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Molecular markers associated with drug resistance in *PFMDR-1* gene of *Plasmodium falciparum* in Gombe Local Government Area, Gombe State, Nigeria

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ABSTRACT

Malaria is a serious deadly disease in Nigeria, and for long prevention and treatment of the disease depended heavenly on different classes of synthetic drugs. However, *Plasmodium falciparum* has developed resistance to almost all the classes of the synthetic antimalarial drug in use, including the most recently recommended drugs (artemisinin-based combination therapies). The aim of this research was to identify and characterize molecular markers associated with drug resistance in *pfmdr-1* genes of *P. falciparum* from Gombe Local Government Area (LGA). Two hundred study subjects were recruited for the research from 3 selected hospitals. Blood samples were collected using vein puncture technique and analyzed using Giemsa stain, rapid diagnostic test, and polymerase chain reaction. Zymo DNA extraction kit was used to extract the DNA samples, from which 167 samples confirmed to be positive by microscopy. A NanoDrop spectrophotometer was used to determine the concentration and purity of the extracted DNA. Fifty seven true-positive samples with high DNA concentration and purity were selected and used for the molecular analysis. Nested polymerase chain reaction was used to amplify the portion of the selected genes and the amplicons were sequenced for the actual determination of single nucleotide polymorphism in *pfmdr-1* gene. Single point mutation was observed in four of the variants (N86Y, Y184F, N1042D, and D1246Y) and the highest prevalence (34, 59.64%) was from the Y184F variant. Conclusively, some basic biomolecular makers of *pfmdr-1* gene were reported from Gombe LGA. Further research should be carried out in the local government area using a large sample size and targeting some other candidate bio markers of the gene.

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1. Introduction

Malaria is a life-threatening parasitic disease and is considered a complex and overwhelming public health problem. It has been a common disease and it continues to be one of the most widely spread health hazards in tropical and subtropical regions. In fact, the disease is a tropical disease that poses serious problems to human being in the region where the environment provides conducive grounds for the parasite and the vector to thrive (Ngozi et al., 2018). The disease has been in existence for over 500 decades. The Chinese medical classic *Nei Chin* (the Canon of Medicine) was

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the first established document to document the fact on the disease since 2700 BC (Claudia et al., 2006).

Over 3.5 billion people globally (more than half of the world's population) live in the areas where they remain at risk of malarial infection (Olasehinde et al., 2015). The disease is a mosquito-borne disease and caused by four species of *Plasmodium* parasites (*P. falciparum*, *P. vivax*, *P. mallarie*, and *P. ovale*), with *P. falciparum* and *P. vivax* being the predominant species. The parasite (*P. falciparum*) is transmitted through the bite of an infected female *Anopheles* mosquito during blood meal (Muhammad and Naphtali, 2014). Recently, a fifth species *Plasmodium knowlesi*, which is a zoonosis, was diagnosed by microscopy and represents a small portion of the infection.

Children under the ages of five and pregnant women are the most vulnerable to malaria, wherein pregnant women result in serious complications and conditions such as maternal anemia, fever, fatal anemia, abortion, still-birth, and even death of the child or mother before birth or soon after delivery (Emmanuela et al., 2014; Patrick et al., 2003). The devastating effect of the parasites arises when the asexual form of the parasites (merozoite) invades the red blood cells and destroys them, thereby leading to severe complications and resulting in fever, anemia, lactic acidiosis, organ failure, cerebral damage, and in some cases coma and death. In addition, asexual multiplication of the malaria parasites in human blood is associated with morbidity and mortality due to the disease (Cabrera and Cui, 2015; Kalyan et al., 2017). According to the World Health Organization (WHO), half of the world's population are at risk of malaria. It is estimated that there are about 300–500 million new cases of malaria each year, with 1.5–2.7 million deaths, worldwide. This translates to a death from malaria every 30 seconds, rendering it an eminent disease particularly in Africa, where about 90% of the global malaria cases are recorded (Patrick et al., 2003). The number of deaths decreased substantially between 2000 and 2012 by more than 45% globally and 49% in African countries, resulting in an estimated 3.3 million deaths prevented over this period (Zahra et al., 2012).

In the absence of a suitable vaccine treatment (Wiriyaporn et al., 2014), prompt, effective, and well-tolerated treatment, through the use of antimalarial drugs, remains one of the cornerstones in the disease management (Eric et al., 2015). Three main classes of antimalarial drugs are recommended by the WHO: quinolines, antifolates and artemisinins. Artemisinin-based combination therapy (ACTs) is the current first-line treatment as they are extremely safe and effective after 3 days of dosing, and currently there is no alternative to artemisinin for treatment of malaria. Drug resistance has been documented in three of the five species known to affect humans in nature: *P. falciparum*, *P. vivax*, and *P. malarie.* The emergence and spread of *P. falciparum* resistance is now one of the greatest challenges facing effort to control malaria. Resistance to currently available drugs is still a cause of therapeutic failure and of the persistence of the high level of morbidity due to malaria (Halima et al., 2006).

One major cause of resistance is genetic mutations that usually occur on adverse drug reaction genes (ADR genes), thereby rendering the antimalarial drugs ineffective in most of the patients affected by the parasites. Generally, eight of these genes, *P. falciparum* ABC transporter (*pfabc*), *P. falciparum* putative metabolite/drug transporter (*pfmt*), *P. falciparum* dihydropteroate synthase (*pfdhps*), *P. falciparum* multidrug resistance-1 (*pfmdr-1*), *P. falciparum* multidrug resistance-2 (*pfmdr-2*), *P. falciparum* sodium/hydrogen exchanger-1 (*pfnhe-1*), *P. falciparum* Dihydrofolate reductase thymidylate synthase (*pfdhfr-ts*), and *P. falciparum* choloroquine resistance transporter (*pfcrt*), are associated with resistance in *Plasmodium*, and geographical distribution of resistance to any one particular drug vary greatly. Therefore, the aim of this research was to isolate, characterize, and identify some molecular markers associated with drug resistance in *pfmdr-1* genes of *P. falciparum* from Gombe Local Government Area of Gombe state.

2. Methodology

2.1. Study area

The study was conducted in Gombe local government area, Gombe state, Nigeria. The local government area lies between longitudes 11°14'07"E and 11°4'42"E, and latitudes 10°16'48"N and 10°17'24"N, with a total land mass 52 km2. Gombe local government area has a projected population figure of 367,500 people (3.3% annual change) (National Population Commission, 2006). The vegetation of the local government is typical of that of Gombe state which is Sudan savannah and experience two distinct season: dry season, which normally commences from November to March, and rainy season, from April to October, with a mean annual rainfall of 863.2 mm. Agriculture is the major occupation in the region (mostly peasant farmers), while some engage in business and few are civil servants. With the local government area being the state capital, both the tertiary (Federal Teaching Hospital) and the secondary (Gombe State Specialist Hospital)

health facilities of the state are domiciled. This is also in addition to the primary healthcare centers that are strategically located in each ward of the local government area. Also, there are quite a number of private hospitals providing different services including malaria diagnosis and treatment.

As it is in the other part of the country (Nigeria), malaria infection is endemic in the local government area, with *P. falciparum* accounting for majority of the reported cases, and very few *P. malaria*. Malaria transmission is all-year round in the local government area with it peak during the rainy season (August–October). The malaria vector (female *Anopheles* mosquito) thrives very well in the local government due to conducive climatic conditions (warm and humid) coupled with the attitude and lifestyle of the people in the local government area, which gives the vector a better chance of survival and rapid multiplication. Figure 1 shows the map of the study site.

2.2. Ethical consideration

Before the commencement of the research, the research proposal was submitted to the Gombe State Ministry of Health for approval. After which, the approval was communicated via letter MOH/ADM/621/VOL.I/222, dated 21 February 2020.

2.3. Study subjects

Human beings of all age groups and gender who willingly and voluntarily agreed to participate in the study were used as the study subjects for the research. Three recruitment centers were selected; they are Gombe Town Maternity (Gidan Magani), Sunnah Clinic, and Idi Children and Women Hospital Gombe. Therefore, a total of 200 volunteers were recruited from the 3 centers.

2.4. Consent of the subjects

Before collecting blood samples from the study subjects, verbal and/or written consent of the subjects was sought after briefing them on the research and the need for them to participate. In a situation whereby the subjects were not mature enough, consent of his/her parents/guardian were sought. All the subjects were assured that all information collected from them will be strictly used for the purpose of the research and will be treated with all levels of confidentiality. In addition, quality control and quality assurance will be assured when handling and treating each of the samples.

2.5. Inclusion and exclusion criteria

Only patients who reported themselves to the selected hospitals [Gombe Town Maternity (Gidan Magani), Sunnah Clinic and Idi Children and Women Hospital Gombe] with (presumed to be malaria-positive) symptoms of malaria (fever) or history of fever in last 24 hours; referred by a physician for the screening of malaria infection; and, in addition, they have not used any antimalarial drugs 28 days prior to the

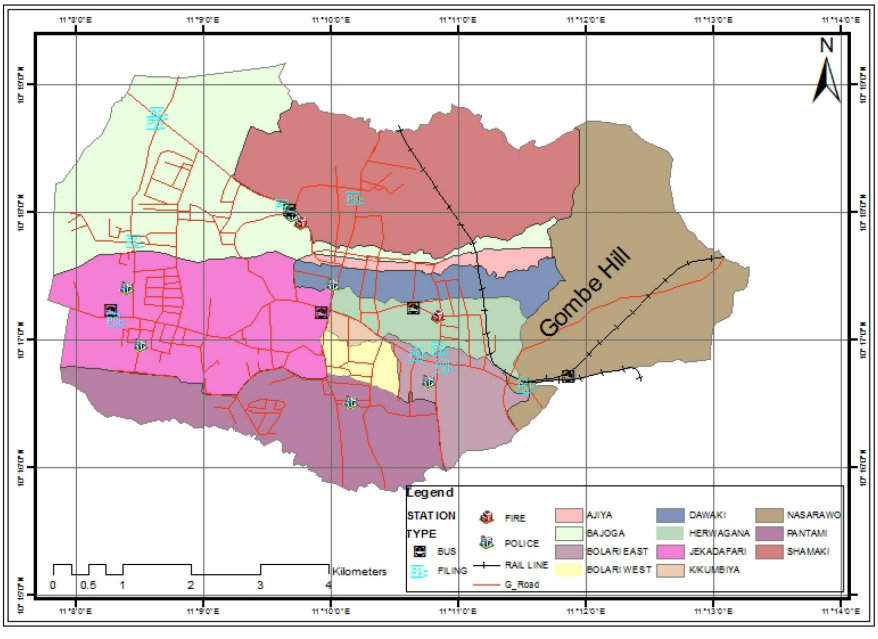

Figure 1. Map of Gombe Local Government Area**.** Source: GIS Laboratory, Geography Department, Gombe State University.

data collection were considered. All subjects having multiple infections were not recruited in the research; only subjects with *P. falciparum* monoinfection and true-positive were recruited for the research.

For molecular analysis, only DNA samples with a very good concentration (2.0 ng and above) and high level of purity (A260/280 value between 1.8 and 2.0) were used.

2.5.1. Blood sample collection and analysis

Vein puncture technique was used to collect venous blood. The blood samples were collected with the help of a medical personnel and the method employed was venepuncture. In this technique, a soft tubing tourniquet was fastened onto the upper arm of the respondents to enable the index finger to feel a suitable vein. The puncture site was then cleaned with methylated spirit (methanol) and venepuncture was made with the aid of a needle attached to a 5 ml syringe. When sufficient blood had been collected, the tourniquet was removed and the needle was removed immediately, after which the blood was transferred into an EDTA container and transferred to the laboratory. Three different methods (microscopy, rapid diagnostic test, and polymerase chain reaction) were used to analyze the blood sample in order to have a true-positive *P. falciparum*.

2.5.2. DNA extraction and PCR for the confirmation *of P. falciparum* using small sub-unit ribosomal RNA gene

The DNA was extracted using the Zymo DNA extraction kit; techniques and procedures outlined and recommended by the manufacturers were strictly adhered to with slight modification. A NanoDrop spectrophotometer was used to determine the concentration and purity of the DNA extracted. Molecular confirmation of *P. falciparum* was carried out using a pair of primers: F5'AACAGACGGGTAGTCATGATTGAG3' R5'GTATCTGATCGTCTTCACTCCC3'. The reaction was carried out in a 25 µl reaction containing 0.5 µl each of forward and reverse primers, 12.5 µl of the polymerase chain reaction (PCR) 2× Master mix (containing dNTPs, Mgcl2, and Taq DNA polymerase), and 6.5 µl distilled water. The gene was amplified by setting an initial denaturation at 95°C for 7 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, while annealing at 57°C for 30 seconds and extension at 72°C for 30 seconds. The final extension was carried out at 72°C for 10 minutes. The amplified gene was subjected to electrophoresis in 2% agarose stained with ethidium bromide and visualized under UV light. Distilled water was used as a negative control. A 100 bp ladder was used to determine the expected band size of 290 bp of the amplicon.

2.5.3. Amplification of *Plasmodium falciparum* multidrug resistance-1 *(pfmdr-1)* gene

Nested PCR was used to amplify the gene from the extracted DNA. Both the primary and the secondary (Nest 2) PCRs were completed in 30 cycles. The reaction was carried out in a 25 µl (DNA template 5 µl, PCR master mix 12.5 µl, 0.5 µl each of reverse and forward primers, and 6.5 µl of distilled water). 5'AGAGAAAAAAGATGGTAACCTCAG3' and 5'ACCACAAACATAAATTAACGG3' were used as the primers for the primary PCR. The primary PCR was completed under the following cyclic conditions: 94°C for 2 minutes initial denaturation, 94°C for 30 seconds denaturation, 50°C for 60 seconds, annealing at 72°C for 60 seconds extension, followed by the final extension at 72°C for 15 minutes. From this primary PCR, the band size was 610 bp; this provided the required codon positions of 86 and 184 of the *pfmdr-1* gene. For the secondary PCR, 5'GCGGAGTTTTTGCATTTAGTTCAGATGATG3' and 5'AGCAGCAAACTTACTAACACGTTTAACATC3' were the primers used for the amplification of the gene. 5 µl of the first PCR product (primary PCR) was used as the template for the secondary PCR (Nest 2) and the same cyclic conditions were used. The band size for the secondary PCR amplicon was 968 bp as expected. This corresponds to the position of codons 1,034, 1,042, and 1,246 of the *pfmdr-1* gene.

3. Results

3.1. Demographic and clinical characteristics of the study subjects

A total of 200 study subjects were used, consisting of 114 (57.0%) and 86 (43.0%) males and females, respectively. The age of the subjects ranged from 5 to 55 years, with the mean of 28.60 ± 10.6. The mean ambient body temperature of the subjects ranged from 33°C to 43°C, with the mean of 37.77 ± 1.92. For molecular analysis, the concentration of the DNA sample extracted ranged from 1.10 to 6.2 ng/µl of the sample, and the mean concentration was 3.55 ± 1.03. For purity, the mean value of A260/280 was 1.72 ± 0.55 and it ranged from 0.7 to 5.11. Table 1 summarizes the basic characteristics of the subjects and the samples used for the molecular analysis.

3.2. Results of the blood analysis

Out of the 200 blood samples collected, 167 (83.5%) samples were positive by microscopy, and 132 (79.04%) and 105 (62.87%) were positive by rapid diagnostic test and polymerase chain reaction, respectively, as shown in Figure 2. Also, 6 (3.59%) and 13 (7.78%) were invalid when tested with rapid diagnostic test (RDT) and PCR, respectively.

3.3. Molecular detection of mutations

Based on the concentration of the parasite's DNA and it is purity, 105 (62.87%) isolates were confirmed malaria positive by PCR and 57 (54.29%) isolates were selected for molecular detection of mutations in *pfmdr-1* gene.

3.4. Single nucleotide polymorphisms (SNPs) at codons 86, 184, 1,042, and 1,246 of pfmdr-1 gene of Plasmodium falciparum

Table 2 shows the results of the *pfmdr-1* mutations at some selected locations of the gene. Among these, four single nucleotide polymorphisms were recorded from N86Y, Y184F, N1042D, and D1246Y variants of the gene. The results revealed that N86Y variant had the highest level mutation prevalence of 22 (44%), while Y184F, N1042D, and D1246Y variants recorded a prevalence of 34 (14%), 09 (4%), and 08 (2%) mutations, respectively, but there was no S1034C mutation recorded in the entire isolate genotyped.

4. Discussion

The *pfmdr-1* gene is located on chromosome number 5 and the selected portion of the gene (96,5661-966529nt, which corresponds to codons 84, 186, 1,034, 1,042, and 1,246) is similar (99.84% identical) to the portion of the genes already deposited in GenBank by Wamae et al. (2019), with the accession number LR131294. In addition, other deposited portions of the gene are 99.67% identical with the following accession numbers: MN375897, MK884183, MK884180, and MK884071. In this study, four different single

Table 1. Demographic and clinical characteristics of the study subjects and basic characteristics of the DNA sample.

Characteristics	Mean	Range	Male	Female
Age	28.60 ± 10.60	5-55 years	114 (57.0%)	86 (43.0%)
Body temperature	37.77 ± 1.92	33°C–43°C		
DNA concentration	3.57 ± 1.03	1.10–6.00 ng/l		
A260/280	1.72 ± 0.55	0.7–5.11		

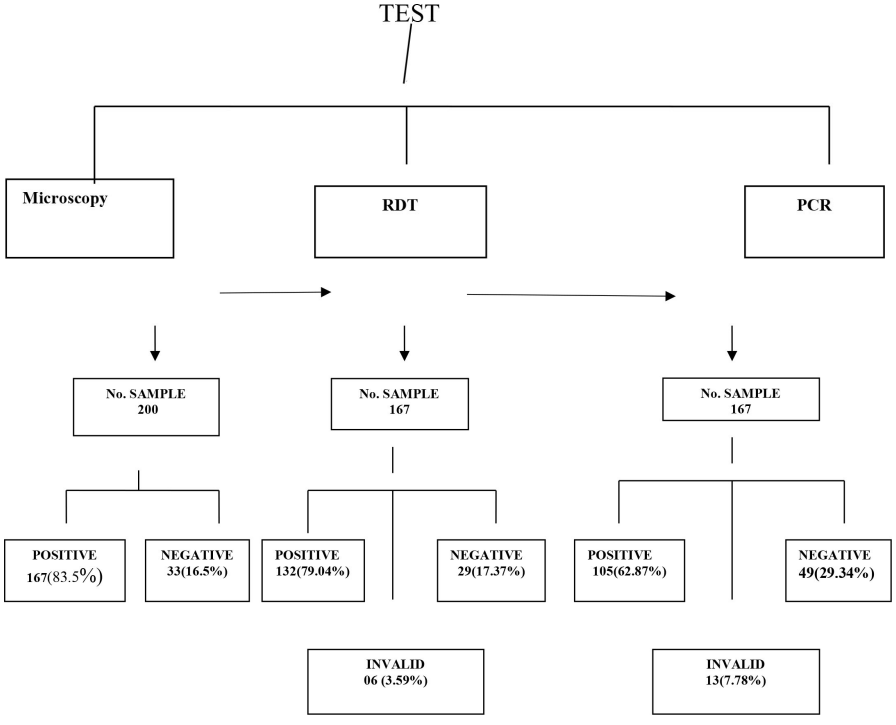

Figure 2. Results of the blood analysis using the three techniques (microscopy, RDT, and PCR).

Table 2. Results of SNPs at codons 86, 184, 1034, 1042, and 1246 of *pfmdr-1* gene of *P. falciparum.*

Codons	N86Y	Y184F	S1034C	N1042D	D1246Y
Wild genotype	**A**AT	T**A**T	AGT	**A**AT	**G**AT
Amino acid	Asparagine	Tyrosine	Serine	Asparagine	Aspartic acid
No. of isolates with wild type	35(61.40%)	23(40.35%)	57(100%)	48(84.21%)	49(85.96%)
Mutant genotype	**T**AT	T**T**T	AGT	**G**AT	**T**AT
Amino acid	Tyrosine	Phenylalanine	Serine	Aspartic acid	Tyrosine
No. of isolates with mutant alle	22 (38.60)	34 (59.64%)	0.00 (0%)	09 (1.79%)	08 (14.04%)

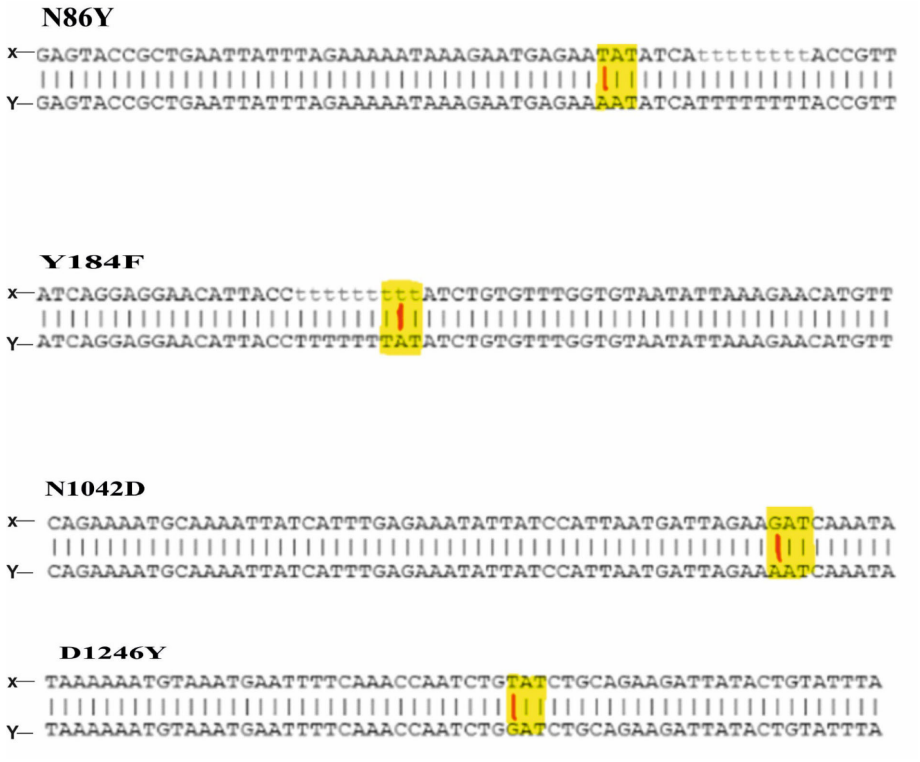

Figure 3. BLAST results comparing the query sequence (*X*) with the reference sequence from the gene bank (*Y*).

nucleotide polymorphisms were identified from four different codon positions (N86Y, Y184F, N1042D, and D1246Y) (Fig. 3). All the reported mutations were not new, they were reported somewhere else, for example, Sabyasachi et al. (2014) reported similar mutations at N86Y and D1246Y codon positions with the following accession numbers: KM056980 and KM056979, respectively. Similarly, 88% of the N86Y mutation was reported by Basco et al. (2002) from Cameroon, and this by far is higher than the 38.0% recorded in this study and 23.1% reported by Fatemeh et al. (2008) from Iran. The differences observed in prevalence rate of N86Y mutation are in a normal fashion, and this is because the geographical distribution of molecular makers of resistance to any particular drug varies greatly, but it may be influenced by other factors such as drug pressure, treatment compliance, and the spread of those resistant alleles to other individuals (Mebrahtu, 2015). In addition, N86Y mutation was reported from northeast and southwest Nigeria and Iran by Adamu et al. (2020), Olasehinde et al. (2014), and Fatemeh et al. (2007), respectively. Zhaoqin et al. (2011) also reported a case of single nucleotide polymorphism in N86Y, Y184F, and N1042D from Myanmar, while Anyirékun et al. (2016) reported mutations in *Pfmdr-1* N86Y and Y184F from symptomatic malaria patients in Burkina Faso.

A prevalence of 59.64% was recorded in this study for Y184F mutation; this is similar to the 65.4% reported by Abel et al. (2019) from Lagos State and

lower than 38.88% reported by Moses et al. (2019a and 2019b) from asymptomatic individuals in south-eastern Nigeria. The differences observed could be attributed to the number of positive samples already involved for molecular determination of makers of resistance, as the latter the study subjects were asymptomatic already, while in former the subjects were symptomatic and some had already developed some of the basic symptoms of the disease. In this study, N1042D variant recorded a very low prevalence (1.76%), which is lower than the 3.12% reported by Thunchanok et al. (2019) from southern Thailand. This low prevalence of the N1042D variant might be attributed to the fact that the marker is not a very prominent marker for *pfmdr-1*. In addition, D1246Y variant had a prevalence rate of 14.04%, and this is by far lower than the 64.0% reported by Irène et al. (2018) from southeastern Gabon. In this study, none of the isolate had S1034C mutations. This is similar to the findings of Thunchanok et al. (2019) and Nathalie et al. (2014), who reported 0.00% mutation prevalence in S1034C from southern Thailand and Dakar, respectively. Low prevalence of N1042D and D1246Y and even zero prevalence of S1034C variants might be attributed to the fact that these variants are not predominantly found in west Africa, instead they are very rare, unlike N86Y and Y184F which are the most predominant variants in the region with also very few cases of N86Y/Y184F (Isabel et al., 2016).

5. Conclusion

Conclusively, some basic molecular makers of PFMDR-1 gene were reported from Gombe L.G.A, this may affects the susceptibility of different classes of antimalarial drugs in the local government. Further research should be carried out in the local government using large sample size and target some other candidate bio markers of the gene.

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