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Original Article

Determining Molecular Markers Associated with Drug Resistance in *DHFR* and *DHPS* genes of *Plasmodium Falciparum* from Gombe L.G.A. Gombe State, Nigeria

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Abstract

Background: Malaria Chemoprevention depends on synthetic drugs, but the parasite is continuously developing resistance to the antimalarial armament, hence a consequential need for surveillance studies on the sensitivity of the drugs is felt. Therefore, the aim of this paper was to determine the presence of biomarkers associated with drug sensitivity in DHFR and DHPS gene of *Plasmodium falciparum*.
Methods: 200 blood samples were collected using vein puncture technique and they were analysed using Microscopy, RDT and PCR. DNA was extracted using Quick-DNA™ Miniprep extraction kit. Purity and concentration of the DNA were determined using Nanodrop Spectrophotometer. 57 samples were selected for molecular analysis. Nested PCR was used to amplify PFDHFR and PFDHPS genes; all PCR reactions were carried out in 25 µL reaction mixture (5 µL DNA template, 1 µL Primer, 6.5 µL distilled water and 12.5 µL Master mix). The PCR products were subjected to electrophoresis using 2% agarose gel. The amplicons were purified, sequenced and subjected to BLAST software.
Results: Mutations were recorded from A16V 05(8.77%), N51I 18(31.58%), C59R 03(5.26%), I164L 12(21.05) variants of DHFR gene, while in DHPS gene, mutations were recorded from K540E 6(10.52%) variant.
Conclusion: Basic Biomarkers of resistance in DHFR and DHPS gene were recorded from Gombe.

Keywords: Gombe, Resistance, DHFR, DHPS, Malaria, *Plasmodium falciparum*..

1. Introduction

Malaria is a life threatening mosquito borne disease with substantial negative repercussions for the global population and is also regarded as a serious public health problem [1]. The disease is a major serious threat to health systems in sub-Saharan Africa where morbidity and mortality, due to the disease, are high in the absence or inadequate surveillance systems to better control the spread of the disease [2], thus make the situation worse. Five parasite specie in the genus *Plasmodium* including *Plasmodium falciparum, P.malarie, P.vivax, P.ovale* and *P.knowlesi* are responsible for clinical manifestation of the disease [3] where more than 90% of the occurrences in Nigeria is caused by *Plasmodium falciparum* [4]. The global burden and economic cost of malaria infection are also huge [5], where it causes about 50% reduction in the per capita GDP of malaria endemic countries compared with a country not affected by malaria [6]. According to World Health Organisation (WHO), in the year 2020, there were about 230 million clinical cases of malaria with about 410 thousand deaths globally [3], where 85-92% of fatal cases were recorded in Sub-Saharan Africa [7] and Nigeria alone accounted for 25% of malaria burden in Africa [8] and 23% of malaria death globally [9].

In Nigeria, malaria chemoprevention in vulnerable and high risk group like infants, children, and pregnant women heavenly relies on Sulfadoxine-Pyrimethamine [10] and in fact, it is the recommended drug for intermittent preventive treatment during pregnancy as they are very cheap (freely given during antenatal) compared with other antimalarial drugs, relatively safe and treatment requires only a single dose. In addition, chloroquine, which was considered to be effective, is completely lost to resistance, which forced most countries to abandon it as first-line treatment and shift to Sulfadoxine-Pyrimethamine (SP) prior to the recommendation of Artemisinin based combination therapy (ACT). Sulphadoxine-Pyramethamine act by attacking folate biosynthetic pathway of the parasite through inhibiting the proper functioning of Dihydrofolate Reductase (DHFR) and Dihydropteroate Synthase (DHPS), both coding for vital enzymes [11].

Malaria cases increases in the past three decades; one of the major causes and contributions to this global problem and especially in Africa mainly attributed to the development of resistance by the parasite. Antimalarial drug resistance is of serious concern because it affects and limits the sensitivity of the most commonly recommended antimalarial drugs like Sulphadoxine-Pyramethamine [12-16]; this has also compelled different nations to constantly change their national malaria control programmes and antimalarial drug policy [8]. However, resistance to this drug has emerged as a result of treatment failures reported in Africa, Asia, Indonesia and South America [17-20]. Quite a number of research studies from most part of the world have convincingly revealed significant correlation between anti-malarial drug sensitivity and mutation in the adverse drug reaction gene of the parasite [21]. Specifically sensitivity or resistance of Sulphadoxine-Pyramethamine is influenced by single nucleotide polymorphism in two enzymes, which include *Plasmodium falciparum dihydrofolate reductase* (PFDHFR), which confers resistance to Pyrimethamine, and *Plasmodium falciparum dihydropteroate synthetase* (PFDHPS), which confers resistance to Sulfadoxine [22]. These mutations are usually associated with Sulphadoxine-Pyramethamine (SP) treatment failures, as such they could be used as molecular

markers for SP resistance [21]. These biomarkers include N51I, C59R, S108N, and I164L [23] in the *plasmodium falciparum dihydrofolate reductase* (PFR) gene, and resistance to Sulphadoxine is associated with single nucleotide polymorphism at codons position S436A/F, A437G, K540E, A581G, and A613S/T of *plasmodium falciparum dihydropteroate synthase* (PFDS) gene [24]. The mutation may involve single, double in DHFR or triple or quadruple in DHPS [12]. It is thought that an important driver of the rapid spread of resistance to Sulfadoxine–Pyrimethamine in Africa has been sub-curative dosing of the drug in children with *falciparum* malaria [25].

Drug resistance monitoring and pattern of mutations are among the basic fundamental factors in malaria control policy and elimination efforts in the modern era, which allow early detection and subsequent prevention of the spread of the resistance [22, 26-29]. Therefore, identifying genetic mutations that mediate antimalarial resistance is also a key to understanding how the parasites evade treatments and tracking these molecular markers in clinical samples can help evaluate the emergence of resistance in a particular region and inform recommendations for first line therapy [30]. In addition, continuous monitoring of the effectiveness of anti-malarial drugs in disease-endemic areas is important for early detection of parasites with reduced susceptibilities to the drugs [31]. Therefore, the aim of this paper was to determine the presence of biomarkers of resistance associated with PFDHFR and PFDHPS gene of *Plasmodium falciparum* in Gombe Local Government Area.

2. Methodology

2.1. Study area

The study was conducted in Gombe Local Government Area, Gombe State, Nigeria (Figure 1). The Local Government lies between 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 of 52 km^2 and a projected population of 367, 500 people (3.3% annual change) according to National Population Commission. The vegetation of the local government is Sudan Savannah with two distinct seasons: Dry season which normally spans from November to March and rainy season from April to October with 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. The local government being the capital of the state, both the tertiary (Federal Teaching Hospital) and the secondary (Gombe State Specialist Hospital) health facilities of the state are domiciled in the Local Government. This is also in addition to the primary health care centres that are strategically located in each wards of the local government, also there are quite a number of private hospitals providing different services including malaria diagnosis and treatment.

2.2. Ethical consideration and consent of the subject

Proposal of the research was submitted to Gombe State Ministry of Health for approval, after which the approval was communicated via a letter MOH/ADM/621/VOL.I/222 dated 21st February 2020. Verbal and or written consent of the 200 subjects were sought after briefing them on the research and inviting them to participate. In a situation where the subjects were less than 18 years of age, consent of their parents/guardian were sought. All the subjects were assured that all information and samples collected were strictly used for the purpose of the

research and were treated with high level of confidentiality. In addition, quality control and quality assurance were assured when handling and treating each of the samples.

2.3. Study Subjects and Inclusion Criteria

The people who willingly and voluntarily agreed to participate in the study were used as the study subject. 200 volunteers from three (3) recruitments centres, namely Gombe town maternity (Gidan Magani) 105(52.50%), Sunnah clinic 45(22.50%) and Idi children and Women Hospital Gombe 50(25.00%), actively participated in the study.

Only patients with symptoms of malaria, i.e. presumed to be malaria positive or have the history of fever in last 24hours and visited by a physician for the screening of malaria infection in facility's laboratories were included. In addition, they had not used any anti-malarial drugs 60 days prior to the data collection. Only subjects with *Plasmodium falcifarum* mono-infection were recruited, also true positive samples with a very good DNA concentration (200 ng and above) and high level of purity (A260/280 between 1.8-2.0) were included for molecular analysis.

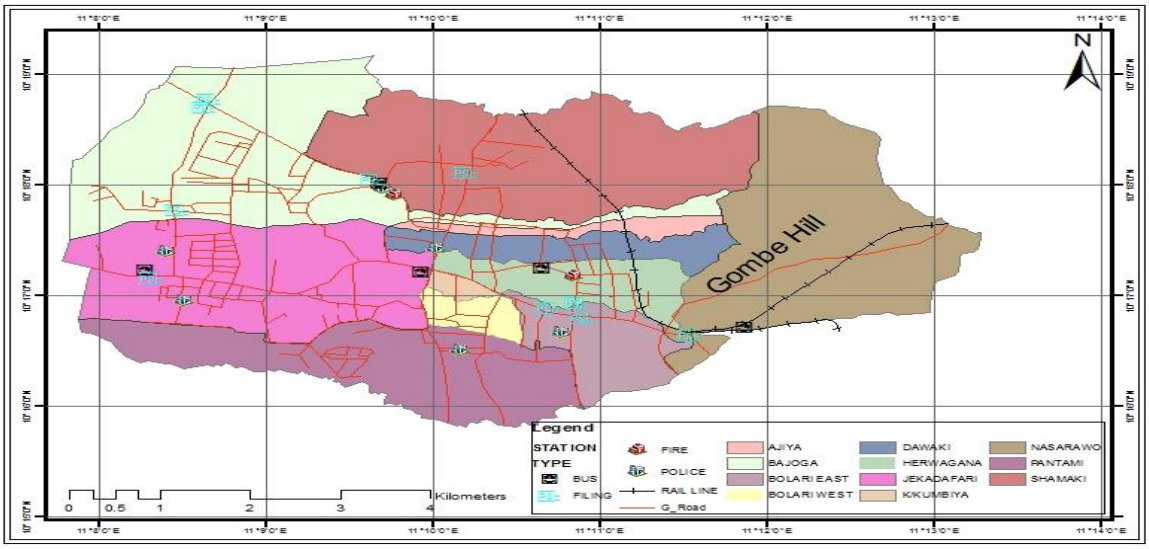

Figure 1. Map of Gombe Local Government Area

Source. GIS Laboratory, Geography department, Gombe State University

2.4. Blood sample collection and analysis

Vein puncture technique was used to collect Venus blood and analysed using three different techniques: Rapid Diagnostic Techniques (RDT), Conventional Microscopy and Polymerase Chain Reaction (PCR) to obtain true positive sample.

2.5 DNA Extraction and determination of concentration and purity

The DNA was extracted using Quick-DNA™ Miniprep Plus Kit with catalog No. D4069 from Zymo research, following manufacturer's instruction with slight modification. Nanodrop Spectrophotometer was used to determine the concentration and purity of the DNA extracted.

2.6 Primers and Molecular Confirmation of *Plasmodium* species

All primers used in this research were validated and supplied from Inqaba

Biotec™ Africa's genomic company. The primers were reconstituted/diluted by using the recommended dilution factor as specified by the manufacturer and stored at -4 °C as stock solution. The actual working solution was obtained by diluting 10 µL of the stock in 90 µL of nano pure water.

For the molecular confirmation of *Plasmodium falciparum,* 18 S Portion of small sub unit ribosomal RNA gene on chromosome 13 was amplified in Classic DW-K960 thermo-cycler. The amplification was carried out in 25 µLreaction mixture containing 5 µL of DNA template, 1 µL of primer (0.5 µL each of F5'AACAGACGGGTAGTCATGATTGAG3' R5'GTATCTGATCGTCTTCACTCCC3'), 6.5 µL distilled water and 12.5 µL of the PCR Master mix (dNTPs, $Mgcl_2$ and T*aq* DNA Polymerase). The forward primer is species-specific, for that it hybridized only with *Plasmodium falciparum* DNA, while the reverse primer is genus-specific, thus hybridized with all the four *Plasmodium* species. The thermo cyclic conditions were set as 95 °C, 15min initial denaturation, 95 °C, 45 sec, denaturation, 60 °C, 90 sec. annealing, 72 °C, 1 min. extension and 72 °C, 5 min. for final extension; the amplification was completed in 40 cycles as shown in Table 1 below.

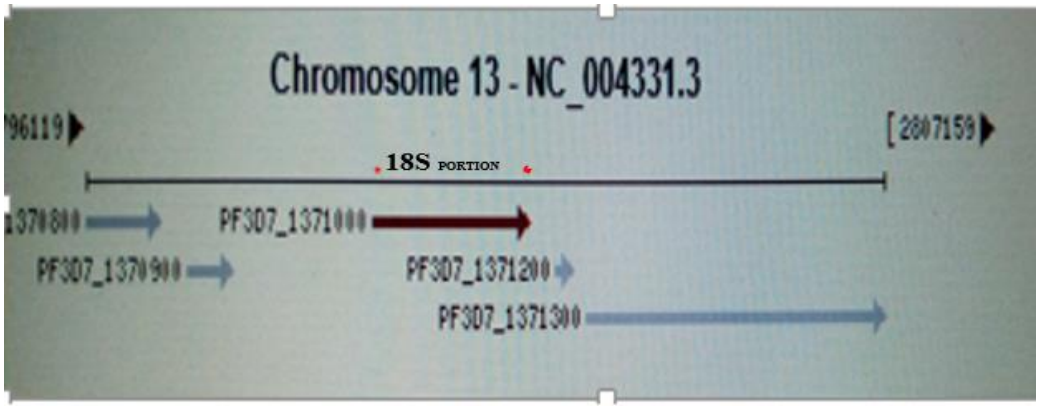

Figure 2. Chromosome 13 of *Plasmodium falciparum* indicating 18 S Portion of Ribosomal RNA gene

2.7. Gel Electrophoresis

The PCR products were subjected to electrophoresis on 2% agarose gel and stained with ethidium bromide. All gels were allowed to run for a period of 1 hour at 100 mA, after which the gel was visualised using UV transilluminator.

2.8. Amplification of the genes

2.8.1. Amplification of plasmodium falciparum dihydrofolate reductase (PFDHFR) gene

Nested PCR was used for the amplification of PFDHFR gene, and for the primary PCR, the reaction was carried out in 25 µL reaction mixture, containing 5 µL of the extracted DNA sample as template, 0.5 µL each of the reverse and the forward primers (F5'TTTATATTTTCTCCTTTTTAT3' and R5'TTACTAGTATATACATCGCTAACAG3'), 6.5 µL of distilled water and 12.5 µL of the master mix (dNTPs, $Mgcl_2$ and T*aq* DNA Polymerase). The thermocyclic conditions were programmed as initial denaturation at 94 °C for 90 sec., denaturation at 94 °C for 30 sec, annealing at 57 °C for 30 sec, extension at 68 °C for 45 sec and final extension at 68 °C for 5 sec. The secondary PCR was carried out in 25 µL reaction, containing 5 µL of the first PCR product (amplicon) as template, 0.5 µL each of forward and reverse primers (F5'ATGATGGAACAAGTCTGCGAC3'

R5'TTACTAGTATATACATCGCTAACAG3'), 6.5 µL of distilled water and 12.5 µL of the master mix (dNTPs, $Mgcl_2$ and T*aq* DNA Polymerase). The cyclic conditions were initial denaturation at 94 °C for 90 sec, denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 66 °C for 45 sec. and final extension at 67 °C for 5 min.

2.8.2. Amplification of Plasmodium falciparum Dihydropteroate Synthase (PFDHPS) gene

The gene was amplified using nested PCR, where F5'GGTATTTTTGTTGAACCTAAACG3' and R5'TCCAATTGTGTGATTTGTCCAC3' primers were used for the primary PCR (Nest 1). Five microliters (5 µL) of the extracted DNA was used as template and the reaction was carried out in 25 µL reaction mixture containing 0.5 µL each of the reverse and the forward primers, 6.5 µL of distilled water and 12.5 µL of the master mix. The thermo cycler was programmed under the following cyclic conditions: 94 °C for 60 sec. in initial denaturation, 94 °C for 30 sec. in denaturation, 59 °C for 30 sec. in annealing, followed by extension at 67 °C for 30 sec. and then final extension at 68 °C for 10 sec. For the secondary PCR (Nest 2), 5 µL of the Amplicons obtained from the primary PCR was used as a template, using F5'GAATGTGTTGATAATGATTTTAG3' and R5'TCCAATTGTGTGATTTGTCCAC3' as the forward and reverse primers, respectively. Both the reaction mixture and the thermocyclic conditions were the same as nest 1 except for the annealing and extension, which were respectively raised to 55 °C and 68 °C.

3. Result

3.1. Demographic and clinical characteristics of the study subject

A total of 200 study subjects were used, comprising 114(57.0%) and 86(43.0%) males and females, respectively. The age of the subjects ranged from 5 to 55 with the mean of 28.60±10.6. The mean ambient body temperature of the subjects ranged from 33 to 43 ^{0}C with the mean of 37.77±1.92. For the 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 below summarises the basic characteristics of the subjects and the sample used for the molecular analysis.

Table 1. Demographic and Clinical characteristic of the study subject 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	33-43 °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 below summarises the results of the blood analysis using the three techniques. Out of the 200-blood sample collected, 167(83.5%) samples were positive by microscopy, 132(79.04%) and 105(62.87%) were positive by Rapid diagnostic test and Polymerase chain reaction, respectively, as shown in Figure 4.1 below. 06(3.59%) and 13(7.78%) were invalid when tested with RDT and

PCR, respectively. In addition, 80(40%) of the collected blood samples were true positive, confirmed positive by the three techniques (Microscopy, RDT and PCR) and also 57(71.25%) of sample met the inclusion criteria for the actual molecular analysis.

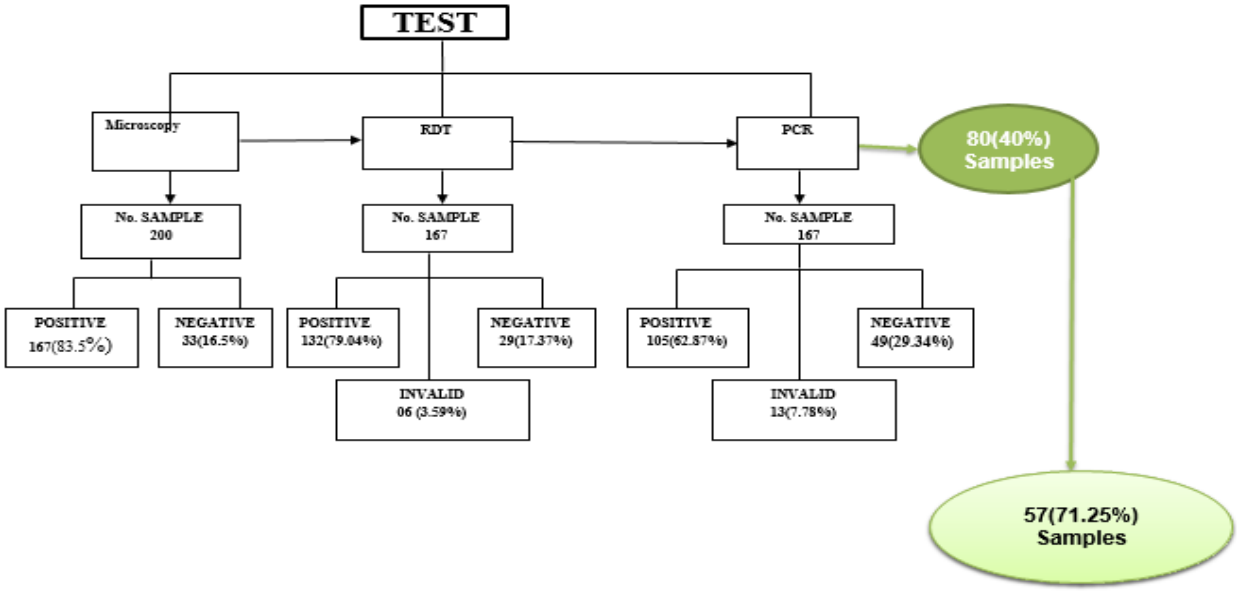

Figure 3. Flow chart of the results of blood analysis

3.2. Result of SNPs at codon 16, 51, 59, 108 and 164 of PFDHFR gene of P. falciparum

In *PFDHFR* gene of the *Plasmodium falcifarum,* the highest prevalence of mutation of 18 (31.58%) was recorded at codon position N51I, while codon C59R had the least prevalence of 03(5.26%). The prevalence of 05(8.77%) and 12(21.05%) were respectively recorded from A16V and I164L variants and there was no mutation at codon S108N. Table 4 below shows the detailed nature of the mutation, where in codon 16 the mutation was due to a change in the nucleotide sequence of Alanine (A) GCA where cytosine (C) in the alanine was substituted with Thymine (T), thereby led to the formation of Valine (V) GTA. In C59R variant the mutation was as a result of a change in the amino acid Cysteine (C) to Argenine (R). This was due to the substitution in the nucleotide sequence of the amino acid Cysteine (C) TGC, where Thymine (T) is substituted with Cytosine (C) thereby led to the formation of the amino acid Argenine (R) CGC.

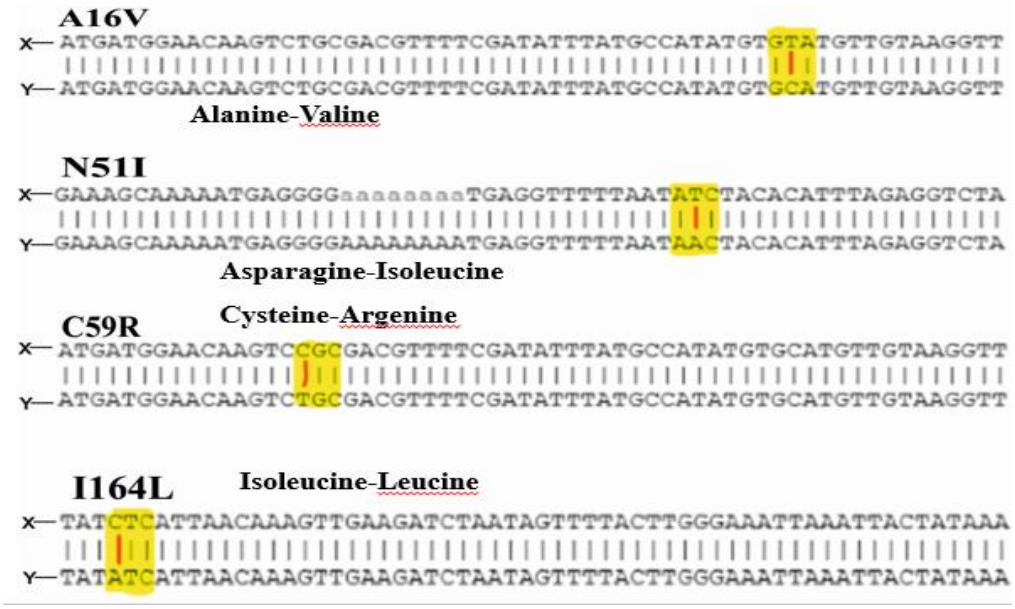

Figure 4. BLAST result of PFDHFR
X=Query Sequence
Y=Reference sequence from the gene bank

Table 2. Single Nucleotide Polymorphism at codon 16, 51, 59, 108 and 164 of PFDHFR gene of *Plasmodium falciparum*

Codons	A16V	N51I	C59R	S108N	I164L
Wildtype genotype	G**C**A	A**A**C	**T**GC	**TCG**	**A**TC
Amino acid	Alanine	Asparagine	Cystein	Serine	Isoleucine
No.of Isolate with wild type	52(91.23%)	39(68.43%)	54(94.74%)	00(0.00%)	45(78.95%)
Mutant genotype	**GTA**	ATC	**C**GC	**TCG**	**C**TC
Amino acid	Valine	Isoleucine	Argenine	Serine	Leucine
No. of Isolate with mutant alleles	05(8.77%)	18(31.58%)	03(5.26%)	0(0.00%)	12(21.05%)

3.3. SNPs at Codon K540E, A581G and S613T of PFDHPS of Plasmodium falcifarum

In *PFDHPS* gene, out of the 57 sample successfully genotyped for single nucleotide polymorphism, only one form of mutation was recorded at codon position K540E with the prevalence of 6(10.52%). The prevalence of 0.00% was observed in A581G and S613T variants. The mutation at codon K540E was as a result of change in the amino acid Lysine (K) to Glutamic acid (E), which was due to a substitution of a single nucleotide' Adenine (A)'in Lysine (K) **A**AA with Guanine (G). This led to the formation of Glutamic acid (E) **G**AA as shown in Table 5 below.

Table 3. Prevalence of Single Nucleotide Polymorphism at K540E, A581G and S613T of PFDHPS gene of *Plasmodium falciparum*

Codon	K540E	A581G	S613T
Wild type	**A**AA	G**C**A	TCG
Amino acid	Lysine	Alanine	Serine
No. of Isolate with wild type	51(89.47%)	57(100%)	57(100%)
Mutant	**G**AA	G**C**A	TCG
Amino acid	Glutamic acid	Alanine	Serine
No. of isolate with mutant allels	06(10.53%)	00(0.00%)	00(0.00%)

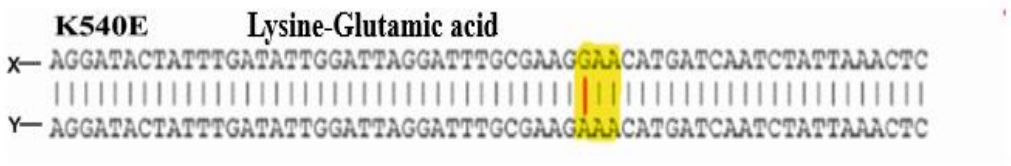

Figure 5. BLAST result of PFDHPS
X=Query Sequence
Y=Reference sequence from the gene bank

4. Discussion

Sulphadoxine-Pyrimethamine is widely available antimalarial, used for prophylaxis and treatment, to which parasites have developed resistance in recent years. Single nucleotide polymorphisms in the *Plasmodium falciparum* genes for dihydropteroate synthase (*DHPS*) and dihydrofolate reductase (DHFR) have been associated with resistance to Sulphadoxine and Pyrimethamine respectively [32, 33]. One of the key mutations in the *DHPS* gene that confers resistance to sulfadoxine is at codon 437 (Ala→Gly). Additional mutations at codons 436, 540, 581, and 613 increase the level of resistance to sulfadoxine. Previous studies have demonstrated the correlation between these point mutations and resistance to antifolate drugs [34].

The sequence of PFDHFR used in this study was 99.85% identical to the portion of the gene deposited in the gene bank with the accession number KJ716210. Similarly, similar portion of the gene with accession number KJ716211, and LR129689 deposited in the gene bank were found to be 99.69% and also 99.85% identical, respectively. Similarly, the sequence of PFDHFR was 99.79% identical to the portion of the gene deposited in the gene bank with the accession number of LR131488 and Z30664. 99.58% similarity was also found with some other portions of the gene already deposited with accession of MN373792 and LR131377. In addition, KX575351 and EU717690 showed 99.57 and 99.36% similarity, respectively, with the portion of the gene used in this study. The high similarity index reported in this study confirmed that the DNA and also genes worked with were from *Plasmodium falciparum*.

Mutations on PFDHFR gene (S108N, C59R, N51I, and I164L) are related to an increasing resistance to pyrimethamine while those occurring on PFDHPS gene

(A437G, K540E, A581G, and A613S/T) are associated with sulfadoxine resistance. In West Africa wild PFDHPS K540 commonly occur with triple DHFR mutations and single 437G DHPS mutation [35]. In this study, five 5 different single nucleotide polymorphisms (4 from PFDHFR and 1 PFDHPS) were recorded at different codon position of the genes, but no new mutation was recorded. The codon position from which these were recorded included A16V, N51I, C59R and I164L for PFDHFR and K540E of PFDHPS gene. This result is similar to the findings in previous research [36] who reported Single Nucleotide Polymorphism in the same codon position of the genes from Mauritanian border. On the other hand, the findings of this study is in contrast to those of previous research [21], reporting eleven different variant from Thailand between 2016-2018. There is also a report on the presence of A16V, N51I, C59R and I164L as molecular makers for antimalarial drug sensitivity in PFDHFR and A581G in FDPHPS from India and Thailand [37], but unlike the findings of this study, S613T variant was obtained together with other additional variants that were found in this study. These differences might be attributed to the sample size used in the two studies, as in the present study only 57 isolate were successfully genotyped while in other study almost 140 isolate were genotyped from the two study sites (Thailand and India); therefore, the chances of obtaining different makers may be higher, so this research was designed to target quite a number of biomarkers.

K540E and N51I variants of FDPHPS and PFDHFR, respectively, had the highest prevalence as compared with other variants. This finding is in contrast to those of previous research from Maiduguri [38], reporting S108N and A437G in PFDHFR and FDPHPS, respectively, as the most prevalent maker for Sulphadoxine and Pyramethamine resistance in *Plasmodium falciparum.* In N51I and K540E variants, the prevalence of 93.0% and 86.9% was respectively recorded by Schönfeld *et al.* (2007) [34] from south-west Tanzania. In variants A581G and S613T of PFDHPS, the prevalence of 26.9% and 0.9% were respectively recorded from Qatar by Bansal *et al.* (2019)[5]. Similarly, the findings in this study is by far lower than 60%, 90% 92% and 42% in N51I, C59R , S108R and I164L, respectively, and 92.6% and 25.9% in K540E variant from Myanmar in previous research [39]. There is the reflection of the prevalence of 67.8% and 26.2% in K540E and A581G variant from Sudan between 2017-2017 [17] [40]. The prevalence of 88.7%, 78.3% and 93.4% N51I, C59R, S108R variants of PFDHFR, respectively, 91.5% in K540E variant of PFDHPS was reported from Kenya in previous research[24]. The prevalence of 95.1%,96.3% and 96.7% in N51I, C59R and S108R variant of PFDHFR, respectively, and 1.2%, 52% and 70% in K540E, A581G and S613T variant of PFDHPS, respectively, from Lagos by Quan *et al*., (2020) [30]. The absence of point mutation on S1613T variant is similar to the findings of previous research [11], reporting zero prevalence in same variant from Democratic Republic of Congo, but in terms of prevalence of K540E (2.1%) and A581G (2.1%) the result differs greatly.

Generally, the very low prevalence of point mutations in PFDHPS and PFDHFR genes were recorded in this study. This might be attributed to the level and mode of sulphadxine- pyrimethamine utilization, as in this study area sulphadxine-pyrimethamine is usually used by exclusively pregnant women as it is given freely during antenatal in most government primary health facility as Intermittent Preventive Treatment (IPT)

and in some clinic is not even available. Unlike in other places where the drug, i.e. sulphadxine-pyrimethamine, is readily available and usually recommended as prophylaxis and treatment of uncomplicated malaria. Another factor that might be responsible for the difference in massive usage of the drug in most of those areas as prophylactic drugs, while in the area of this study there was less attention to prophylaxis as much of the attention is toward treatment with the recommended Artemisinin Based Combination Therapies (ACTs). Therefore, the chances of reporting PFDHPS and PFDHFR molecular makers for resistance could be low. Interestingly, no any double, triple or quintuple mutations was reported from this study. Triple PFDHFR mutation N51I, C59R, and S108N in combination with the double A437G and K540E PFDHPS mutant formed a quintuple mutant haplotype, which confers a high risk of treatment failure with Sulphadoxine-Pyrimethamine [41]. Therefore, with this train, these drugs may be still very relevant in prophylaxis and treatment of malaria in the area, especially among pregnant women who depend on free-Sulphadoxine Pyrimethamine prophylaxis. Though, unlike other antimalarial drugs like chloroquine in which the resistance took many years to develop, resistance to antifolates develops much faster. The genetic mechanism of resistance to antifolates is more straightforward in comparison to Chloroquine resistance, with single point mutations in the genes encoding either DHFR or dihydropteroate synthase (DHPS) in response to sulpha drugs[30].

Conclusion

In a nutshell, some basic biomarkers of resistance at low prevalence were reported from five different variants of PFDHPS and PFDHFR genes from Gombe, where four isolates from PFDHFR were mutant and only S108N was found to wild type, but in PFDHPS, all the isolates were wild type except K540E variant. Fortunately, all the biomarkers reported in the study were not new, instead they were equally reported from other part of the globe and even Nigeria, as no any new biomarker of PFDHPS and PFDHFR was reported in this study from Gombe.

Acknowledgements

We really appreciate and commend TETfund for providing us with the fund to carry out the research. In addition, special regards and appreciation go to Dr. Adamu Saidu from University of Maiduguri Nigeria for supplying us the DNA extraction kit at a very critical moment (Covid-19 Period) when we were about to give up.

Ethics Approval and Consent to Participate

Permission was sought through Health, Research and Ethical committee of ministry of health, Gombe state. The ethical approval was communicated via a letter with the following code MOH/ADM/621/VOL.I/222.

Conflict of interest

The authors declare that there is no conflict of interest

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