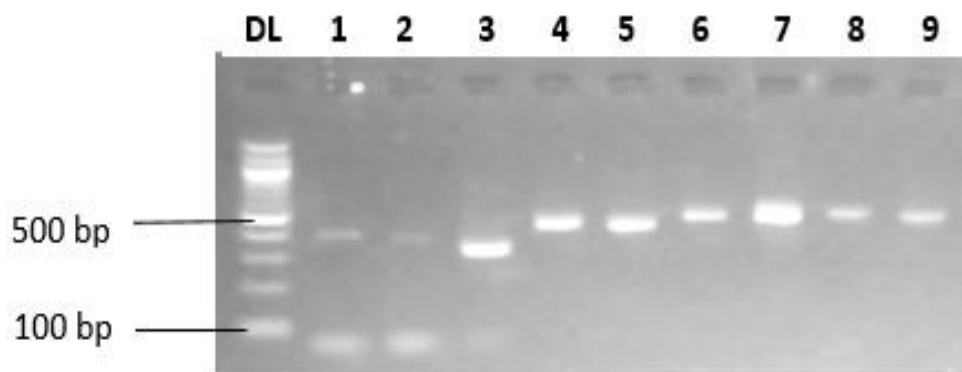
Plate 1. MSP 2 Clones: Lanes 1, 2 and 3 = FC27 Clones, Lanes: 4 -9 = 3D7 Clones
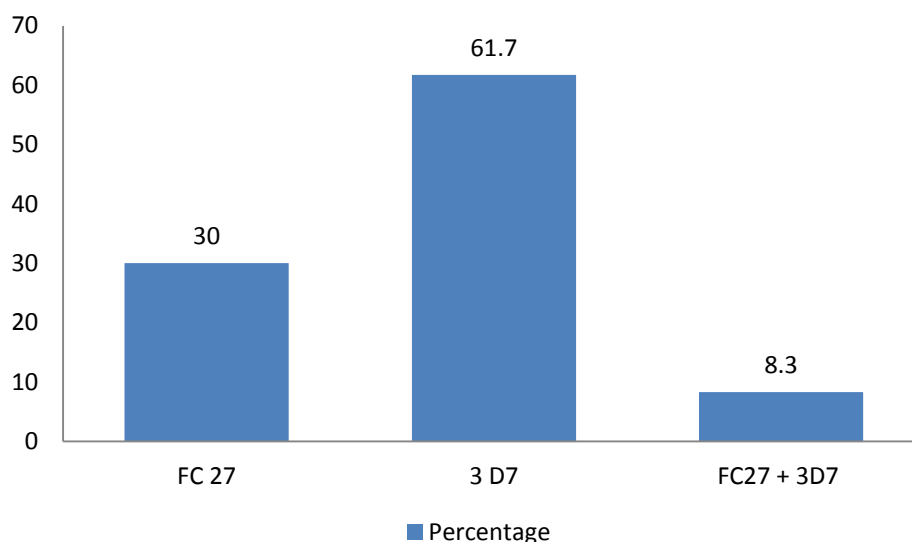
Fig. 1. Frequency of Genetic diversity of *Plasmodium falciparum* using MSP2
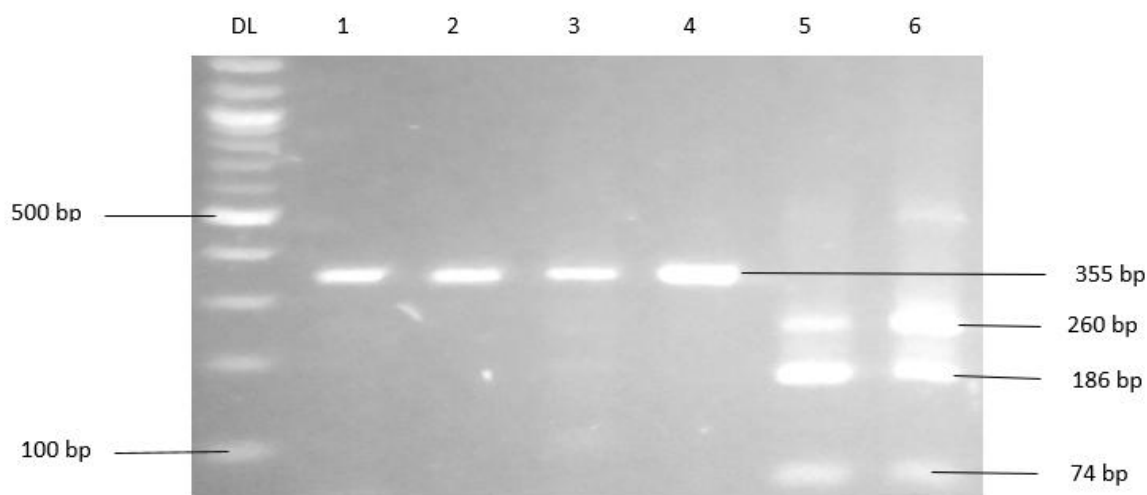
Plate 2. Restriction endonuclease digestion of *P. falciparum* PCR amplicons for detection of antimalarial drug resistant mutations
Dl = 100 bp DNA size marker,
Lanes 1 and 2, 355 bp fragments amplified with MDR 5/MDR 6 primers, and digested with Eco RV HF restriction enzyme remains uncut (mutant)
Lanes 3 and 4: 355 bp fragments amplified with 754 F/754 R primers and digested with Nsp 1 restriction enzyme remains uncut (mutant)
Lanes 5 and 6 260 bp fragment of Kelch 13 gene amplified with F0/R1 primers and digested with Alu 1 Restriction enzyme cuts the fragment to 186 bp + 74 bp (wild type)

Table 3. Status of mutation in antimalarial drugs among patients in Benue State

Gene locus and codon	Mutant	Wild type	Total
PfMdr1 N86Y	60 (100.0%)	0(0.0%)	60 (100.0%)
PfMdr1 DI246Y	60 (100.0%)	0 (0.0%)	60 (100.0%)
*Pf*Kelch13(G449A)	0(0.0%)	60 (100.0%)	60 (100.0%)
Total	120 (66.7%)	60 (33.3%)	180 (100.0%)

X^2= 180.00; df=2; p=.000

4. DISCUSSION

The genetic diversity of *P.falciparium* infections in Benue State was determined by polymerase chain reaction (PCR) using the merozoite surface protein two (MSP2).The results of this study revealed that the 3D7 parasite clones were more abundant, compared to FC27 clones. Similar results was reported in Lafia North Central Nigeria by Oyedeji et al. [5]. Also, the result corroborates with earlier reports from Jharkhard India, Tripura Northeast India, and Cameroon by Hussain et al. [9]; Patgiri et al. [7] and Metoh et al. [26. In contrast, the reports from Ogun State, south western Nigeria by Olasehinde et al. [4]; Lagos State by Oyebola et al. [6] and Minna, Nigeria by Omalu et al. [10] reported that FC27 had higher frequency than 3D7 alleles. In Sabah, East Malaysia and Bioko Island in Equatorial Guinea, it was shown that FC27 had higher frequency than 3D7 alleles in those areas [11,27].

The observed differences could be due to differences in the rate of malaria transmission at these locations. It could also be due to natural selection which is more efficient when acting on the Msp-2 families. Hussain et al. [9] observed that differences in parasite types could be attributed to host immune selective pressure on particular types or spatiotemporal changes in the availability of different mosquito species that can transmit specific parasite types in a particular area and at a given time or seasons, with areas that have the same mosquito species tending to have similar parasite types.

Usually, the high intensity of malaria transmission in an areas is characterized by high parasite diversity, and infected individuals usually carry multiple parasite genotypes [10]. Thus in low malaria transmission areas parasite populations may have few genetic diversity leading to most infections being monoclonal [6]. Also, it could be as result of antimalarial treatment by the patients prior to their presentation to secondary health facilities.The *Msp2* is a highly polymorphic marker that has been used for genotyping recurrent parasitaemia in anti-malarial drug trials, but one argument is that in such trials, treatment is likely to reduce the number of genotypes in an infected individual, which may compromise the suitability of the *Msp2* antigenic markers as a genotyping tool for drug efficacy tracking [28]. The acquisition of immunity, the spread of the drug resistance, the condition of transmission, and the design of effective vaccines against *P. falciparum* and control strategies are some useful applications that may require the knowledge about *Msp2* diversity in a given area [9,27].

Resistance to anti-malarials is a major public health problem worldwide. Mutations in *P.falciparum* genes, including *Pfmdr1* and *Pfk13* are associated with variation in parasite sensitivity to a range of drugs [13,29] . The *Pfmdr1* mutation is known to modulate *P.falciparum* susceptibility to various antimalarial drugs by regulating the influx of the drugs into the parasite's digestive vacuole [30]. Single nucleotide polymorphisms in the *Pfmdr1* gene such as the N86Y and D1246Y mutation lead to changes in the physicochemical properties of the transporter thereby altering its ability to bind and transfer the target drugs [31,7]. The present study revealed 100% mutation in*Pfmdr1* gene for N86Y and D1246Y, which may be a sign of resistance to partner drugs that are used in combination with artemisinin such as amodiaquine and lumefantrine. However, there was 100% wildtype alleles recorded in *Pfkelch13*gene at codon 449, indicating the absence of the G449A mutation for Artemisinin. The implication is that while the artemisinin or its derivative in artemisinin based combination drugs used in the area may appear to lack resistance developed against it, the fact that the partner drug such as amodiaquine and lumefantrine may have resistance developed against it will expose the artemisinin or its derivative to early risk of parasite resistance since it may be acting alone. Thus there may be a need to carry out an *in vivo* drug sensitivity study with the artesunate/amodiaquine and artemether lumefantrine combinations in the study area to observe the current efficacies of these artemisinin drug combination therapies which are currently used for the treatment of uncomplicated malaria in the area of study.

The above argument is consistent with the recent observation that six mutations in *Pfkelch13* gene (E433G, F434I, F434S, I684N, I684T, and E688K) were identified in northern Nigeria, among which E433G and E688K were identified from isolates with the delayed clearance artemisinin based combination [18]. With the current use of ACTs for *P. falciparum* malaria throughout the world, none of these mutations detected in Nigerian isolates has been fully validated *in vivo* or *in vitro* for resistance to ACTs.

5. CONCLUSION

Conclusively, the genetic diversity of *P. falciparum* isolates in Benue State and the very high level of key mutations (N86Y and D1246Y) associated with antimalarial drugs resistance in the *Pfmdr1* gene in this study is of great concern. This is because it could threaten the efficacy of partner drugs in the ACTs and thus expose artemisinin or artemether to be a stand-alone drug in ACTs that are being used in Nigeria. If this were to persist, it would lead to early development of resistance to ACT combinations. Thus, diligent surveillance is needed to monitor the susceptibility of artemisinin-based combination therapies in the Nigeria.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT AND ETHICAL APPROVAL

Permission was sought for and obtained from the Ethical Committee of the Hospitals Management Board, Makurdi. Patients presenting themselves for Laboratory test in the selected General hospitals were duly informed on the significance of the study. Informed consent of adults and parents/ guardians of the children were obtained before blood sample collection for the tests.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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