Open Access Library Journal
2023, Volume 10, e10783
ISSN Online: 2333-9721
ISSN Print: 2333-9705

Molecular Detection of *Plasmodium falciparum* and *Plasmodium falciparum* Chloroquine Resistance Transporter Gene from Microscopy-Confirmed Malaria in Febrile Patients at the Federal Teaching Hospital Gombe Nigeria

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How to cite this paper: Baba, F.A., Adamu, H., Maigana, Z.S. and Saleh, N. (2023) Molecular Detection of *Plasmodium falciparum* and *Plasmodium falciparum* Chloroquine Resistance Transporter Gene from Microscopy-Confirmed Malaria in Febrile Patients at the Federal Teaching Hospital Gombe Nigeria. *Open Access Library Journal*, **10**: e10783.
https://doi.org/10.4236/oalib.1110783

Received: September 20, 2023
Accepted: October 28, 2023
Published: October 31, 2023

Abstract

Malaria is a parasitic disease caused by the protozoan parasites *Plasmodium species,* and usually transmitted through the bite of a female *Anopheles species* mosquito. *Plasmodium falciparum* is regarded as the most lethal of the *Plasmodium species* and most efforts are geared towards its control. Hitherto, with no available vaccines, an antimalarial drug such as Chloroquine was the main stay in the clinical management of uncomplicated *P. falciparum* malaria. Chloroquine clinical efficacy was eroded with the emergence of *P. falciparum* bearing the mutant form of *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene. The aim of the study was to molecularly confirm through PCR, *P. falciparum* malaria and the presence of *pfcrt* gene from light microscopy-confirmed malaria patients at the Federal Teaching Hospital Gombe Nigeria. 100 whole blood samples from light-microscopy confirmed *P. falciparum* malaria from patients of all age groups and genders were collected. A thick and thin smear of finger-prickd blood was made to detect *P. falciparum* malaria microscopically, EDTA preserved whole blood, DNA integrity, purity and concentration prior to nested-PCR to confirm *P. falciparum* and *pfcrt* gene. DNA with high integrity with an average DNA yield of 34.7 µg/mL and about 99% free from impurities able to interfere with PCR. Through nested PCR methods, the prevalence of *P. falciparum* malaria infection (52%) is nearly identical to non-*P. falciparum* malaria infections (48%) ($p < 0.0025$); though light microscopy detected *P. falciparum* malaria preva-

lence of 63%. 75% of *P. falciparum* strains were positive for *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene. All the *P. falciparum* strains from age groups 41 - 50, 51 - 60, 61 - 70 and 71 - 80 years were *crt* positive. The implication of the findings shows *P. falciparum* malaria prevalence is on the decline; there is the misdiagnosis of *P. falciparum* malaria with light-microscopy method and an increased chloroquine-sensitive *P. falciparum* strains.

Subject Areas

Biotechnology

Keywords

Artemisinin-Based Combination Therapy, Chloroquine Resistance Transporter Gene, Malaria, *Plasmodium falciparum*, Polymerase Chain Reaction

1. Introduction

Malaria is a life-threatening parasitic disease transmitted through the bite of infected female *Anopheles* mosquitoes. Malaria is endemic in tropical and subtropical countries of the world but reports of global prevalence are emerging- in temperate countries, it is associated with international traveller's diseases. Nigeria accounts for the highest proportion of cases globally (27%), followed by the Democratic Republic of the Congo (10%), India (6%) and Mozambique (4%) [1]. In 2017, it is estimated that 219 million malaria cases with 435,000 malaria deaths occurred in 91 countries with ongoing malaria transmission [2]. 80% of global malaria cases and deaths took place in fifteen countries 90% and 91% of malaria cases and malaria deaths, respectively, occur in Sub-Saharan Africa including Nigeria (the vast majority of malaria cases 95% are reported in Africa) [3]. The disease is caused by *Plasmodium* species, the protozoan parasite belonging to the Apicomplexa and *Plasmodiidae* family. The genus comprises of five species of medical importance in humans, namely: *P. falciparum, P. malariae, P. ovale, P. vivax and P. knowlesi* (parasite of malaria in animals with humans occasionally infected) [4]. *P. falciparum* the most lethal of all the *Plasmodium* species is the cause of 92% deaths due to malaria in all age and risk groups. Rapid diagnosis and treatment are essential in avoiding severe illness or even fatal outcomes [5]. Microscopy is considered the gold standard in diagnosis but rapid diagnostic tests (lateral flow Assay) and molecular approaches are equally effective [4].

Children less than 5 years, the elderly and immuno-compromised individuals are considered high-risk groups for malaria diseases and risk factors driving *Plasmodium* spp. transmissions include population immunity, social and economic status (housing conditions), application of mosquito control measures, and environmental factors [6]. In rare occasions, malaria can be transmitted congenitally from a mother to an unborn child (*in utero*), as well

as through blood transfusion, organ transplantation and unhygienic needle sharing [7].

Chloroquine (CQ), an aminoquinoline, was discovered in 1934 and introduced into clinical practice in 1947 [8]. CQ was the first-line drug for the treatment of uncomplicated malaria caused by *P. falciparum* in Nigeria until 2005 when the Malaria Treatment Guideline switched to Artemisinin-based Combination Therapy (ACT) due to reports of clinical treatment failure associated with CQ resistance [9]. CQ resistance emerged worldwide as early as the late fifties in Southeast Asia and South America [5]. In Africa, CQ resistance was reported in Sudan in the late 1970s [10] and in Nigeria in 1977 [11]. Though CQ, is a derivative of the quinoline antimalarial drug quinine, which was first isolated from the extract of the bark of the Cinchona tree (Cinchona *officinalis*), was used by the indigenous people in Peru in the treatment of high chills. Despite the switch to ACT in the treatment of uncomplicated malaria, CQ remained a drug of choice in certain settings in Nigeria and other parts of Africa, because of its accessibility and affordability [9]. The genomic mechanisms of CQ resistance have been mapped to Single Nucleotide Polymorphisms (SNPs) in two genes *P. falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance 1 (*pfmdr1*) genes [12] [13]. The persistence of CQ resistant genes despite the change in Malaria Treatment Guideline or conversely in the absence of CQ selective pressure remains not completely understood. Reversion to CQ-sensisitive *P. falciparum* (wild type) after the complete withdrawal of CQ in the population in Malawi and Kenya has been reported [14].

With malaria vaccine yet to be deployed, therapeutic intervention with antimalarial drugs remains the cornerstone in the control of malaria. Prevention and control of malaria diseases relies on the epidemiological burden of the disease, treatment efficacy and understanding of the vector biology as well as transmission pathways. In this regard, the present study aimed to survey the prevalence of chloroquine-resistant *P. falciparum* strains isolated from patients with malaria in Federal Teaching Hospital Gombe State North-East, Nigeria.

2. Methodology

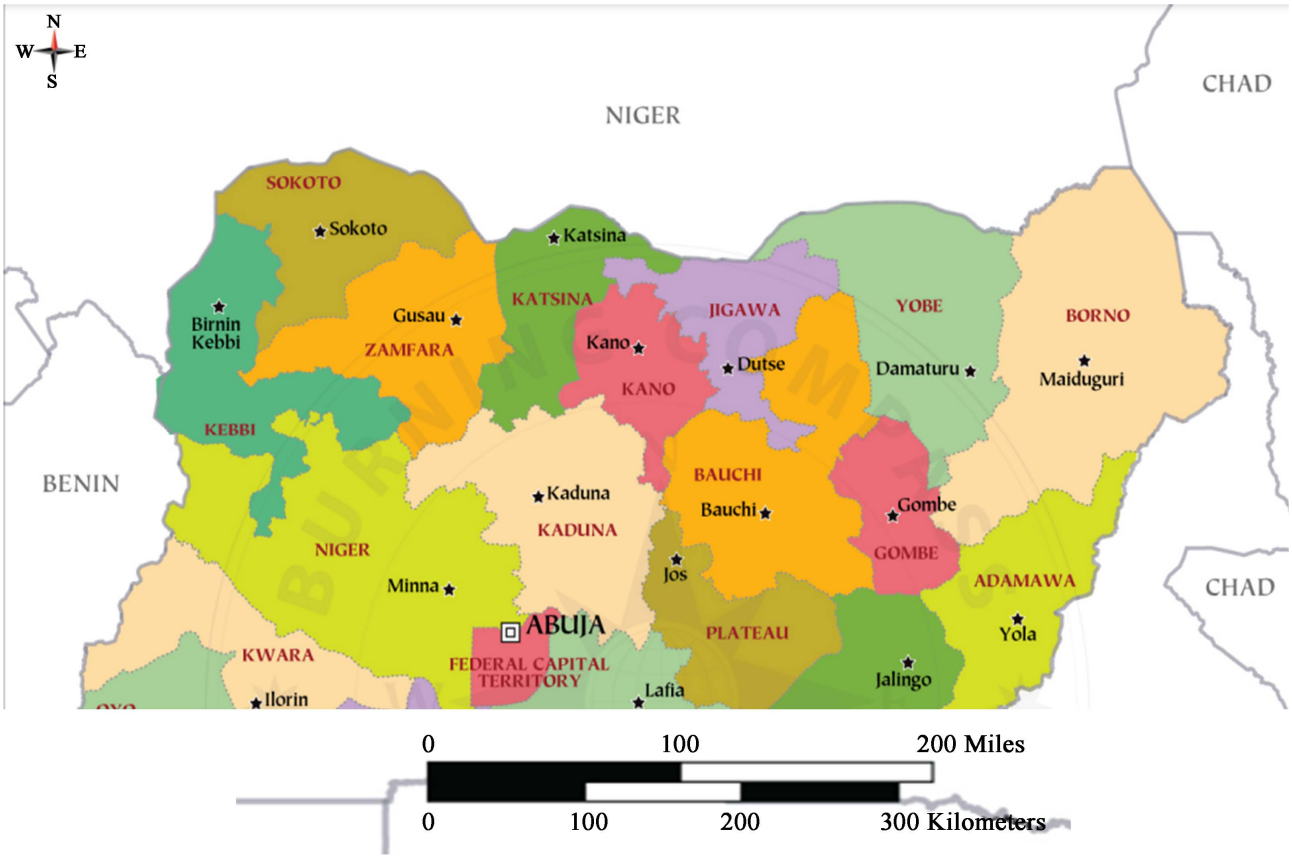
2.1. Study Design and Study Area

The study was a hospital-based, prospective and cross-sectional study targeting febrile patients attending FTH Gombe and was conducted between October and November 2021. The FTH Gombe is a Federal Government of Nigeria established 530-bed tertiary hospital, located in Gombe the capital of Gombe State. The hospital operates clinics with 34 specialties, catering to patients from States in North-East and North-West Nigeria such as Gombe, Bauchi, Borno, Yobe, Jigawa, Adamawa and Taraba States. Gombe State is located in the Guinea and Sudan Savannah ecological zone between latitudes 10.15˚N and 11.17˚E. Gombe State records the highest temperature of 40˚C in the month of April with the coldest period in December with about 11˚C. The state has a tropical climate

with two marked seasons, the dry and wet season. The dry season starts from October to April while the rainy season starts from May to September with an average rainfall of 850 mm. The State shares a border with Yobe State in the North, Adamawa State in the South, Borno State in the East and Bauchi State in the West (Figure 1). The State estimated population according to the 2006 census of 2,365,000 inhabitants with a land area of 20,265 km^2. The State is multi-ethnic and multi-lingual with the Fulani, Hausa, Tarawa, Bolewa, Kanuri, Tangale, Tula, Waja and Jukun as indigenous tribes (GIS).

2.2. Study Participants

Study participants were recruited from the routine health delivery system at FTH Gombe. Self-presenting febrile patients attending outpatient or inpatient clinics and of all age groups were eligible for enrolment. Any patient with suspected malaria was approached for consent immediately after receiving their laboratory result. For those who consented to be part of the study, approximately 5 - 10 µl finger-prick and 10 mL venous blood samples were collected. No additional clinical assessment was carried out at the time of recruitment. Adults and children meeting the age inclusion criteria were screened for *P. falciparum* infection by examining prepared thick and thin smears stained with 10% Giemsa



(Source: https://www.burningcompass.com/countries/nigeria/maps/nigeria-state-map.pdf accessed 19th August 2023).

Figure 1. The Map of the Study Area—Gombe State North-East Nigeria.

and by PCR for the molecular identification of *P. falciparum* and *P. falciparum* chloroquine-resistant transporter gene. Patients treated with antimalarial for the past two months were excluded from the study.

2.3. Ethics Statement

Ethical approval with reference number NHREC/25/10/2013 was obtained from the FTH Gombe Nigeria, Hospital Research and Ethics Committee for the study titled Parasitemia in Chloroquine-Sensitive and Chloroquine-Resistant *Plasmodium falciparum* in Malaria Patients Attending FTH Gombe Nigeria. Informed written consent and assent (in the case of children between 12 and 18 years of age) were obtained from each patient or a parent/legal guardian. The study participation was voluntary and had no influence on the treatment provision at the respective health facilities. All patient information and records were kept confidential and in line with the requirements of Nigerian National Health Research Ethics Committee guidelines.

2.4. Sample Collection

10 µL finger-pricked blood was obtained for each patient and placed on a slide for thin and thick blood smear. About 2mls of Venous blood was collected into EDTA tubes from each patient aseptically using a syringe by applying a tourniquet after disinfecting the collection site. The blood sample was then stored in a refrigerator at 2˚C - 8˚C for the study until used.

2.5. Stain Preparation and Examination

Using an automatic pipette, 6 µL and 2 µL of finger-prick blood from a patient were placed at different sites on the same slide for the thick and thin films respectively. The blood smears were air-dried, fixed in methanol and subsequently stained for 20 - 30 minutes in 3% (w/v) Giemsa [15]. Buffer of pH 7.2 was used to wash off the stain. All the positive slides were subsequently examined for malaria parasites by counting against 200 leucocytes. The degree of parasitaemia was graded thus < 1000/µL or +, 1000 - 9999/µL or++ and ≥10,000/µL or +++ [14]. At least 100 thick field films with an oil immersion lens at 100X magnification were examined before a slide was considered negative. The stained thick smear was used for the detection of the presence of the malaria parasite, whereas the thin film was used for species identification. All blood slides were examined by two WHO-trained microscopist to cross-check each slide before the results for a specimen were finalized. For quality control, 10% of the blood slides were re-examined by an independent microscopist unaware of the initial results.

2.6. Genomic DNA Extraction from Blood

Total genomic DNA from samples was extracted using Quick-DNA Miniprep Kit™ (Zymo Research). Briefly, 50 µL of fresh venepuncture blood samples, or preserved blood in EDTA, were used, and blood was collected from hospital pa-

tients by clinicians or medical laboratory scientists. Blood sample frozen was thawed at room temperature and homogenised. 200 µL of Genomic lysis Buffer was added to 50 µl of blood (4:1 ratio) in 1.5 mL microcentrifuge tube, the sample was mixed by vortexing for 4 - 6 seconds, and then the mixture was allowed to stand for 10 minutes at room temperature. The mixture was then transferred to Zymo-Spin™ IC column in a collection tube, which was centrifuged at 13,500 revolution per minute (rpm) for one minute. The collection tube with the flow through was discarded and the spin column was transferred to a new collection tube. 200 µL of DNA Pre-Wash buffer was added to the spin column followed by centrifugation at 13,500 rpm for one minute. 500 µL of g-DNA Wash Buffer was added to the spin column followed by centrifugation at 13,500 rpm for one minute. The spin column was transferred to a clean autoclaved microcentrifuge tube. 35 µl of DNA Elution Buffer was added to the spin column and allowed to Incubate for 5 minutes at room temperature and then DNA eluted by centrifugation at 13,500 rpm for one minute. Eluted DNA was checked for integrity in a 0.35% (w/v) agarose, purity and quantity using the NanoDrop 1C spectrophotometer (Thermo Fischer Scientific, Massachusetts) at the Africa Centre for Excellence in Phytomedicine Research and Development (ACEPRD) University of Jos Plateau State Nigeria.

2.7. Nested PCR for the Molecular Identification of *P. falciparum* and *P. falciparum* Chloroquine Resistant Transporter (*pfcrt*) Gene

PCR was performed with DNA that meets the quality for PCR with Taq 2X Master Mix M0270 (New England Biolabs UK/Inqaba Biotech Ibadan Nigeria) with established primers (F 5-'TTAAAATTGTTGCAGTTAAAACG-3') and (R 5'-CCTGTTGTTGCCTTAAACTTC-3') according to Zhou *et al* 2020 (Table 1). Briefly, for the primary (1st round) of PCR *pfcrt*1, 2.0 µL genomic DNA template, 12.5 µL Taq 2X Master Mix- final concentration 1X, 1.0 µL forward primer (10 µM)- final concentration 0.4 µM, 1.0 µL reverse primer (10 µM) (final

Table 1. Species-specific primers used in this study for Nested-PCR for the detection of *P. falciparum* and *pfcrt* gene.

Target	Nested-PCR Stage	Primer Label	Primer Sequence	Size (bp)	Reference
P. falciparum	Nest-I	fPLU	5'-TTAAAATTGTTGCAGTTAAAACG-3'	1100	Orlov *et al.* 2015
		rPLU	5'-CCTGTTGTTGCCTTAAACTTC-3'		
	Nest-II	fFAL	5'-TTAAACTGGTTTCGGAAAACCAAATATATT-3'	205	
		rFAL	5'-ACACAATGAACTCAATCATGACTACCCGTC-3'		
pfcrt	Nest-I	OuterP1	5'-CCGTTAATAATAAATACACGCAG-3'	537	Zhou *et al.* 2016
		OuterP2	5'-CGGATGTTACAAAACTATAGTTACC-3'		
	Nest-II	InnerP1	5'-TGTGCTCATGTGTTTAAACTT-3'	145	
		InnerP2	5'-CAAAACTATAGTTACCAATTTTG-3'		

concentration 0.4 μM), and molecular grade H_2O (up to 25.0 μL) were mixed and subjected to the following program: initial denaturation at 95˚C for 30 seconds, followed by 35 cycles of 95˚C for 30 s, 50˚C for 30 s, and 72˚C for 1 min, and a final extension at 60˚C for 5 min. Five microliters of products from the 1st round of PCR amplicons were separated in 1.5% agarose (w/v) gel electrophoresis. For the secondary (2nd round) of PCR *pfcrt*2, 2.0 μL products from the 1st round of PCR with 12.5 μL Taq 2X Master Mix-final concentration 1X, 1.0 μL forward primer (F 5'-TTAAACTGGTTTCGGAAAACCAAATATATT-3'), (R 5'-FACACA ATGAACTCAATCATGACTACCCGTC-3') (Table 1) (10 μM), 1.0 μL reverse primer (10 μM)—all primers at final concentration 0.4 μM—and nH_2O (up to 25.0 μL) were mixed and subjected to the following program: initial denaturation at 95˚C for 30 second and followed by 35 cycles of 95˚C for 30 s, 50˚C for 30 s, and 72˚C for 60 s, and a final extension at 72˚C for 5 min. Five microliters of products from the 2nd round of PCR amplicons were separated in 1.5% agarose(w/v) gel electrophoresis [16].

2.8. Statistical Analyses

The data obtained was analyzed using Microsoft Excel. Statistical analysis was carried out using P-values from a chi-square test for proportions using P-value of 0.05, comparing the prevalence of *P. falciparum* between different age groups. The analysis was done using SPSS 20.0.

3. Results

DNA extraction from EDTA-preserved blood samples resulted in DNA with high integrity (**Supplementary Material (Suppl.) 1**), an average DNA yield of 34.7 μg/mL and about 99% of DNA eluates free from impurities able to interfere with PCR (an aliquot of the impure DNA eluate was diluted in molecular grade water before further use) (Table 1).

The age group of 0 - 20 years accounts for over 60% of *P. falciparum* malaria cases in the study, while the age group 61 - 80 years with 2% prevalence was the least infected with *P. falciparum malaria* cases (Table 2).

In age groups 0 - 40 year *P. falciparum* positive and negative for *pfcrt* were observed. All the *P. falciparum* strains from age groups 41 - 50, 51 - 60, 61 - 70 and 71 - 80 years were *pfcrt* positive. The difference in *pfcrt* presence was not significant ($p < 0.671$) (Table 3).

4. Discussion

The optimal extraction of DNA from diverse protozoal species is an essential step in the downstream molecular biology applications for the analyses of protozoal gene sequences, gene expressions and interspecies differences. Several methods and kits are available for the isolation of protozoal DNA and RNA. The Zymo Quick-DNA Miniprep kit D3024, a spin column-based technology for quick purification of DNA of high quality without the use of proteinase K and

Table 2. Assay for DNA purity and concentration using nanodrop 1C.

Sample Label	Concentration (µg/µL)	A280/A260	A260/A230	Inference
1	33.4	1.88	0.37	Pure
2	20.0	1.94	0.33	Pure
3	22.0	1.93	0.13	Pure
4	47.0	1.86	0.29	Pure
5	36.0	1.86	0.23	Pure
6	16.7	1.83	0.38	Pure
7	57.1	1.82	0.52	Pure
8	25.9	1.80	0.17	Pure
9	49.4	1.82	0.76	Pure
10	31.0	1.83	0.50	Pure
11	81.4	1.73	0.49	Impure
13	36.4	1.97	0.03	Pure
17	33.4	1.88	0.16	Pure
21	3.8	1.81	0.06	Pure
22	30.5	2.57	0.09	Impure
38	34.1	1.82	0.04	Pure
49	32.0	1.85	0.27	Pure

Table 3. Comparison of light microscopy and Nested-PCR in prevalence of *P. falciparum* species stratified by patients age.

Age Group	No. of Samples	Light Microscopy		Nested PCR		P-Value
		P. falciparum (%)	*Non-P. falciparum* (%)	*P. falciparum* (%)	Non-*P. falciparum* (%)	
0 - 10	26	26 (86.7)	04 (13.3)	21 (70.0)	09 (30.0)	<0.0025
11 - 20	16	16 (66.7)	08 (33.3)	13 (54.2)	11 (45.8)	
21 - 30	10	10 (62.5)	06 (37.5)	07 (43.8)	09 (56.2)	
31 - 40	08	08 (57.1)	06 (42.9)	05 (35.7)	09 (64.3)	
41 - 50	02	02 (50.0)	02 (50.0)	02 (50.0)	02 (50.0)	
51 - 60	03	03 (50.0)	03 (50.0)	02 (33.3)	04 (66.7)	
61 - 70	01	01 (50.0)	01 (50.0)	01 (50.0)	01 (50.0)	
71 - 80	01	01 (25.0)	03 (75.0)	01 (25.0)	03 (75.0)	
Total	67	67 (67.0)	33 (33.0)	52 (52.0)	48 (48.0)	

organic denaturants and compatible with anticoagulants such as EDTA, heparin and citrate is ideal for the isolation of high-quality DNA from preserved blood specimens in our setting. The stages in DNA extraction with the Zymo kits are

Table 4. Molecular distribution of Chloroquine Resistant Transporter (*crt*) gene in *Plasmodium falciparum* according to patients age group.

Age Group	Total (%)	*Pfcrt* + ve (%)	*pfcrt* – ve (%)	P-value
0 - 10	21 (40.4)	17 (43.6)	04 (30.8)	0.671
11 - 20	13 (25.0)	08 (20.5)	05 (38.5)	
21 - 30	07 (13.5)	04 (10.3)	03 (23.1)	
31 - 40	05 (9.6)	04 (10.3)	01 (7.7)	
41 - 50	02 (3.8)	02 (5.1)	00 (0.0)	
51 - 60	02 (3.8)	02 (5.1)	00 (0.0)	
61 - 70	01 (1.9)	01 (2.6)	00 (0.0)	
71 - 80	01 (1.9)	01 (2.6)	00 (0.0)	
Total	**52 (100)**	**39 (75)**	**13 (25)**	

steps common to most nucleic acid isolation methods, namely; cell lysis, the use of detergents to dissolve cell membranes and denature proteins, extraction technology that removes PCR inhibitors, DNA precipitated in 95% ethanol (isopropanol), purified DNA eluted in TE buffer or molecular grade water is suitable for PCR and other further analyses.

The study describes the PCR molecular identification of *P. falciparum* from a cohort of microscopy–confirmed *P. falciparum* infection in febrile patients attending Federal Teaching Hospital Gombe North-East Nigeria. The molecular prevalence of *P. falciparum* infection is nearly identical (similar) to non-*P. falciparum* malaria infections. In 100 blood samples examined 52% had *P. falciparum* malaria infections while 48% had non-*P. falciparum* infection. *P. falciparum* malaria infections remain a public health challenge in Nigeria despite the progress in the global reduction of its prevalence and mortality [17]. Hitherto, *P. falciparum* malaria has always taken most of the attention and efforts in the control and elimination of malaria in sub-Saharan Africa [18]. Children are generally known to be a high-risk group for malaria infections including *P. falciparum* malaria and it is estimated that more than half a million children die each year due to *P. falciparum* malaria [19]. Adults with persistent asymptomatic infection due to previous immunity are thought to be reservoirs for malaria infection in children [14].

In a *P. falciparum* malaria prevalence study in Ghana, molecular PCR detected twice *P. falciparum* compared to microscopy; there was an underreporting of *P. falciparum* malaria through microscopy [20]. Microscopic distinction of *P. falciparum*, *P. malariae,* and *P. ovale* was found to be a challenge in Côte d'Ivoire [21], in settings such as Nigeria where malaria is endemic and where all three species are prevalent both in mono-infection and more often in co-infection, *P falciparum* prevalence could be over-reported or underreported (**Table 2**).

Chloroquine was the first line treatment of uncomplicated malaria due to *P.*

falciparum as a result of its efficacy, availability, low toxicity, and affordability. The emergence of clinical failure of treatment with chloroquine in 1960s was first described independently in South East Asia and South America before it spread to Africa led to the resurgence of malaria in several countries [16]. Interest in the mechanistic basis of chloroquine resistance led to the discovery in 2000 of *P. falciparum chloroquine resistance transporter* (*Pfcrt*) gene About 80% of *P. falciparum* strains were positive for chloroquine resistance transporter (*crt*) gene. Overall we detected 25% CQ-sensitive *P. falciparum* in this study which is after 18 years of the change in malaria treatment guidelines from CQ to ACTs. Muhammad *et al.* 2017 documented 17.4% of CQ-sensitive *P. falciparum* 12 years after CQ withdrawal [10]. In the analyses of 103 parasite-positive blood samples, Ikegbunam *et al.* reported 94.5% prevalence of *pfcrt* alleles among 725 non-febrile patients in Nnewi Southeast Nigeria [22]. The *PfCRT* a 49 kDa drug/metabolite transporter protein *PfCRT* localizes to the digestive vacuole (DV) membrane mediating chloroquine efflux out of the DV away from its heme target [8] [23]. The declining efficacy of chloroquine in the treatment of *P. falciparum* malaria resulted in the discovery of several chloroquine derivatives such as piperaquine and other antimalarial utilized as a mono therapy or a combination therapy to slow the onset of resistance [17].

5. Summary, Conclusions and Recommendations

The summary of the research findings is as follows: DNA of high integrity, purity and concentration ideal for downstream molecular biology was obtained with the DNA extraction kit used and the blood samples were collected and preserved. A significant difference was observed in the age-group and the prevalence *P. falciparum* malaria. Similarly, though *pfcrt* prevalence was found to be high, there was no significant difference in *pfcrt* prevalence and *P. falciparum* malaria in different age groups.

In conclusion, molecular nested PCR for *P. falciparum* malaria from light microscopy confirmed prevalence of *P. falciparum* malaria is nearly identical to non-*P. falciparum* malaria in the study area. The light microscopy methods appear to over report *P. falciparum* malaria probably due to light microscopically indistinguishable asexual stages between *P. falciparum* and other non-*P. falciparum* species. Though CQ resistance remains high in *P. falciparum* isolates tested, there is an increase in CQ-sensitive *P. falciparum* in samples tested in an era of ACT therapy.

We recommend the further training of microscopist in the distinction of *Plasmodium species* in thick and thin blood film analyses. The polymorphisms in *pfcrt* in *P. falciparum* and its impact in clinical treatment require further investigation.

Acknowledgements

We want to thank the Dr Manga and the staff of the Medical Laboratory Federal

Teaching Hospital Gombe Nigeria. We also sincerely appreciate Prof Longdet's Molecular Laboratory at the Department of Biochemistry University of Jos as well as the Africa Centre of Excellence in Phytomedicine Research and Development (ACEPRD) University of Jos, Jos Plateau State Nigeria for bench space to carry out molecular biology experiments.

Conflicts of Interest

The authors declare no conflicts of interest.

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