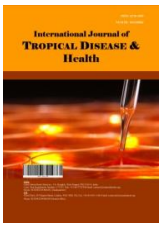

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Molecular Epidemiology of *Plasmodium falciparum* Chloroquine Resistance Transporter Genes among School Children in Kwara State, Southwestern Nigeria

A. O. Oluwasogo[1,2*], H. O. Ismail[3] and D. A. Pelumi[2,4]

[1]Department of Public Health, Kwara State University, Malete, Nigeria.
[2]Infectious Diseases Surveillance and Malaria Cluster Research Group, Kwara State University, Malete, Nigeria.
[3]Zoology Unit, Department of Biosciences and Biotechnology, Kwara State University, Malete, Nigeria.
[4]Department of Medical Laboratory Sciences, Kwara State University, Malete, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author AOO conceived the idea, designed the study, researched the literature and reviewed the final draft of the manuscript. Author HOI wrote the protocol, performed the experiment and wrote the first draft of the manuscript. Author DAP performed the statistical analysis, contributed to review the literature and provided further technical inputs. All authors read and approved the final manuscript.

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ABSTRACT

Background: *Plasmodium falciparum* existence continues to develop resistance to conventional antimalaria drugs in malaria endemic areas. *Plasmodia* often prevent drugs from interacting with the target site, hence, developing resistance to antimalaria drugs. Mutations in the *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*), are the major determinant of chloroquine resistance in human malaria parasite.
Methodology: Malaria infection, *Pfcrt* and *Pfmdr1* genes of isolates among school students within the age range of 11-22 years from four selected rural communities of Kwara state were studied.

**Corresponding author: Email: olalubisogo@gmail.com, oluwasogo.olalubi@kwasu.edu.ng;*

One hundred and eighty seven subjects (187) were selected for the study. Blood samples were collected by finger prick method for malaria screening. Nested PCR and restriction fragment length polymorphism (RFLP) were done to detect alleles of *pfcrt* at codon 76 and *pfmdr1* at codon 86. DNA of isolates was appropriately extracted from the filter paper blots using the methanol fixation method. Logistic regression was performed on the binary observations obtained while linear regression was conducted on the fifty (50) subjects that tested positive to malaria.

Results: Out of 187 subjects screened, 26.7% (50) were positive to *P. falciparum*. Highest malaria parasite count of 36.4% was recorded in 14-16 years age group while 20-22 years age group had the least malaria parasite count (15.4%). The result of the studied isolates indicated that out of 50 isolates analyzed for *Pfcrt* gene, wild type alleles accounted for 32% (16) while mutant alleles accounted for 68% (34). Alakuko Community accounted for the least number of T76 mutant alleles 10% (5) while Apado community recorded the highest number of T76 mutant gene 22% (11). For *Pfmdr1* gene analysis at codon 86, isolates from Apado community showed the highest mutant type alleles (Y86) of 22% (11), while Igbonla community in Ifelodun local government had the least mutant alleles, 6% (3).

Conclusion: The overall result revealed existence of mutant alleles in both the *Pfcrt* and *Pfmdr1* genes which was higher than the wild type gene in both cases. The presence of chloroquine resistance genes among the studied population implies that alternative antimalaria drugs should be designed by pharmaceutical industry.

Keywords: Malaria; Pfcrt and Pfmdr1 mutant genes; epidemiology; nested-PCR and RFLP.

1. INTRODUCTION

Drug *resistance Plasmodium falciparum* has become a critical barricade to the control and elimination of malaria. Long-term anti-malarial monotherapy is capable of springing up drug *resistance* parasite strains and may provide a chance to spread the resistance to sensitive parasite population in epidemic areas under favorable condition for the parasite transmission [1]. Since the early 1940s, chloroquine was continuously used to treat malaria in many countries, which has been confirmed as one of the most important antimalarial drugs with quick metabolism, curative effects, and low price [2]. However, due to excessive drug usage over the years, the CQ resistance of *P. falciparum* isolates was found initially to emerge from Thailand-Cambodia border in Southeast Asia in 1957 and Venezuela-Colombia border in Northern South America in 1959, and eventually spread to other countries around the world [3]. In 2006, the World Health Organization (WHO) recommended artemisinin-based combination therapies (ACTs) as the first-line treatment for uncomplicated *P. falciparum*. ACTs serve as an important therapeutic method to avoid or defer the development of drug resistance for *P. falciparum* infection. Unfortunately, *P. falciparum* resistance to artemisinin, the bedrock of ACTs, was noted in Western Cambodia and subsequently spread across several neighboring countries in Greater Mekong Sub-region (GMS) of Southeast Asia in recent years [4]. The emergence of artemisinin *resistance* indigenous isolates in Africa has been reported more recently, which would be fatal in the future since the suitable replacement drugs are limited. Thus, it is important to monitor the drug *resistance* trend of *P. falciparum*, so as to assess the possibility of reintroducing conventional drugs and also attempt to block the emergence of potential large-scale artemisinin *resistance* transmission [5].

Molecular marker detection for parasites drug resistance is one of several methods for the surveillance of *resistance* prevalence and antimalarial efficacy. The single nucleotide polymorphisms (SNPs) at codons 72, 74, 75 and 76 of *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*), and 86, 184, 1034, 1042, 1246 of *P. falciparum* multidrug resistance 1 gene (*Pfmdr1*) have been shown to be associated with parasite's chloroquine *resistance* (Golassa et al. 2014). The genetic basis of drug resistance is because of polymorphisms in *P. falciparum* genes. For example, mutations in the *P. falciparum* chloroquine *resistance* transporter gene (*Pfcrt*), and *P. falciparum* multidrug *resistance* 1 (*Pfmdr1*) genes are known to confer resistance against chloroquine [6]. However, the *pfcrt* gene has also been associated with artemisinin resistance, whereas parasite *pfmdr1* polymorphisms also confer reduced susceptibility to other antimalarial drugs, including mefloquine, lumefantrine, and quinine [7].

Molecular surveillance of antimalarial drug *resistance* markers has become an important

part of resistance detection and containment. In the present state of multidrug resistance, including resistance to the first-line treatment drug, artemisinin, there is a consensus to upscale molecular surveillance [8]. The most notable obstacle to current surveillance efforts is that skill and infrastructure requirements are not available in many regions. This includes sub-Saharan Africa, where *Plasmodium falciparum* is responsible for most of the global malaria disease burden [8]. New molecular and data technologies have emerged with an emphasis on accessibility. These will pave way for surveillance to be conducted in broad settings where it is most needed, including the primary healthcare level in endemic rural part of countries like Nigeria. This study aimed to study the *Pfcrt* and *Pfmdr* genes of isolates in order to add to the existing malaria surveillance data by surveying for plausible allelic mutations of *Pfcrt* and *Pfmdr* polymorphs from selected rural communities of Kwara State.

2. MATERIALS AND METHODS

2.1 Research Design

It was a Descriptive Survey study involving collection of information concerning the problem from the representative samples and drawing of conclusion based on the information gathered and analyzed from the population of study.

2.2 Study Population

The populations for this study were secondary school students within the age range of 11-22 years that cut across four selected communities in Kwara State.

2.3 Research Setting

The research was conducted in Igbonla (Ifelodun local government), Iponrin community, Apado community and Alakuko community (Ilorin East local government) respectively. This was done during rainy period since this is the period that malaria infection used to be dominant.

2.4 Sample Size

A total of 187 blood samples were collected. The sample size was computed from Taro Yamane Formula which is n= N/ [1+N $(e)^2$]. Where N= 556,700, e signifies the level of precision or margin error which is 0.05 at 95% confidence interval and1 is a constant.

2.5 Sample and Sampling Technique

Stratified random sampling technique was employed to select subjects that would participate in the study. Participants were grouped into four different strata of age, viz: 11-13, 14-16, 17-19, and 20 - 22. Each stratum was used to randomly choose subjects from each school of the selected communities. We randomly chose half of the total number of subjects that represent each stratum in the four different schools by paper balloting to arrive at the sample size obtained.

2.6 Blood Sample Collection

Blood samples of subjects were taken by finger prick of the index finger. Then dropped on a 3 mm Whatman filter paper (Whatman International Ltd., Maidstone, England), kept in individual sealed plastic bags at room temperature for molecular analysis; a drop was also placed on microscope slides for microscopic screening and third drop spotted for rapid diagnostic test (RDT). Safety procedures were adopted for the collection of finger-pricked blood samples by swabbing the area to be pricked with 70% ethanol and allowed to dry before collection.

2.7 Laboratory Investigations

Rapid diagnosis test: Blood obtained from subjects were analyzed in situ with RDT kit for malaria (CareStart Malaria HRP2 from Access Bio, Inc, England). Drops of blood samples were placed in samples holes and wash buffer added in another hole. Two lines both at the control and test side of the test instrument indicate positive for *P. falciparum* malaria while a line only at the control side indicate negative for *P. falciparum* malaria. This was observed within 25 seconds of the test. Students that were positive were referred to community's health centers for proper clinical assessment and treatment.

Microscopy analysis: Thick and thin film stained with Giemsa were prepared for the microscopic examination of the malaria parasite. The thin films were fixed with methanol and all films were stained with 3% Giemsa stain of pH 7.0 for 30 min as recommended by WHO [9]. Blood films were examined microscopically using 100X (oil immersion) objectives as described by Cheesbrough [10]. The thick films were used to determine the parasite densities while thin films were used to identify the parasite species and infective stages.

2.8 Molecular Analysis

DNA extraction: Parasite DNA was extracted from the filter paper blood blots using the methanol fixation method described by (Cortese & Plowe, 1999). Briefly, 3 mm^2 of the blood blot filter paper was incised into a 0.5 ml microfuge tube, followed by the addition of 50 µl of methanol. The tube was incubated for 15 min at room temperature, after which the methanol was discarded. Doubled distilled water, 50 µl, was added and the tube heated for 15 min at 95°C with occasional vortexing. The extract after centrifugation, rich in parasite's DNA, was stored at −20°C until used.

Detection of *Pfcrt K76T* and *Pfmdr1 N86Y* alleles: Genetic characterization of *Plasmodium falciparum* isolates (total genomic DNA of each isolate), including human DNA, were extracted using the MaxMag™DNA MultiSample kit (Applied Biosystems, Warrington, UK) following the manufacturer's instructions. All molecular experiments were performed at the Malaria laboratory in University College Hospital, Ibadan.

Pfcrt single-nucleotide polymorphisms at Codon 76 (K76T, PlasmoDB single nucleotide polymorphism (SNP) ID: Pf3D7_07_v3: 403,625) was genotyped by restriction fragment length polymorphism (RFLP) as detailed by Anderson et al. [11]. *Pfcrt* (PF3D7_0709000) was amplified by semi-nested PCR with fluorescent end labelled primers. First and second rounds of PCR had the same cycling conditions: 94°C for 2 min, 10 cycles of 94°C × 20 s, 50°C × 20 s and 60°C × 30 s, 35 cycles of 94°C × 20 s, 45°C × 20 s and 60°C × 30 s, and a final 5-min extension step at 60°C. The 6-FAM-labelled product (1 µl) was digested with 1.2 units of Apo I (New England Biolabs, Evry, France) at 50°C for 3 h in a 30 µl reaction. Labelled and restricted products were diluted 100× and detected on ABI 3130XL capillary sequencer (Applied Biosystems) using Gene scan 120 LIZ® size standards. Genomic DNA from *P. falciparum* reference clones 3D7/unknown origin (wild-type) and W2/Indochina (mutant) was used as positive controls, and water and human DNA were used as negative controls.

Pfmdr1 (PF3D7_0523000) genotyping was performed by Sanger's method of DNA sequencing. Four pairs of primers were used to amplify two *Pfmdr1* fragments carrying five polymorphisms associated with drug *resistance* phenotype. N86Y and Y184F were on the first fragment MDR1-1 (590 base pairs, bp) and S1034C, N1042D and D1246Y on fragment MDR1-2 (968 bp). The reaction mixture, PCR conditions, amplicons purification and sequencing were described in previous studies of Anderson et al. [11] and Baret, et al. 2012.

Pfmdr1 copy number was determined using TaqMan real-time PCR (7900HT Fast Real-Time PCR system, Applied Biosystems, Courtaboeuf, France) using the single-copy gene β-tubulin (PF10_0084) as the reference.

Each sample was selected on the basis of a sufficient amount of DNA in duplicate. The sequence of oligonucleotide primers and probes to be used, the preparation of reaction mixture, PCR conditions, and evaluation of PCR efficiency were described in detail in the study of (Anderson et al. 2002). DNA extracted from the *P. falciparum* 3D7 reference clone, which has a single copy of each gene, was used as a calibrator, and β-tubulin housekeeping gene was used as a control in all experiments. The number of gene copy was determined using the method outlined by Price et al. [12].

The resulting PCR products were subjected to gel electrophoresis on agarose gels and stained with SYBR green. All amplicons with the correct PCR band were purified using PeqGOLD Cycle pure kit (Classic-Line) (peQLab 682 Drug-*resistance* falciparum malaria (Biotechnologie GmbH, Erlangen, Germany) according to manufacturer's instructions. The purified amplicons was re-amplified, purified with Sephadex TM G-50 and the DNA sequence was determined using an ABI PRISIM 3100 Genetic Analyser (Applied Biosystem). Sequence results were analyzed using the freely available BioEdit Sequenc Alignment Editor software. Single nucleotide polymorphisms (SNPs) were identified by assembling the sequences with each reference sequence using Codon code Aligner 4.0 software and were reconfirmed visually from their respective electropherograms.

2.9 Data Collection

Data collection was done with the aid of a structured questionnaire to extract demographic as well as health information from the subjects. After obtaining informed consents, the respondents were guided on how to fill the questionnaires appropriately.

Inclusion/ Exclusion criteria: Student of one of the four selected schools of study who fell within the age range of 11-22, consistent in school and

willing to participate in the research were enrolled in the study. Similarly, students were biased by ethnic or religious organization to participate in a research, and inconsistent in school were all excluded.

2.10 Data Analysis

The demographic data and other statistical analyses were carried out using SPSS version 16 (SPSS Inc., USA). The distribution of the *resistance* genes between the mutant and wild type with be analyzed by percentage frequency distribution while the significant difference between the parasitaemia density and age of the subjects with be analysed with linear regression with p-value =0.05.

3. RESULTS

3.1 Rapid Diagnostic Test for Malaria

Out of the 187 subjects drawn from the four communities of the two local government areas of interest, 50 (26.7%) were tested positive for malaria parasite with RDT Method, 25 (27.5%) were positive among the age group of 11 – 13 years, 17.6%, 4.4% and 1.1% were invalid, inconclusive and of No result respectively (Table 1). Similarly, among the age group 14 - 16 years old, a total of 44 subjects were diagnosed. Of this number, 36.4% tested positive for malaria parasite, 6.8% of the results were invalid while 4.5% and 2.3% were inconclusive and No result respectively. For age group 17-19, out of 39 subjects, 17.9% tested positive to the parasite, 7.7% and 10.3% were invalid and inconclusive respectively. For the 20-22 age category, from a total of 13 subjects, 15.4% of the subjects tested positive to the parasites (Table 1).

The result of the malaria RDT obviously shows that the percentage of those tested positive to malaria parasite declines as age increases while there is increase in the percentage of subjects tested positive to malaria parasite as age decrease (Fig. 1).

3.2 Malaria Microscopy

The 50 RDT positive for malaria parasite were compared with microscopic method. Simple linear regression with parasite density as dependent variable and age of the subjects as independent variable was obtained (Fig. 2). The p-value of the coefficient of age is 0.337, which is greater than significant level, 0.05. There was no statistically significant between age of the subjects and parasite density. Indirect relationship there is a weak indirect relationship between the malaria parasite density and age of the studied population as demonstrated by scatter plot with slight downward line of negative correlation ($r = -0.1385$) (Fig. 2).

3.3 Evaluation of *Pfcrt* and *Pfmdr1* Genes

A total of 50 blood samples from the four studied communities indicated *P. falciparum*-infection. These samples were examined for the present of *Pfcrt* gene mutation at codon 76 as well as *Pfmdr1* gene mutation at codon 86 respectively.

Table 1. RDT results for malaria parasite within age categories

Age	Malaria parasite: Count (%)			No result	Total
	+VE	Invalid	Inconclusive		
11-13years	25 (27.5%)	16 (17.6%)	4 (4.4%)	1 (1.1%)	91
14 - 16years	16 (36.4%)	3 (6.8%)	2 (4.5%)	1 (2.3%)	44
17 -19years	7 (17.9%)	3 (7.7%)	4 (10.3%)	0	39
20-22years	2 (15.4%)	0	0	0	13
Total	50	22	10	2	187

Table 2. *Pfcrt* gene analysis result

Pfcrt gene (K76T)	T76 (Mutant)	K76 (Wild)
Codon Positon	76	76
Igbonla Community	8 (16%)	3 (6%)
Iponrin Community	10 (20%)	6 (12%)
Apado Community	11 (22%)	2 (4%)
Alakuko Community	5 (10%)	5(10%)
Total	34 (68%)	16(32)

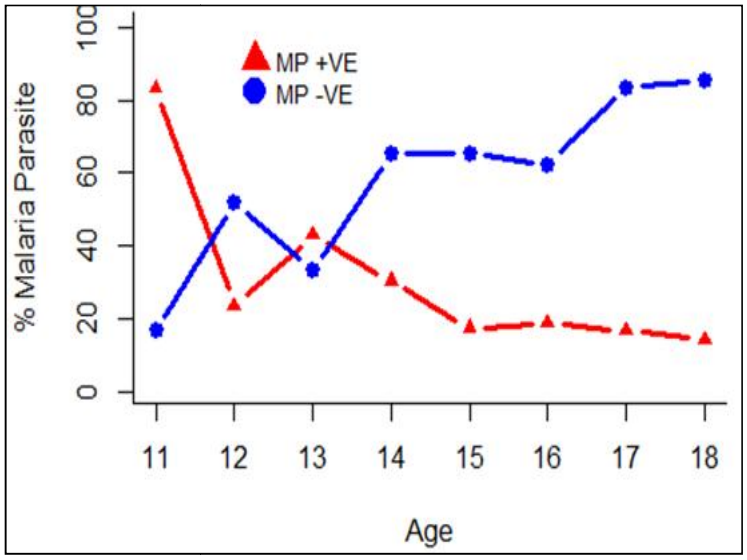

Fig. 1. Graph of Malaria Parasite Positive (MP +VE) and Malaria Parasite Negative (MP -VE) across the studied subjects

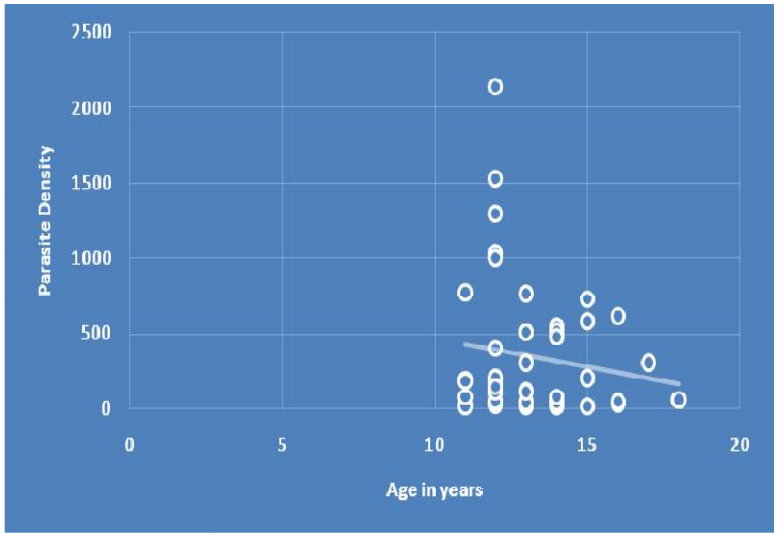

Fig. 2. Scatter plot of parasite density across age categories under study

Table 3. *Pfmdr1* gene analysis result

***Pfmdr1* gene (N86Y)**	**Y86 (Mutant)**	**N86 (Wild)**
Codon Positon	86	86
Igbonla Community	3 (6%)	5 (10%)
Iponrin Community	7 (14%)	9 (18%)
Apado Community	11 (22%)	4 (8%)
Alakuko Community	6 (12%)	5(10%)
Total	27 (54%)	23 (46%)

Among the 50 isolates analyzed for *Pfcrt* gene, wild type alleles accounted for 32% (16) while mutant alleles accounted for 68% (34) all at codon 76 (Table 2). Alakuko Community (Ilorin

East local government) accounted for the least number of T76 mutant alleles 10% (5) while Apado community (Ilorin East local government) indicated the highest prevalence of T76 mutant gene 22% (11). Of the wild type alleles, Iponrin community (Ilorin East) had the highest number, 12% (6) out of 32% (16) while Apado community (Ilorin East) had the least number of the wild type alleles 4% (2) (Table 2).

For *Pfmdr1* gene analysis at codon 86, isolates from Apado community showed the highest mutant type alleles (Y86) of 22% (11), while Igbonla community in Ifelodun local government represented the least mutant alleles, 6% (3) (Table 3). With wild type alleles N86 accounting for 46% (23), the highest of this gene was obtained from Iponrin (Ilorin East), 18% (9) while the least was gotten from isolates representing Apado community (Ilorin East), 8% (4).(Table 3).

4. DISCUSSION

Malaria is the most prevalent tropical disease in the world today and in Sub-Saharan Africa, it is ranked among the most frequent cause of morbidity and mortality, especially among children, and is often the leading identifiable cause of death [13]. Prevalence of *Plasmodium falciparum* which causes the most serious type of malaria especially in sub Saharan Africa was studied among selected communities of Kwara State, Nigeria. One hundred and eighty seven subjects were included in the study on the prevalence of *P. falciparum* infection. Fifty (26.7%) were positive to falciparum malaria. Despite the fact that the study was conducted in rainy season, the result implies a low rate of malaria infection among the studied population compared to other studies conducted in other part of the country. As reported by Nas et al. [14], overall prevalence of 84% was found among 370 subjects studied in Kano State. The High prevalence of malaria in that study was attributed to rainy season where mosquito are actively reproducing and growing and the use of symptomatic patients for the study. The result of this study more so deviate from what [13] studied, where an overall prevalence of falciparum malaria from 4066 subjects as determined by microscopic study in Ogun State to be 62.7%. The result obtained from this study however is in slight agreement with what Nonlan et al. 2014 reported from Abia (Southeast) and Plateau (North Central) states of Nigeria with a prevalence of 36.1% and 36.6% respectively. The low prevalence reported by Nonlan et al. 2014 could possibly arise from the fact that the study was conducted in the dry season where malaria transmission is low. However, a slight low prevalence of malaria noticed in this study can be attributed to the use of insecticide treated mosquito nets by the studied communities which are more common in the rural setting of the country. This study shows no relationship between malaria parasites' density and the age group of the studied population.

We reported Mutations in two genes, the *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*) at codon 76 and at codon 86 of the *P. falciparum* multidrug resistance gene (*Pfmdr1*). This is consistent to WHO [9] report that drug-*resistance* strains have more often evolved out of areas of low malaria transmission then spread across to endemic areas like Africa, where they have contributed to worsening mortality.

The studied isolates revealed the existence of mutant alleles for both *Pfcrt* gene and *Pfmdr1* gene which surpass the number of wild type alleles for the two genes. This implies that chloroquine resistance is available in the studied population since the two genes, *Pfcrt* and *Pfmdr1* are linked to chloroquine resistance. This is in line with what [15] studied, where a prevalence of *Pfcrt* T76 and *Pfmdr1* Y86 in the two locations (Osogbo and Lafia) was 86%, 93% and 35%, 39% respectively from a total isolates of 156. The prevalence of mutant alleles observed from the study, according to Olusola et al. [15], was comparably high in both locations. Furthermore, a study by Michael et al. 2011 revealed similar trend as the *Pfcrt* T76 allele was found present in 95 out of the 98 samples studied in Sagamu, South-Western Nigeria. The existence of high number of mutant alleles in both *Pfcrt* and *Pfmdr1* genes among isolates of Apado community in Ilorin East local government may be attributed to the use of chloroquine medication for malaria treatment. This is in line with what [16] obtained from their studies in Edo State. With 24% and 18.9% for *Pfcrt* and *Pfmdr1*. Okungbowa and Mordi [16] concluded that "the spread of these mutant genes which is associated with the presence of the causative agent (*P. falciparum*) across the country, may have been facilitated by the migration of people carrying the parasite having the *resistance* genes from one place to another for recreational activities, employment or education pursuits and due to disasters". Further stating, [17] noted that *Pfcrt* (95%) mutant gene was higher prevalence

than Pfmdr 1 (45%) mutant gene from isolates studied in Akure. Simon-Oke et al. [17] concluded that "the prevalence of the mutant genes in the study area could be as a result of high indiscriminate use of drugs (drug abuse) for treatment of malaria by people in the study area. Also, it could also be as a result of long time use of chloroquine as antimalarial drugs". The prevalence of the mutants genes over the wild type genes in general from the studied isolates of the four communities may be attributed to weak drug control policy in the country, which allows chloroquine to still be freely available for use within healthcare services even after it withdrawal in 2005 due to high level of treatment failure noticed in the country. According to Sa et al. [18] constant exposure of the parasite to drugs could lead to development of these resistance genes. It is expected that sensitivity to chloroquine will be restored about 10 years after withdrawal as observed in other countries. In this regard, Malawi studies have revealed that the prevalence of the *Pfcrt* T76 allele, which is associated with chloroquine resistance declined rapidly after the withdrawal of chloroquine from 85% in 1992 to undetectable levels by 2001. A clinical trial later confirmed that these changes in the prevalence of resistance-mediated *Pfcrt T76* allele have been accompanied by a dramatic increase in chloroquine efficacy for the treatment of malaria in Malawi Michael et al. 2011. But the opposite is observed in Nigeria.

5. CONCLUSION

Using Nested-PCR and RFLP, this study clearly shows a widespread distribution of *P. falciparum* drug-*resistance* alleles in the studied communities of Kwara State. Regional drug pressure, as well as the use of chloroquine instead of the recommended ACTs probably explains partly the high prevalence of the mutant alleles observed in this study. The future, especially in Africa, will be defined by how well the central tenets of malaria control can be united with the central tenets of control of drug resistance. To prevent resistance to anti-malarial drugs, a high degree of vigilance is required to contain the predisposing factors that facilitate resistance especially in the endemic regions like Nigeria. Also, the level of anti-malarial drug sensitivity of *P. falciparum* should be closely monitored while compliance to anti-malarial drug use should be encouraged. In this regard, the use of *Pfcrt K76T* and *Pfmdr1 N86Y* as molecular marker surveillance tools will help to predict when chloroquine can be reintroduced into the Nigeria healthcare system without detriment of any form.

6. RECOMMENDATIONS

Further studies of broader scope is highly recommended for any individual, research institutes, professional bodies, government institutions, educational institutions, students, or any interested researcher as this will go a long way providing sufficient literature for the management of malaria.

CONSENT

After obtaining informed and written consents, the respondents were guided on how to fill the questionnaires appropriately.

ETHICAL APPROVAL

Permission for this study was obtained from the Ethical Review Committee, Kwara State Ministry of Health, Nigeria. Introductory letters were issued to the community heads and school principals of interest for permission to carry out the research in their communities and schools.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDICES

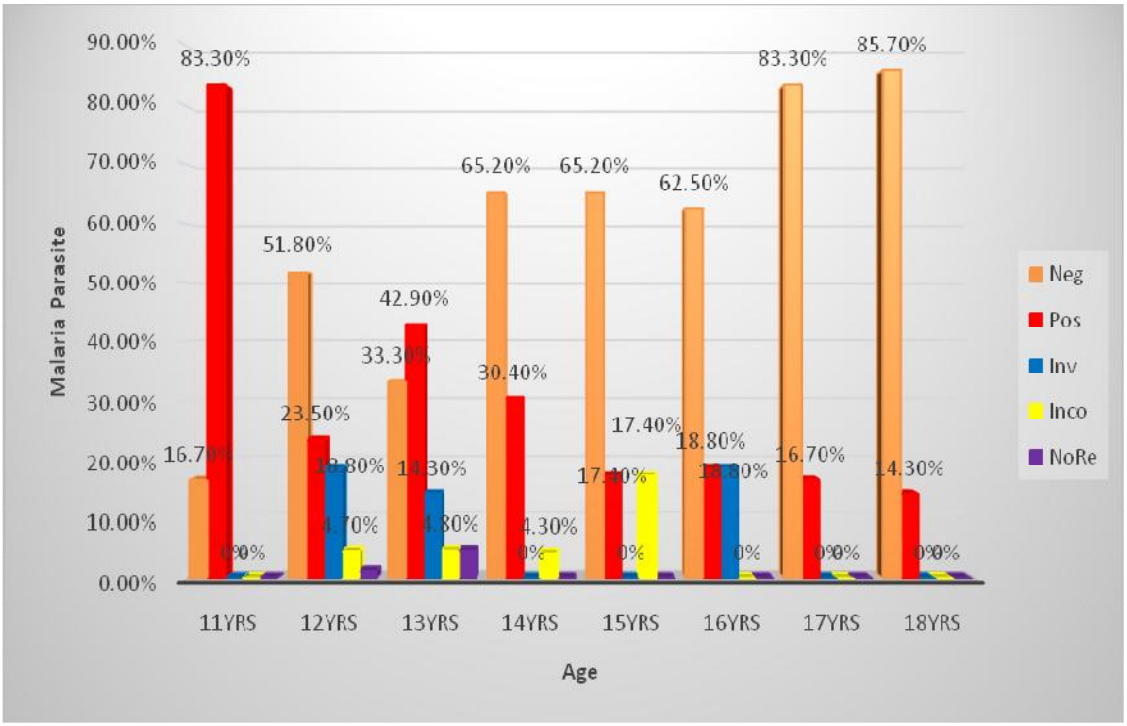

Fig. A1. Malaria parasite across studied age categories

In the chart above, the red bars represent percentage tested positive to *Plasmodium falciparum* parasite, the green bars represent percentage tested negative, the blue bars represent percentage of invalid results, the yellow bars represent percentage of inconclusive results while the purple bars represent percentage of "No result".

Table A2. The output of logistic regression

	Estimate	S.E	Z value	P-value
Intercept	2.2683	1.3767	1.648	0.0994
Age	-0.2233	0.1031	-2.166	0.0303*

Model Accuracy = 67.3%

$$logit\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 age$$

$$logit\left(\frac{p}{1-p}\right) = 2.2683 - 0.2233age$$

The odds ratio of having Malaria parasite changes by a factor of $\exp(\beta_1) = 0.7999$ for each additional year of age. Note that the association is statistically significant (p-value < 0.05) and the estimate of the coefficient of age is negative, therefore, additional age is associated with lower frequency of Malaria parasite in this area.

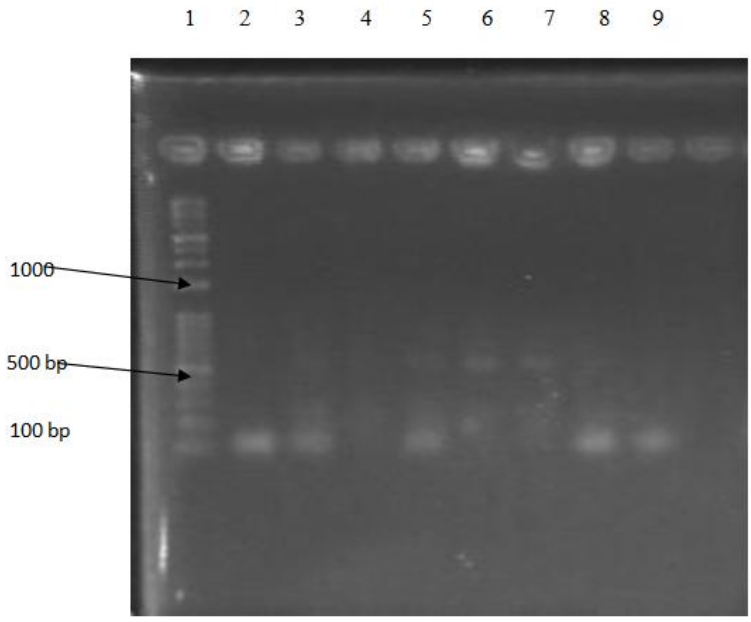

Fig. A3. 2% Agarose gel electrophoresis analysis of nested PCR from the primary amplification of the 535 bp of *Plasmodium falciparum chloroquine resistance transporter (Pfcrt)*

Lanes 2-5 and 8 shows the expected amplicon band size of 220 bp for the K76T mutant Pfcrt, while lanes 7 and 9 were not amplified. Lane 1 is the DNA ladder (1kb +)

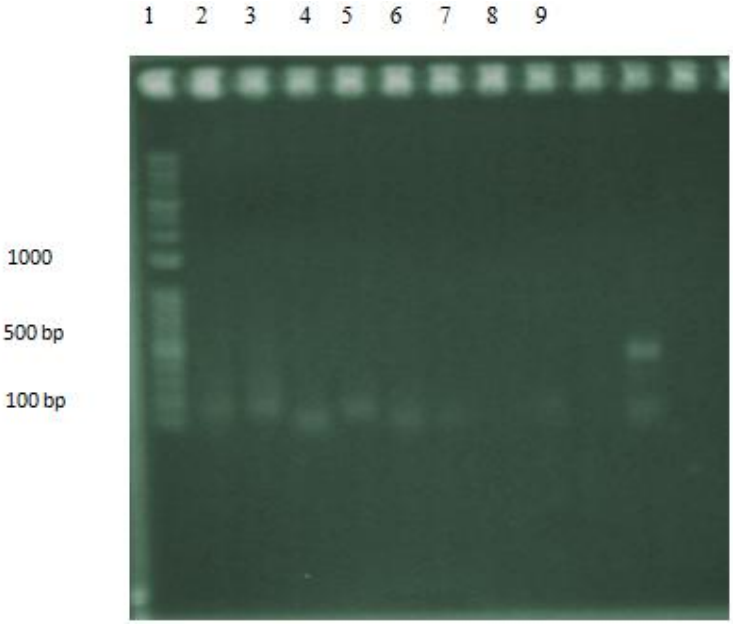

Fig. A4. 2% Agarose gel electrophoresis analysis of nested PCR from the primary amplification of the 535 bp of *Plasmodium falciparum chloroquine resistance transporter (Pfcrt)*

Lanes 2-6 shows the expected amplicon band size of 220 bp for the K76T wild Pfcrt, while lanes 7-9 were not amplified. Lane 1 is the DNA ladder (1kb +)

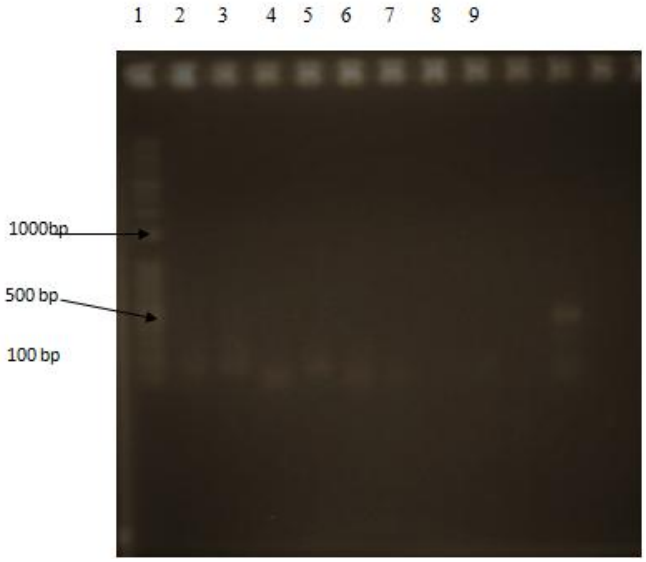

Fig. A5. 2% Agarose gel electrophoresis analysis of nested PCR from the primary amplification of the 535 bp of *Plasmodium falciparum multidrug drug resistance 1 (Pfmdr1)*

Lanes 2-6 shows the expected amplicon band size of 220 bp for the N86Y mutant Pfmdr1, while lanes 7 -9 were not amplified. Lane 1 is the DNA ladder (1kb +)

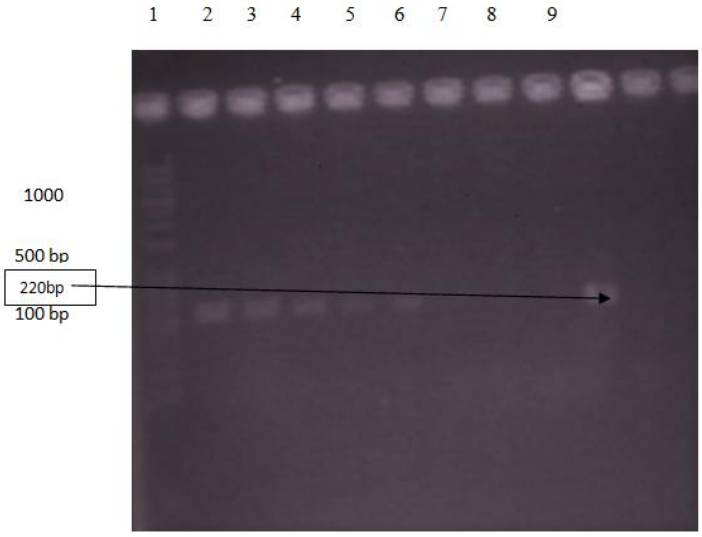

Fig. A6. 2% Agarose gel electrophoresis analysis of nested PCR from the primary amplification of the 535 bp of *Plasmodium falciparum multidrug drug resistance 1 (Pfmdr1)*

Lanes 2-6 shows the expected amplicon band size of 220 bp for the N86Y wild Pfmdr1, while lanes 7 and 9 were not amplified. Lane 1 is the DNA ladder (1kb +)

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