

Original Article



Molecular analysis of bio-makers of Chloroquine resistance in *Plasmodium falciparum* isolate from gombe local government area, Gombe State, Nigeria

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ABSTRACT

Chloroquine was one of the most cheapest and effective chemotherapeutic drugs for *Plasmodium falciparum*-malaria, but for a long, the drug has been officially withdrawn in almost all malaria-endemic countries including Nigeria, due to the development of resistance by the parasite. Withdrawal of the drug may make the drug regains its efficacy. Therefore, this study aimed to determine the presence of Biomarkers associated with chloroquine resistance from Gombe Local Government Area, Gombe State, Nigeria after its withdrawal in 2005. Twenty hundred blood samples were collected from consented study subjects and analysed using Microscopy, RDT and PCR. DNA was extracted using Quick-DNA™ Miniprep (No. D4069), Purity and Concentration of the DNA were determined using Nanodrop Spectrophotometer. 57 true positive samples were selected for molecular analysis. Nested PCR was used to amplify the required codon (C72S, M74I, K76T and N75E) position of PCRT the gene of *P. falciparum*. Both Primary and Secondary PCR was carried out. The PCR products were subjected to electrophoresis in 2% agarose and stained with ethidium bromide. The amplicons were purified and sequenced, after which the sequenced products were subjected to BLAST software. Single Nucleotide Polymorphism was recorded from C72S and K76T with a prevalence of 05(8.80%) and 46(80.70%) respectively. Confirmed biomarkers of Chloroquine resistance are still present in *P. falciparum* isolate from Gombe L.G.A.

Keywords:

PCRT, Sensitivity,
Molecular makers, Single
Nucleotide Polymorphism

1. Introduction

Malaria is regarded as a serious global public health problem, which is partly due to its cosmopolitan distribution and high rate of mortality traced to the disease, especially in sub-Saharan African countries that bear many consequences of the disease[1]. The disease is caused by four parasite species in the genus *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*), recently human infections with the monkey malaria parasite, *P. knowlesi*,

have also been reported from the forested regions of South-East[2]. These parasites are transmitted into the susceptible human host through the bite of an infected female Anopheles mosquito during a blood meal. *Plasmodium falciparum* is responsible for the majority of malaria cases and deaths occurring in sub-Saharan Africa[3] where about 99.7% of all malaria morbidity and mortality are attributed to the species with a disproportionate number of deaths in children under the ages of five[4].

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The disease globally affects over 220 million people with at least 405,000 mortality each year, pregnant women and children are at high risk of the disease where 67% of those deaths occur particularly in children under the age of five[5] and pregnant women[6]. The majority (93%) of the global malaria burden is recorded in Sub-Saharan Africa, and Nigeria alone bears about 25% of this burden[7]. Malaria is endemic in Nigeria where the entire country is at risk[8] with continuous transmission all year round in the southern half of the country, while in the north disease transmission it is more seasonal [9] with a high peak of transmission during the rainy season.

Despite current development and progress made in chemotherapeutics, diagnostics and vector control measures, the disease continues to exert a major impact on human health[10-15], especially in the tropical and sub-tropical countries. In the absence of an effective vaccine, effective antimalarial drugs are the mainstay to effective malaria prevention, control and eradication [12, 16-19]. From the 1940s up to the 2000s, chloroquine was the most widely used drug for treating malaria fever[20], this is partly due to its high efficacy, safety and low cost[21]. Chloroquine is a synthetic product that was first produced in the year 1934 and accepted for clinical use in 1947[22]. The drug has so many potentials for combating the parasite which includes; antimalarial, antiparasitic, anti-inflammatory, antibacterial and antiarrhythmic activity as well as stabilization of lysosomal membrane[23]. unfortunately, the parasite has developed resistance to most of the antimalarial armament, thus increasing the burden of the disease and also posing serious challenges in the global fight against the disease[24].

The level of susceptibility or resistance of *P. falciparum* strains to quinoline antimalarial drugs such as Chloroquine, amodiaquine, lumefantrine and mefloquine has been associated with to single nucleotide polymorphism (mutations)[25] in some of the adverse drug reaction genes of the parasites specifically Pfcrt and Pfmdr1 genes. A series of Single Nucleotide Polymorphisms are associated with increased rates of clinical

failure[26]. It has been reported that mutations in the region of Pfcrt encompassing codons 72-76 are a key biomarker of *P. falciparum* chloroquine resistance[27]. Chloroquine resistance is also attributed to the reduction in the concentration of chloroquine that accumulates in the digestive vacuole, which is the chloroquine site of action. The mechanism for this decreased accumulation is controversial, some studies have shown that the decrease in drug efflux[27]. This is because research has shown that the resistant *Plasmodium falcifarum* parasite releases chloroquine 40-50 folds more rapidly than the susceptible parasite[9].

Chloroquine resistance emanates from Southeast Asia[28] and South America[29] as early as the late fifties[30] and within two decades the resistant strain spread to the vast majority of malaria-endemic countries[31] including Sub Saharan Africa and particularly Nigeria[32]. This led to a decline in the efficacy of chloroquine, as a result, almost all countries changed their treatment policy from chloroquine to other alternative antimalarials drugs with high efficacy, such as antifolates, mefloquine and artemisinin derivatives[33].

Chloroquine was the recommended as a first-line drug for the treatment of uncomplicated malaria in all age groups in the year 2005, but 17 years later, its usage was officially banned in Nigeria[34] due to the emergence of a massive resistant-chloroquine strain of the parasite, with the hope to reduce drug pressure and with full hope that it may regain its susceptibility after its discontinuation usage in the country. Therefore this surveillance study aimed to evaluate the presence of molecular markers of resistance in the PCRT gene of *P. falciparum* isolates from Gombe L.G.A, Nigeria after the drug has been officially suspended for treating uncomplicated malaria.

2. Materials and Methods

2.1. Study Area

The study was conducted in Gombe Local Government Area, Gombe State, Nigeria. 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 landmass of 52 km².

Gombe Local government 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 experiences two distinct seasons, the dry season which normally commences from November- to March and the rainy season from April-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 a civil servant. The local government being the state 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 centers that are strategically located in each ward of the local government, also there are quite several private hospitals providing different services including malaria diagnosis and treatment.

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 a later MOH/ADM/621/VOL.I/222 dated 21st February 2020.

2.3. Consent of the Subjects

Before collecting the blood samples from the study subjects' verbal and or written consent of the subject 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 was sought. All the subjects were assured that all information collected from the subjects will be strictly used for the research and will be treated with a high level of confidentiality. In addition, quality control and quality assurance were assured when handling and treating each of the samples[35].

2.4 Study Subjects and Inclusion Criteria

Human beings of all age groups and gender who willingly and voluntarily agreed to participate in the study were used as the

study subject for the research. Three recruitments centers were selected; these are Gombe town maternity (Gidan Magani), Sunnah clinic and Idi children and Women Hospital Gombe, where a total of 200 volunteers actively participated in the study.

Only patients who reported themselves to the selected hospitals (Gombe Town Maternity (Gidan Magani), Sunnah Clinic and Idi Children and Women Hospital Gombe.) with a (presumed to be malaria positive) symptoms of malaria (fever) or history of fever in last 24hours and referred by a physician for the screening of malaria infection and in addition they have not used any anti-malarial drugs 60 days prior to the data collection. All subjects having multiple infections were not recruited in the research; only subjects with *Plasmodium falcifurum* mono-infection were recruited. For molecular analysis, only true positive samples with a very good DNA concentration (2.0ng and above) and high level of purity (A260/280 between 1.8-2.0) were used[36].

2.5 Blood sample collection and Analysis

The blood sample was collected and analysed [9], where the Vein puncture technique was used to collect Venus blood. The blood samples were collected with the help of medical personnel and the method employed was venepuncture techniques. In this technique, 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 5ml syringe. When sufficient blood samples had been collected, the tourniquet was removed and the needle removed immediately, after which the blood was transferred into an EDTA container and transferred to the laboratory. The blood samples were analysed using Conventional microscopy, Rapid Diagnostic Techniques (RDT) and Polymerase Chain Reaction [37].

2.6 DNA Extraction and Purity and Concentration determination

The DNA was extracted using Quick-DNA™ Miniprep (No. D4069). Techniques and procedures outlined and recommended by the

manufacturers were strictly adhered to. Nanodrop Spectrophotometer was used to determine the purity and concentration of the DNA extracted[36].

2.7 Primers and Confirmation of *Plasmodium* species

All validated Primers were supplied from Inqaba Biotec™ Africa's genomic company. All primers were Reconstituted/ diluted by using the recommended dilution factor (appropriate amount of distilled water) as specified by the manufacturer (i.e Inqaba Biotec™ Africa's genomic company.) and stored at -4°C as stock solution. The actual working solution was obtained by diluting 10µl of the stock in 90 µl of nano pure water (distilled water) making (10%).

For the molecular confirmation of *P. falciparum* method of Londono et al., [38] was adopted, where 18S Portion of small subunit

ribosomal RNA gene was amplified in Classic DW-K960 thermal cycler using the following set of primers(Table 1). The forward primer is species-specific, for that it is hybridized only with *P. falciparum* DNA, while the reverse primer is genus-specific, thus hybridized with all the four *Plasmodium* species. The reaction was carried out in a 25 µl reaction mixture containing 5µl of the extracted DNA as the template, 1µl of primer (0.5µl each of reverse and forward primers), 6.5µl distilled water and 12.5µl of the PCR Master mix (Containing dNTPs, MgCl₂ and Taq DNA Polymerase). The gene was amplified by setting an initial denaturation at 95°C for 15minutes then followed by forty (40) cycles of denaturation at 94°C for 45seconds while annealing at 60°C for 90seconds and extension at 72°C for 1minute. The final extension was carried out at 72°C for 5minutes.

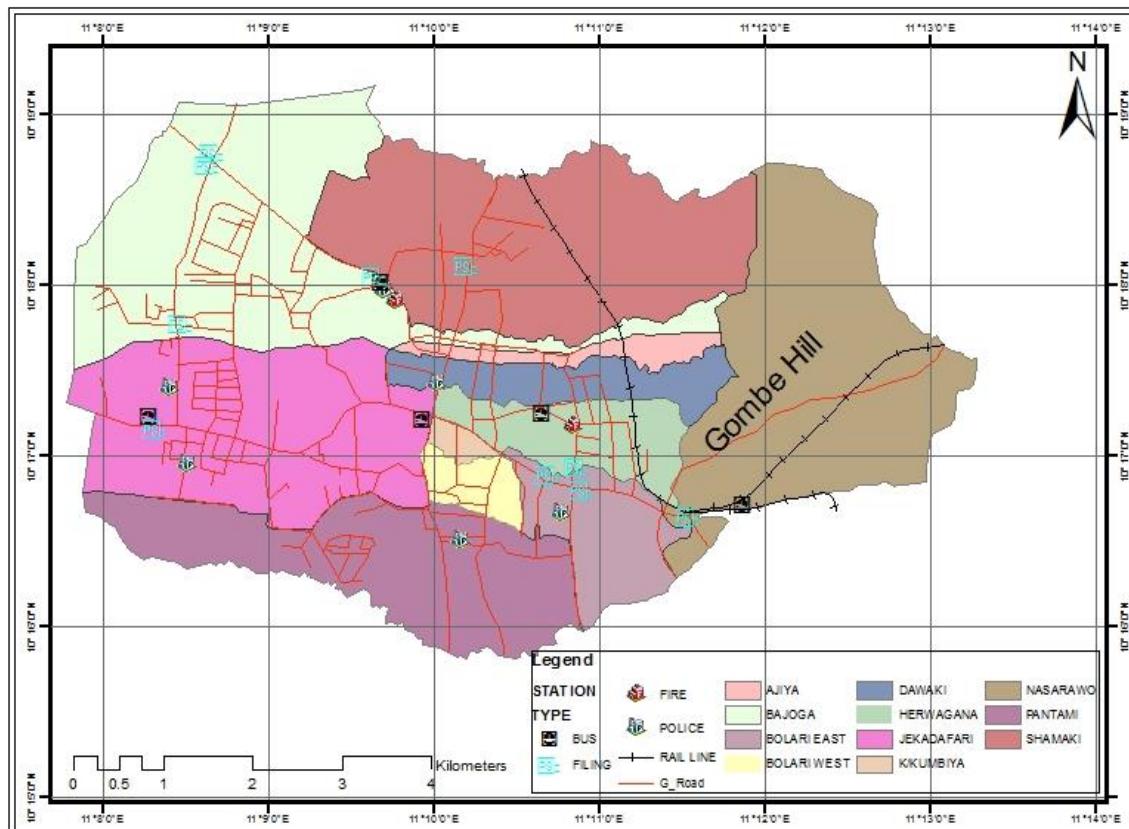


Figure 1: Map of Gombe Local Government Area

Source: GIS Laboratory, Geography Department, Gombe State University

Table 1. PCR Cycling Condition for the confirmation of *P. falciparum*

Primer Sequence	Cycling condition	Cycles
*F(5'AACAGACGGTAGTCATGATTGAG3')	95°C(15min); 95°C(45sec), 60°C(90sec), 72°C(1min); 72°C(5min)	40
*R(5'GTATCTGATCGTCTTCACTCCC3')		

*; F(Forward), R(reverse)

2.8. Amplification of *P. falciparum* Chloroquine Resistance Transporter (PfCRT) gene

The gene was amplified in 35 cycles using Nested PCR according to the method described by previous research [39]. For the Primary PCR (Nest 1) 5µl of the extracted DNA was used as a template. The reaction was carried out in a 25 µl reaction mixture, containing PCR master mix 12.5µl, primers 1µl (0.5µl each of F5'GCGCGCGCATGGCTCACGTTAGGTGGAG3' and R5'GGGCCGGCGGATGTTACAAACTATAG3') and 6.5µl distilled water. The different cyclic conditions for nest 1 were 94°C initial denaturation for 2minutes, 94°C denaturation for 30seconds, annealing at 54°C for 30seconds, extension at 68°C for 20seconds and final extension 68°C for 5minutes. For the second PCR (Secondary), 5µl of the amplicons obtained from the primary PCR were used as the template. The reaction was also completed in 25µl reaction volume, using 5'TGCTGTCATGTGTTAACTT3' and 5'CAAACTATAAGTTAACCAATTG3' primers.

The cyclic conditions were 94°C, 60sec. initial denaturation, 94°C 30sec denaturation, 54°C 30sec annealing, 68°C 15sec extension and 68°C 5min. final extension

2.9. Electrophoresis

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

2.10. Sequencing of the PCR Product and BLAST

The entire amplified PCR products were sent to Cambridge Genomic Services, the UK for sequencing. Afterwhich all sequenced amplicons were subjected to BLAST (Basic Local Alignment Search Tool) in order to determine the position of mutation and the nature of the mutation.

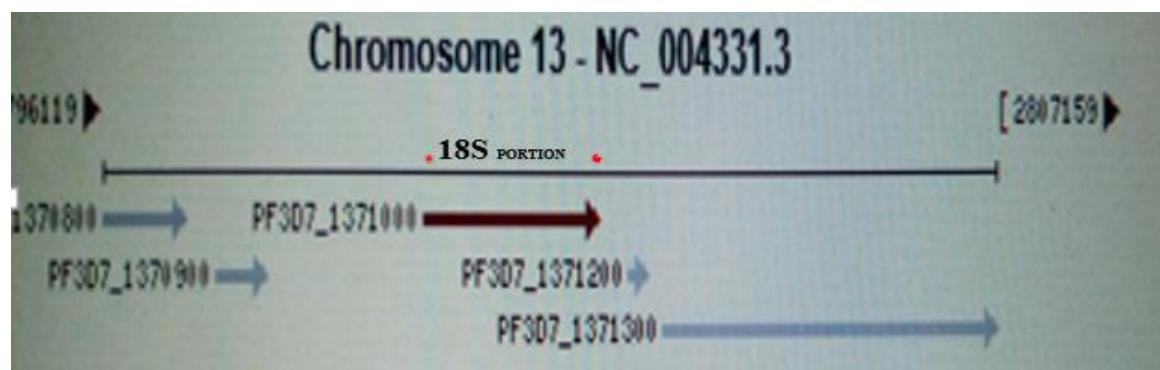
**Fig. 2.** Chromosome 13 of *P. falciparum* indicating 18S Portion of Ribosomal RNA gene

Table 2. Gene, Chromosome number, Primer Sequence and PCR Thermo cyclic Conditions

Gene	Chromosomes No.	PCR	Primer	Primer sequence	Cyclic condition
PCRT	Seven	Primary	PCRTN1F	F5'gcgccgcgtggctacgttttagtgag3'	94°C(2 min); 94°C(30 s), 54°C(30 s), 68°C(20 s); 68°C(5 min)
			PCRTN1R	R5'ggcccgccatgttacaaaactatag 3'	
	Secondary		PCRTN 2F	F5' tgctgtcatgtttaaactt 3'	94°C(60 sec.); 94°C(30 sec), 54°C(30 sec.), 68°C(15 sec); 68°C(5 min)
			PCRTN 2R	R5' caaaactatagtaaccatttt 3'	

3. Results

3.1 Demographic and Clinical characteristics of the study subject

A total of 200 study subjects were used, comprised of 114(57.0%) and 86(43.0%) male and female respectively. The age of the subjects ranges from 5 to 55 with a mean of 28.60 ± 10.6 . The mean ambient body temperature of the subjects ranges from 33-43°C with a mean of 37.77 ± 1.92 . For the molecular analysis, the concentration of the DNA sample extracted ranges from 1.10-6.2ng/ μ l 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 ranges from 0.7-5.11. Table 3 below summarises the basic characteristics of the subjects and the sample used for the molecular analysis.

3.2 Prevalence of SNPs at C72S, M74I, K76T and N75EA PCRT gene of *P. falciparum*

Table 4 below shows that out of the fifty-five samples genotyped, the K76T variant had the highest prevalence of 46(80.70%), while the C72S variant recorded a prevalence of 05(8.80%). The other variants M74I and N75T had no mutation completely. The mutation at K76T was as a result of a substitution of the nucleotide Adenine (A) in Lysine (K) AAA with nucleotide Cytosine (C), thereby leading to the formation of Threonine (T) ACA. Similarly, the mutation in the C72S variant was due to the substitution of Thymine (T) in Cysteine (C) TGT with Adenine (A), this single substitution led to the formation of Serine (S) AGT.

Table 3. Demographic and clinical characteristics 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	1.10-6.00ng/l		
A260/280	1.72 ± 0.55	0.7-5.11		

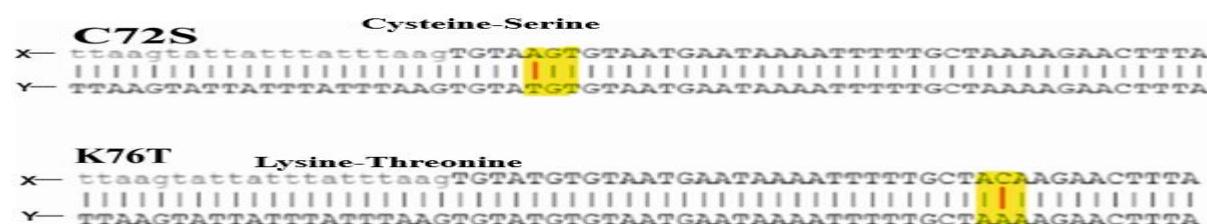
**Fig. 3.** BLASTn result comparing the Query Sequence (X) with the Reference sequence from the gene bank (Y)

Table 4. Prevalence of SNPs at C72S, M74I, K76T and N75E in a PCRT gene of *P. falciparum*

Codons	C72S	M74I	K76T	N75E
Wild type	TGT	AGT	AAA	AAT
Amino acid	Cysteine	Methionine	Lysine	Asparagine
No. of Isolate with wild type	52(91.20%)	57(100%)	11(19.30%)	57(100%)
Mutant	AGT	AGT	ACA	AAT
Amino acid	Serine	Methionine	Threonine	Asparagine
No. of isolate with mutant alleles	05(8.80%)	00(0.00%)	46(80.70%)	00(0.00%)

4. Discussion

Chloroquine was previously regarded as the most effective drug for malaria treatment globally until it was no longer the drug of choice in most malaria-endemic countries as a result of the emergence and spread of resistant parasite strains[40]. Resistance to chloroquine, the cheapest and most widely available anti-malarial, has reached significantly high levels leading to replacement with artemisinin-based combination therapy (ACT) in many malaria-endemic countries[27]. Notwithstanding, chloroquine resistance from several studies has suggested among other factors that mutations in two genes - *P. falciparum* chloroquine-resistant transporter (*Pfcrt*) and *P.falciparum* multidrug-resistant (*Pfmdr1*) genes are associated with chloroquine resistance[33]. It is therefore important to continue to monitor for the presence of markers known to confer CQ resistance, in view of the possible re-introduction of CQ for malaria treatment, in addition to investigating markers. This is because of its rapid onset of action, low toxicity and is well tolerated[41].

The portion of the *PCRT* gene used in this study which covers codon positions 72, 73, 74, 75 and 76 is 99.31% similar to the portion of the gene obtained in previous research [42] with the accession number MN419879, MW274873 and MW267868 respectively. MK884834 and LC498195 also show the same percentage of 99.31% with the same portion of the gene used in this study.

In this study, two variants K76T (80.7%) and C72S (8.8%) of the *PCRT* gene were reported as the molecular markers for chloroquine resistance. This result is lower than the 95.0% positive isolate for *PCRT*-gene

from Akure in previous research [20]. The difference observed might be attributed to the differences in the drug pressure specifically chloroquine exerted by the parasite (*P. falciparum*) from the two study areas (Gombe and Akure). Contrary to the findings of this study, it was reported [39] five different variants (K76T, Q271E, N326I, I356T and R371I) from Pahang, Malaysia. The difference observed in the number of additional variants from Malaysia, might be attributed to the fact that Chloroquine was still in use or regarded as a first-line treatment for uncomplicated malaria. Unlike in the present study, where chloroquine usage was stopped more than twenty years ago. Therefore, the chances of getting different makers for chloroquine resistance could be higher in Malaysia. In addition, the prevalence of 80.7% in the K76T variant recorded in this study is by far higher than 12.4% in north-western Nigeria[1]. In this present study only two variants (K76T and C72S) of the *PCRT* gene were reported, this is contrary to the findings in previous research [43] which also reported five different variants(C72S, M74I, N75E/D K76T and A220S) of *PCRT* gene from India.

On the other hand, the prevalence of 80.7% in the K76T variant recorded in this study is similar to the findings in previous research [44] which reported a 78.0% prevalence of point mutation associated with *PCRT* from Chhattisgarh, Central India but contrary to the findings in previous research [45] who reported a prevalence of 29.80% from isolating imported from Africa to China and also it was reported [46] a prevalence of 56.7% in K76T variant from India. The difference in the prevalence of *pcrt* mutation might be attributed to the level of Choroquine exposure by the study subjects used in the

two studies. In this present study, the subjects were exclusively Nigerians who had been exposed to the adverse quenciquencies of chloroquine for a long period. While in the study [45], the subjects might be a mix-up of Nigerians and foreigners (Especially Chinese) and for long China had eliminated malaria, therefore, the subjects may not have had the adverse impact of chloroquine (resistance). Similarly, it was reported [47] the lower prevalence of 54.7% and 60.0% in the K76T variant from eastern India and Oyo state, Nigeria respectively.

The high prevalence of the K76T variant in this study and other several studies is attributed to the fact that the variant is the most predictive point mutation for chloroquine resistance[48]. This finding is contrary to the previous result [26] which reported Zero prevalence of Chloroquine-resistance genotyped from Zambia. This difference might be attributed to the fact that the usage of chloroquine for malaria treatment was for long discontinued officially in the country and there was almost complete compliance. Unlike here where up to now some consider chloroquine as an effective drug in malaria treatment. This is because partly, the parasite is still sensitive to chloroquine, especially in South-Western Nigeria[49]. The prevalence of 80.70% in K76T recorded in this study is lower than 90.30% reported in previous research [50] in Yunna province from China and 100% recorded in previous research [51] from endemic malaria areas in Rondonia and Pará State, Brazilian Amazon Region. The result is also contrary to the findings in previous research [52] from southeast Nigeria where 100% and 94.5% prevalence were recorded in C72S and M74I, N75E and K76T variants respectively. Similarly, it was reported [53] 100% mutation in K76T from Guyana, South America. Therefore all these justify the immediate need and reason for the withdrawal of the drug (Chloroquine) as the first-line drug in the treatment of malaria.

The absence of single nucleotide polymorphism on M74I and N75E variants reported in this study is similar to previous research [40] which reported the absence of any other variant apart from K76T (70.4%)

from two communities (Lekki and Ijede of Lagos state) Nigeria and it was reported [54] a zero prevalence of M74I and N75E variants and any other variant except K76T(70.4%) variant in Toronto among study subject who returned from malaria-endemic areas. Similarly, according to previous research [55] from western Kenya, a prevalence of 65% was reported in the K76T variant of the PCRT gene. Over dominance of the K76T variant in this study and several other studies elsewhere is attributed to the fact that the K76T variant, unlike other variants which are in most cases regarded as candidate markers, K76T variant is a confirmed molecular bio-maker [56]. This is because the K76T variant is often found in almost all Chloroquine-resistant parasites and clinical isolates. Therefore, for that, it has been regarded as the main molecular marker in order to monitor chloroquine resistance in the field [57]. Another factor that could be responsible for the high prevalence of the K76T variant as compared to the variants might be due to the much attention given to the K76T variant by several researchers especially because of its synergistic effect with Y184F of PFMDR-1[58].

On the other hand, the finding of this study is contrary to previous research [48] which reported the prevalence of 26.9% each of M74I and N75E variants from Nigeria. Although these variants were not reported in the present study, the parasite may still not be sensitive to the drugs (Chloroquine) in the study area. This is because these bio makers are regarded as candidate bio makers, not confirmed makers. In addition, their absence in this study may be attributed to the limited number of samples collected, the study area covered coupled with the limited number of centres used for sample collection.

5. Conclusion

Confirmed biomarkers of Chloroquine resistance are still present in *P. falciparum* isolate from Gombe L.G.A. with K76T isolate having a high prevalence. For that the parasites are still resistant to the drug (chloroquine) in the study area, therefore the continued ban on the usage of some antimalarial drugs like chloroquine should be sustained, with continuous monitoring of its status.

Conflict of Interests

All authors declare no conflict of interest.

Ethics Approval and Consent to Participate

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

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' Contribution

All authors had equal role in study design, work, statistical analysis and manuscript writing.

Informed Consent

The authors declare not used any patients in this research.

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