Occurrence of *Pfcrt* SNP at Codon 72&76 and *Pfmdr1* SNPs at Codons 86&184 and 1034,1042&1246 in Out-Patients from a Malaria Endemic Region in Southwest, Nigeria.

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Article

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Abstract

The research identified, ipso facto, Pfcrt gene SNP at codon 72&76 and Pfmdr1 gene SNPs at codons 86 &184 and 1034,1042 &1246 from malaria patients in Ifedore LGA in Ondo State, Nigeria. Thick blood film microscopy was used to examine blood samples from 2,063 febrile malaria patients within the study area. Four hundred positive samples were used to make Dry Blood Spots (DBS) on Whatmann No.3 paper. The parasite DNA was extracted from the DBS samples using spin column-based DNA purification kit. Identification and genotyping of the Pfcrt and Pfmdr1 mutant genes of the parasite were done using Nested PCR method. Molecular analysis of the 400 positive samples yielded 352 positive results after testing with various plasmodia markers with only Plasmodium falciparum detected in the study area. Results obtained from genotyping the mutant genes showed that of all the 352 P. falciparum isolates examined, Pfmdr1 SNP at codons 86&184 recorded the highest prevalence (11.08%), followed by Pfmdr1 SNP at codon 1034,1042&1246 (7.95%) while Pfcrt SNP at codon 72&76 recorded the lowest prevalence (3.41%). A prevalence of 19.32% coexistence of the three SNPs of the mutant genes was observed among the study population ($p<0.05$). The existence of the Pfcrt and Pfmdr1 mutant genes suggests resistance of P. falciparum to most malaria drugs used in the treatment of malaria in the study area. Therefore, it is important to monitor resistance to treatment regimes and therapeutics to aid management of malaria in endemic areas.

Introduction

Malaria remains a public health threat in developing countries of the tropical and subtropical regions of the world[1]. The disease is caused by the protozoan parasite of the genus *Plasmodium*[2] and vectored by female Anopheles mosquitoes in endemic regions[3]. Five different species of *Plasmodium* are capable of infecting human; *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium falciparum* and *Plasmodium knowlesi*[2]. Of all the *Plasmodium* species, *P. falciparum* has been incriminated to cause the most severe cases of malaria especially in Africa region[4,5].

In 2019, an estimated number of 229 million malaria cases were reported by the World Health Organization to have occurred in 87 endemic nations with about 215 million cases recorded in the WHO African region accounting for 94% of the cases. Globally, it was reported that Nigeria had the highest burden (27%) of malaria cases[6]. Till date, malaria prevention and control are basically dependent on vector control and use of antimalarial drugs[7] which have been proven to be effective over the years; however these do not prevent recrudescence. Currently, of all the several antimalarial regimes, Artemisinin-Based Combination Therapy (ACT) has been the major antimalarial regime used to combat malaria infection after chloroquine and sulfadoxine/pyrimethamine were proven to be less effective due to emergence of drug resistance[8]. About 183 million ACTs were distributed to the public health sector in 2019 out of which 90% went to the sub-Sahara Africa regions[6]. In Nigeria, Artemether–lumefantrine and Artesunate-amodiaquine are the major ACTs that are recommended for malaria treatment and have been proven to reduce malaria prevalence in the country[9]. However, emergence of resistance by *Plasmodium* species to ACTs and other antimalarial regimes has been a major public health problem mitigating against efforts towards malaria control. Emerging resistance to antimalarial regimes can be assessed through Single Nucleotide Polymorphisms (SNPs) Studies. In recent studies, resistance to some malaria drugs has been linked to genetic polymorphisms in *P. falciparum* (SNPs)[10]; in *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt* (codon 72&76)) and *Plasmodium falciparum* multidrug resistance (*Pfmdr1*) mutant genes, which encode putative transport proteins, affect responses to multiple drug[11].

Several studies have reported that polymorphism in the *Pfcrt* gene with substitutions at codon 72 to 76, 97, 220, 271, 326, 356 and 371 has a relationship with in vitro and in vivo resistance to amodiaquine and chloroquine[12,13]. Polymorphism of *Pfcrt* gene with substitution at codon 76 (K76T) have been reported to be the most predictive point mutation for chloroquine resistance[12]. As regards the *Pfmdr1* mutant gene, the N86Y, Y184F, S1034C, N1042D and D1246Y mutations are the *Pfmdr1* amino acid mutations associated with the multidrug resistant phenotypes[14]. *Pfmdr1* mutant gene has been reported in several studies to increase resistance to multiple drugs such as chloroquine, amodiaquine, mefloquine and halofantrine[11,15,1,14]. Also, *Pfmdr1* gene has been reported to enable malaria parasite to resist ACTs[16].

In Nigeria, there are reports about the occurrence and spread of *Pfcrt* and *Pfmdr1* mutant genes in the country[17-21]. Despite available reports on these mutant genes globally, there is a need for continuous monitoring to know the global spread especially in some parts of Nigeria including Ondo State where there is a dearth of information about malaria drug resistance and distribution of genes for chloroquine and multiple drug resistance. Therefore, this research aimed at determining the occurrence of *Pfcrt* (codon 72&76) gene SNP at codon 72&76 and *Pfmrd1* gene SNPs at codons 86&184 and 1034,1042&1246among malaria patients in Ifedore Local Government Area (LGA) of Ondo State, Nigeria.

Materials And Methods

Study Area

The study was carried out in Ifedore Local Government Area (LGA) of Ondo State, Southwestern region of Nigeria. Ifedore LGA ($7^0$27□20□□N; $5^0$10□10□□E) occupies an area of 295 km□ with a population of 176,327[22]. The area enjoyed two climatic seasons; the rain (wet) season, from March to October and the dry season, from November to February with an average annual rainfall of 1500mm. Ifedore had ambient temperature ranging from 21.4^{0C} to 31.1^{0C} and relative humidity of 80%[23]. The weather condition of the study area favoured agricultural activities thus most dwellers engaged in farming while others engaged in trading and commerce, and civil service, The study was conducted in some selected Hospitals and Health Centres at the following towns within the study area; Owena, Ibuji, Igbara-Oke, Isarun, Eroo, Ilara, Ipogun, Ibule, Aaye, Ijare and Irese (Fig. 1).

Study Design

A cross-sectional survey was conducted between January, 2019 to May, 2020 among febrile malaria patients attending some selected Health Centres and Hospitals in 11 major communities within Ifedore LGA of Ondo State, Nigeria. Prior to the commencement of the study, an advocacy visit was carried out to all selected Health Centres and Hospitals to inform the Public Health coordinators and Chief Medical Directors about the aims and objectives to the study. Quantitative data were collected from patients of all ages visiting the Health Centres and Hospitals using a well-structured closed-ended questionnaire. Blood samples were collected from each patient for malaria test using thick blood film microscopy method and samples positive for malaria parasite were used to make Dry Blood Spots (DBS) for molecular analysis of the samples. DNA was extracted from the DBS samples and *Plasmodium* species identification and genotyping of the resistant genes were done using Polymerase Chain Reaction (PCR).

Ethical Consideration

Prior to the commencement of the research, approval was sought from Ministry of Health of Ondo State (ethical clearance was issued with protocol number: OSHREC/11/12/2018/079), Ondo State Primary Health Care Development Board (OSPHCDB), Health Care Coordinator of Basic Health Centres in Ifedore LGA, Officers in Charge (OICs) and the Chief Medical Director (CMD) of various Health Centres and Hospitals where samples were being collected. Also, written informed consent was obtained from the volunteers whose samples were used for the study while volunteers less than 6 years of age were consented through their parents/guardians.

Sample Size Determination

The sample size was determined using classical statistical method with the use of Raosoft sample size calculator[24] at 5% margin of error and 95% confidence level[25]. The sample size obtained for each of the studied town varied with the varying population size of each town. Despite the 384-sample size that was generated, a total of 2,063 blood samples were analyzed for malaria parasite. A total number of 400 samples positive for malaria parasite were collected randomly across the LGA.

Sample Collection

Blood samples were collected through the help of qualified Phlebotomists from febrile malaria Patients of all ages visiting the selected Hospitals and Health Centres in each of the towns for malaria test. Samples from each patient was collected though venipuncture with the use of sterile needle and syringe and transferred directly into an Ethylenediamine tetra-acetic acid (EDTA) bottle to prevent coagulation of the blood sample.

Questionnaire Survey

A well-structured closed-ended questionnaire was administered to volunteers to obtain information on their demographic, socioeconomic and environment as well as their attitude and practices towards malaria. Each volunteer’s information was

obtained through oral interview. Interpretation to local language was done through an interpreter. However, volunteers less than 6 years of age were interviewed through their parents/guardians who knew their information and status on malaria.

Malaria Parasite Screening

Think blood film was used to examine the blood samples for malaria parasite. For every sample, the film was prepared on a clean grease-free glass slide and allowed to air dry for 15 minutes, and was stained with Giemsa for 15 minutes. After15 minutes, the stain was washed off rapidly using distilled water and left to air dry. Two drops of immersion oil was added to the film and observed under the microscope at x100 objective for characteristic feature of malaria parasite[26]. It was ensured that the slides that were negative were re-examined for accuracy.

Molecular Analysis

Two to three drops of blood samples positive for malaria parasite were spotted on 3mm whatmann filter paper inscribed between labeled cardboard papers. The blood spots on the whatmann filter papers were allowed to dry properly under room temperature. The Dried Blood Spots (DBS) were placed in a desiccated ziplock pouch to prevent reaction with air. The desiccated ziplock pouches containing the DBS were placed in plastic containers which were later kept in a refrigerator at 4^0C. The samples were transported to the Molecular Entomology and Vector Control Research Laboratory of the Nigeria Institute of Medical Research (NIMR), Lagos for molecular analysis.

Deoxyribonucleic Acid (DNA) Extraction

DNA was extracted from DBS samples on the 3mm Whatman filter paper using Spin Column Based DNA Purification Kit containing all protocol, necessary materials and reagents for extraction. The extracted DNA samples were stored at -20^0C to prevent the degradation of the DNA samples till further use[27].

DNA Samples Screening for *Plasmodium falciparum* Identification

The DNA samples extracted were screened for *P. falciparum* using PCR technique[28] for confirmation of the microscopy test. A Nested Polymerase Chain Reaction (PCR) approach (involving Nest 1 and Nest 2 reactions) was used for the molecular screening of DNA samples of *P. falciparum.* The Nest 1 reaction detected *Plasmodium* spp. in the samples while the Nest 2 reaction detected the specific species of *Plasmodium* in the samples (Table 1).

The SNP of *Pfcrt* mutant gene amplified was at codon 72&76 while the two SNPs of *Pfmdr1* mutant gene amplified were *Pfmdr1* gene at codon 86&184 and codon 1034,1042&1246. A Nested PCR approach was also used for the amplification of the mutant genes (Table 2).

Agarose Gel Electrophoresis

All products of the PCR Nest 2 reaction were subjected to Agarose gel (2%) electrophoresis.

Table 1: PCR primer, sequence and conditions for *Plasmodium* Species Identification

Species	Primers	Sequence (5′–3′)	PCR Conditions	Size (Base Pair, bp)	References
Plasmodium (Nest 1)	RPLU;Forward Reverse	5′-CCTGTTGTTGCCTTAAACTTC-3′ 5′-TTAAAATTGTTGCAGTTAAAACG-3′	Initial denaturation at 94^0C for 3 minutes; 30 cycles for denaturation at 94^0C for 30 seconds, Annealing at 47.4^0C for 30 seconds, elongation at 72^0C for 45 seconds; final elongation at 72^0C for 5 minutes and post hold at 8^0C	1100	28
P. falciparum (Nest 2)	RFAL;Forward Reverse	5′-TTAAACTGGTTTGGGAAAACCAAATATATT-3′ 5′-ACACAATGAACTCAATCATGACTACCCGTC-3′	initial denaturation at 95^0C for 3minutes; 35 cycles for denaturation at 94^0C for 30 seconds, Annealing at 56.8^0C for 30 seconds, elongation at 72^0C for 45seconds; final elongation at 72^0C for 5 minutes and post hold at 8^0C	205	28

Table 2: PCR primer, SNPs codons, sequence and conditions for *Pfcrt* and *Pfmdr1* Mutant Genes amplification

Mutant Genes	SNPs codons	Primers	Sequence (5′–3′)	PCR Conditions	Size (Base Pair, bp)	References
Pfcrt (codon 72&76) (Nest 1)	72&76	Forward Reverse	5′-CCGTTAATAATAAATACACGCG-3′ 5′-CGGATGTTACAAAACTATAGTCC-3′	Initial denaturation at 95^0C for 3 minutes; 35 cycles for denaturation at 94^0C for 30 seconds, Annealing at 50.5^0C for 30 seconds, elongation at 62^0C for 1 minute, final elongation at 72^0C for 5 minutes and post hold at 4^0C	NA	29
Pfcrt (codon 72&76) (Nest 2)	72&76	Forward Reverse	5′-AGGTTCTTGTCTTGGTAAATTTGC-3′ 5′-CAAAACTATAGTTACCAATTTTG-3′	Initial denaturation at 95^0C for 3 minutes; 35 cycles for denaturation at 94^0C for 30 seconds, Annealing at 49.3^0C for 30 seconds, elongation at 62^0C for 1 minute, final elongation at 72^0C for 5 minutes and post hold at 4^0C	410bp	29
Pfmdr1 (codon 86&184) (Nest 1)	86&184	Forward Reverse	5′-AGGTTGAAAAAGAGTTGAAC-3′ 5′-ATGACACCACAAACATAAAT-3′	Initial denaturation at 95^0C for 3 minutes; 35 cycles for denaturation at 94^0C for 30 seconds, Annealing at 44.6^0C for 30 seconds, elongation at 62^0C for 1 minute, final elongation at 72^0C for 5 minutes and post hold at 4^0C	NA	29
Pfmdr1 (codon 86&184) (Nest 2)	86&184	Forward Reverse	5′-ACAAAAAGAGTACCGCTGAAT-3′ 5′-AAACGCAAGTAATACATAAAGTC-3′	Initial denaturation at 95^0C for 3 minutes; 35 cycles for denaturation at 94^0C for 30 seconds, Annealing at 48.3^0C for 30 seconds, elongation at 62^0C for 1	580bp	29

				minute, final elongation at 72^0C for 5 minutes and post hold at 4^0C		
Pfmdr1 (codon 86&184) (Nest 1)	1034,1042&1246	Forward Reverse	5-GTGTATTTGCTGTAAGAGCT-3 5-GACATATTAAATAACATGGGTTC-3	initial denaturation at 95^0C for 3 minutes; 35 cycles for denaturation at 94^0C for 30 seconds, Annealing at 47.9^0C for 30 seconds, elongation at 62^0C for 1-minute, final elongation at 72^0C for 5 minutes and post hold at 4^0C	NA	29
Pfmdr1 (codon 86&184) (Nest 2)	1034,1042&1246	Forward Reverse	5-CAGATGATGAAATGTTTAAAGATC-3 5-TAAATAACATGGGTTCTTGACT-3	initial denaturation at 95^0C for 3 minutes; 35 cycles for denaturation at 94^0C for 30 seconds, Annealing at 48.1^0C for 30 seconds, elongation at 62^0C for 1 minute, final elongation at 72^0C for 5 minutes and post hold at 4^0C	864bp	29

Data Analysis

All data collected were recorded on Microsoft excel sheet and then exported to Statistical Package for Social Sciences (SPSS) version 26 (IBM SPSS Statistics) where the data were analyzed. Test for significance was done using Pearson's Chi-Square, Phi and Cramer's V Tests at P=0.05. All charts were created using Microsoft Excel.

Coordinate Collection and Map Construction

The coordinate of each town was taken using Geographical Positioning System (GPS) device (2Kit consulting, Dusseldorf, Germany ©2006-2013 Google Inc. Version 2.0). While map was constructed using Microsoft ArcGIS software (Esri® ArcGIS Desktop 10.7.1 Version 10.7.0.10450).

Results

A total of 2,063 malaria Patients across the eleven major towns in Ifedore LGA participated in the research, out of which 1,619 (78.5%) tested positive for malaria. Table 3 presents the prevalence of malaria infection across the eleven towns

respectively. Statistical analysis revealed a significant difference in the prevalence of malaria among the different towns ($P<0.05$).

Of all the samples positive for malaria parasite during microscopic analysis, 400 samples positive for malaria parasite were collected randomly across the LGA base on the population size of each town.

Molecular Screening of Samples for Identification of *Plasmodium falciparum*

The 2% agarose gel electrophoresis analysis of *P. falciparum* (Fig. 2) showed distinctive separate bands migration of varying base pairs (bp). The first wells loaded with DNA ladder signifying the molecular weight marker showed bands migration at intervals of 100bp. The second wells loaded with the control positive samples showed band migration at specific base pairs of 205bp. The third wells that were left blank serving as the negative control showed no band migration. Subsequent wells loaded with the PCR Nest 2 reaction products showing bands migration at the same level with the positive control bands at 205bp were positive for *P. falciparum* while those that showed no migrating bands were negative for *P. falciparum.* It was observed that of all the 400 samples screened for *P. falciparum*, only 352 samples were positive for *P. falciparum*.

Molecular Screening of Samples for Mutant Genes

The 2% agarose gel electrophoresis analysis of *Pfcrt* gene at SNPs codon 72&76 (Fig. 3) showed distinctive separate bands of varying base pairs with the first well loaded with DNA ladder showed migrating bands at intervals of 100bp while the second wells that were left blank serving as the negative control showed no band migration. In other wells loaded with PCR nest 2 reaction products, some of the wells showed band migrations at 410bp indicating they were positive for *Pfcrt* gene at SNPs codon 72&76. However, some of the wells showed no band migration indicating they were negative for *Pfcrt* gene at SNPs codon 72&76.

Gel electrophoresis result of *Pfmdr1* gene SNPs at codon 86&184 (Fig. 4) revealed distinctive separate bands of varying base pairs with the first well loaded with DNA ladder (molecular weight marker) showing migrating bands at intervals of 100bp while the second wells that were left blank (negative control) showed no band migration. For the wells loaded with PCR nest 2 reaction products, some showed band migrations at 580bp indicating they were positive for *Pfmdr1* gene SNPs at codon 86&184. However, some of the wells showed no band migration indicating that they were negative for *Pfmdr1* gene SNPs at codon 86&184.

Result of the gel electrophoresis analysis of *Pfmdr1* gene SNPs at codon 1034,1042&1246 (Fig. 5) showed distinctive separate bands of varying base pairs with the first well loaded with DNA ladder (molecular weight marker) with migrating bands at intervals of 100bp while second wells that were left blank serving as the negative control showed no band migration. In subsequent wells loaded with PCR nest 2 reaction products, some of the wells showed band migrations at 864bp indicating they were positive for *Pfmdr1* gene SNPs at codon 1034,1042&1246. However, some of the wells showed no band migration indicating that they were negative for *Pfmdr1* gene SNPs at codon 1034,1042&1246.

Table 3: Prevalence of malaria infection in Towns of Ifedore LGA

Towns	Number Examined	Positive	Negative	Prevalence (%)
Aaye	81	77	4	95.1
Ero	101	53	48	52.5
Ibuji	120	107	13	89.2
Ibule	209	153	56	73.2
Igbara-Oke	403	271	132	67.2
Ijare	281	249	32	88.6
Ilara	224	131	93	58.5
Ipogun	196	168	28	85.7
Isharun	79	74	5	93.7
Owena	208	187	21	89.9
Irese	161	149	12	92.5
Total	2063	1619	444	78.5

χ^2 =217.211, df = 10, P = 0.001, Phi= 0.001, Cramer's V= 0.001

Prevalence of Mutant Genes among Patients in the Study Area.

Of the 352 *P. falciparum* positive isolates examined, *Pfmdr1* gene SNPs at codon 86&184 recorded the highest prevalence (11.08%), followed by *Pfmdr1* SNP at codons 1034,1042&1246 (7.95%) while *Pfcrt* SNP at codon 72&76 recorded the lowest prevalence (3.41%) (Fig. 6). However, there were records of coexistence of the three SNPS of the mutant gene among the study population. Examples of the latter include occurrence *Pfcrt* SNP at codon 72&76 and *Pfmdr1* SNP at codons 1034,1042&1246; *Pfmdr1* SNPs at codon 86&184 and *Pfmdr1* SNP at codons 1034,1042&1246; and *Pfcrt* SNP at codon 72&76 and *Pfmdr1* gene SNPs at codon 86&184 recorded the prevalence of 19.32%, 19.03%, 17.90% and 8.24% respectively (Fig. 7). Statistical analysis showed a significant difference in the prevalence of the mutant genes among the Study population ($p<0.05$).

Prevalence of the mutant genes in the studied towns within the LGA is presented in Table 4. Result showed that the mutant genes were distributed across the eleven (11) studied towns at varying prevalence. For the *Pfcrt* gene, Aaye town presented the highest prevalence of 69.7% while the least prevalence (29.0%) was recorded in Ero town. The highest prevalence (84.2%) and lowest prevalence (38.7%) where recorded for *Pfmdr1* gene at codon 86&184 at Ilara and Ero town respectively while Ilara (97.4%) and Igbara-Oke (33.3%) towns presented with the highest and lowest prevalence of *Pfmdr1* gene at codon 1034,1042&1246 respectively. Statistical analysis revealed a significant difference in the prevalence of the mutant genes across the towns ($p<0.05$).

Out of the 223 female and 129 male patients that tested positive for *P. falciparum*, 108 (48.4%) female patients and 68 (52.7%) male patients were positive for *Pfcrt* mutant gene, 120 (53.8%) female patients and 79 (61.2%) male patients were positive for *Pfmdr1* gene at codon 86&184 while 136 (61.0%) female patients and 90 (69.8) male patients were positive for *Pfmdr1* gene at codon 1034,1042&1246. It was further observed that the male patients presented higher prevalence in all the three (3) SNPs of the mutant genes compared with their female counterparts. Statistical analysis showed no significant difference in the prevalence of the mutant genes in respect to the sexes of the patients ($p> 0.05$) (Fig. 8).

The prevalence of the mutant genes in relation to the marital status of the patients is presented in Fig. 9. As regards the *Pfcrt* gene, result showed that unmarried patients (51.7%) had a higher prevalence compared with those that were married. For the *Pfmdr1* gene at codon 86&184 the unmarried and the married patients presented prevalence of 57.8% and 54.6% respectively while for the *Pfmdr1* gene at codon 1034,1042&1246, unmarried and the married patients presented prevalence of 70.1% and 55.3% respectively.

Prevalence of mutant genes in relation to age (Table 5) revealed that age groups 61- above (57.9%) and 51-60 (38.1%) presented with the highest and the least prevalence for *Pfcrt* mutant gene. For *Pfmdr1* gene at codon 86&184, patients within the age of 10 years and below (64.2%) and 41-50 (46.4%) presented the highest and the least prevalence respectively while for the *Pfmdr1* gene at codon 1034,1042&1246, the highest prevalence of 70.8% was recorded among patients of age 10 years and below with the least prevalence of 36.8% recorded among patients within the age of 61 years and above. Statistical analysis revealed no significant difference in the prevalence of the mutant genes in relation to the different age groups ($p>0.05$).

Table 4: Prevalence of Mutant genes in the studied towns within the LGA

Towns	Number Examined	*Pfcrt* (codon 72&76) Positive (%)	*Pfmdr1* (codon 86&184) Positive (%)	*Pfmdr1*(codon 1034,1042&1246) Positive (%)
Aaye	17	10 (58.8)	12 (70.6)	15 (88.2)
Ero	31	9 (29.0)	12 (38.7)	15 (48.4)
Ibuji	27	17(63.0)	16 (59.3)	26 (96.3)
Ibule	33	23 (69.7)	27 (81.8)	22 (66.7)
Igbara-Oke	57	26 (45.6)	24 (42.1)	19 (33.3)
Ijare	41	12 (29.3)	20 (48.8)	19 (46.3)
Ilara	38	26 (68.4)	32 (84.2)	37 (97.4)
Ipogun	27	14 (51.9)	12 (44.4)	18 (66.7)
Isharun	28	12 (42.9)	12 (42.9)	14 (50.0)
Owena	29	15 (51.7)	13 (44.8)	19 (65.5)
Irese	24	12 (50.0)	19 (79.2)	22 (91.7)
Total	352	176 (50.0)	199 (56.5)	226 (64.2)

Pfcrt (codon 72&76): χ^2 =26.205, df = 10, P = 0.003, Phi = 0.003, Cramer's V= 0.003; *Pfmdr1* (codon 86&184): χ^2 = 42.073, df = 10, P = 0.001, Phi= 0.001, Cramer's V= 0.001; *Pfmdr1* (codon 1034,1042&1246): χ^2 = 77.774, df = 10, P = 0.001, Phi= 0.001, Cramer's V= 0.001

Table 5: Prevalence of Mutant Genes in relation to age groups of patients in the study area

Age Groups	Number Examined	*Pfcrt* (codon 72&76) Positive (%)	*Pfmdr1* (codon 86&184) Positive (%)	*Pfmdr1* (codon 1034,1042&1246) Positive (%)
≤ 10	120	65 (54.2)	77 (64.2)	85 (70.8)
11-20	54	23 (42.6)	27 (50.0)	37 (68.5)
21-30	72	37 (51.4)	38 (52.8)	47 (65.3)
31-40	38	19 (50.0)	20 (52.6)	23 (60.5)
41-50	28	13 (46.4)	13 (46.4)	15 (53.6)
51-60	21	8 (38.1)	13 (61.9)	12 (57.1)
61-Above	19	11 (57.9)	11 (57.9)	7 (36.8)
Total	352	176 (50.0)	199 (56.5)	226 (64.2)

Pfcrt (codon 72&76): χ^2 =3.881, df = 6, P = 0.693, Phi = 0.693, Cramer's V= 0.693; *Pfmdr1* (codon 86&184): χ^2 = 5.856, df = 6, P = 0.439, Phi= 0.439, Cramer's V= 0.439; *Pfmdr1* (codon 1034,1042&1246): χ^2 = 11.014, df = 6, P = 0.088, Phi= 0.088, Cramer's V= 0.088

Table 6 shows the prevalence of the mutant genes in relation to the different ethnic groups in the study area. Results obtained showed that the Hausa/Fulani tribe (northern Nigeria tribe) and those that belong to other ethnic groups excluding the Yoruba and Ibo tribes had the highest and least prevalence of 100% and 36.4% respectively for the *Pfcrt* gene. As regards the *Pfmdr1* gene at codon 86&184, the highest prevalence (72.7%) was recorded among other ethnic groups (not belonging to Yoruba, Ibo and Hausa/Fulani tribes) while the Hausa/Fulani presented no prevalence. However, 100% and 62.5% prevalence were recorded among the Hausa/Fulani and Yoruba tribes respectively for the *Pfmdr1* gene at codon 1034,1042&1246. Statistical analysis showed no significant difference in the prevalence of the mutant genes among the different ethnic groups ($p>0.05$).

The prevalence of the mutant genes in relation to occupation of patients in the study area is shown in Table 7. Result for the *Pfcrt* gene, showed that Retirees (66.7%) presented the highest prevalence while Traders (41.7%) presented with the least prevalence. However, for the *Pfmdr1* gene at codon 86&184, the highest prevalence was also recorded among the Retirees (100.0%) while the lease prevalence was recorded among Civil servants (50.0%). It was observed that Students (71.9%) and the Civil servants (54.5%) presented the highest and least prevalence respectively for *Pfmdr1* genes at codon 1034,1042&1246. Statistical analysis showed no significant difference ($p>0.05$) in the prevalence of the mutant genes in relation to the occupation of the patients in the study area.

Malaria treatment seeking habit of patients in the study area is presented in Fig. 10. For the *Pfcrt*, patients who used herbs only, those who used antimalarial drugs only and those who combined antimalarial drugs with herbs presented with prevalence of 100.0%, 89.7% and 81.1% respectively. In the same order of groups as listed above, prevalence result for *Pfmdr1* gene at codon 86&184 were 61.5%, 56.9% and 40.5% respectively, and for *Pfmdr1* gene at codon 1034,1042&1246, prevalence was 92.3%, 72.4% and 73.1% respectively. It was observed that for all the mutant genes, patients who make used of herbs only for malaria treatment presented with highest prevalence followed by the antimalarial drugs only while those that combined both presented with the least prevalence with no significant difference ($p>0.05$).

Table 6: Prevalence of Mutant Genes in Relation to Different Ethnic Groups in the Study Area

Ethnicity	Number Examined	*Pfcrt* (codon 72&76) Positive (%)	*Pfmdr1* (codon 86&184) Positive (%)	*Pfmdr1* (codon 1034,1042&1246) Positive (%)
Yoruba	299	151 (50.5)	172 (57.5)	187 (62.5)
Igbo	40	19 (47.5)	19 (47.5)	29 (72.5)
Hausa/Fulani	2	2 (100.0)	0 (0.0)	2 (100.0)
Others	11	4 (36.4)	8 (72.7)	8 (72.7)
Total	352	176 (50.0)	199 (56.5)	226 (64.2)

Pfcrt (codon 72&76): $\chi^2 = 2.948$, df = 3, P = 0.400, Phi = 0.400, Cramer's V= 0.400; *Pfmdr1* (codon 86&184): $\chi^2 = 5.223$, df = 3, P = 0.156, Phi= 0.156, Cramer's V= 0.156; *Pfmdr1* (codon 1034,1042&1246): $\chi^2 = 3.020$, df = 3, P = 0.389, Phi= 0.389, Cramer's V= 0.389

Table 7: Prevalence of Mutant Genes in Relation to Occupation of Patients in the Study Area

Occupation	Number Examined	*Pfcrt* (codon 72&76) Positive (%)	*Pfmdr1* (codon 86&184) Positive (%)	*Pfmdr1* (codon 1034,1042&1246) Positive (%)
Artisans	46	26 (56.5)	27 (58.7)	30 (65.2)
Civil servants	22	10 (45.5)	11 (50.0)	12 (54.5)
Farmers	42	19 (45.2)	23 (54.8)	26 (61.9)
Retirees	3	2 (66.7)	3 (100.0)	2 (66.7)
Students	153	81 (52.9)	88 (57.5)	110 (71.9)
Traders	48	20 (41.7)	26 (54.2)	22 (45.8)
Total	314	158 (50.3)	176 (56.7)	202 (64.3)

Pfcrt: 2 = 3.529, df = 5, P = 0.619, Phi = 0.619, Cramer's V= 0.619; *Pfmdr1* (codon 86&184): 2 = 2.999, df = 5, P = 0.700, Phi= 0.700, Cramer's V= 0.700; *Pfmdr1* (codon 1034,1042&1246): 2 = 12.022, df = 5, P = 0.034, Phi= 0.034, Cramer's V= 0.034

Discussion

Emergence of resistance by malaria pathogen to malaria drugs remains a public health problem in endemic regions of the world especially in Nigeria despite all control efforts to curtail the disease. During the study, of all the total number of 400 malaria positive samples confirmed through microscopy, only 352 samples were confirmed by PCR analysis carried out on the DNA samples extracted from the DBS of the 400 samples to be positive for *P. falciparum.* On the contrary, [21]in their study at Akure, Nigeria reported that all samples positive for malaria by microscopic analysis were also positive for *P. falciparum* when subjected to PCR analysis. However, knowing well that the primers used for *Plasmodium* species identification in this study were specific to *P. falciparum*¸ the negative samples were further probed with specific primers of other *Plasmoduim* species (*P. vivax, P. ovale, P. malariae, and P knowlesi*) but negative for other *Plasmoduim* species indicating that the negative results could have been as a result of errors in interpretation of result during microscopic analysis.

Result obtained from agarose gel electrophoresis for *P. falciparum* species identification revealed the molecular weight of *P. falciparum* to be 205bp. Similar molecular weight (205bp) was reported of *P. falciparum* by[30,28,31] in their various studies. However, [32-34]in their respective research revealed the molecular weight of *P. falciparum* as 206bp. Variations may be due to the molecular weight of *P. falciparum* in this study and the timing and electric charge of the gel electrophoresis could have influence on migration of the DNA samples[28].

Agarose gel electrophoresis analysis of *Pfcrt* (codons 72&76) and *Pfmdr1* (codons 86&184 and 1034,1042&1246) mutant gents revealed their molecular weights to be 410bp, 580bp and 864bp respectively. This result correlates with the findings of[29]. However, [33,21]in their respective studies at Indian and Akure, Nigeria reported the molecular weight of 264bp and 603bp for *Pfcrt* and *Pfmdr1* mutant genes respectively. Reason for the difference in the molecular weight obtained for the mutants genes in the different studies could be as a result of the PCR technique used. [33,21]in their studied used conventional PCR approach for genotyping of the mutant genes while nested PCR approach was employed for genotyping of the mutant genes in this study[35].

Occurrence of *Pfcrt* (codons 72&76) and *Pfmdr1* (codons 86&184 and 1034,1042&1246) mutant genes in the study area has also been reported previously in different parts of Nigeria. For instance, [36,37, 20,21] in their studies reported the occurrence of the mutant genes in North-Eastern Nigeria, Ibadan, Edo State, and Akure respectively. In the current study, the mutant genes occurred at very high prevalence in the different communities sampled. High prevalence of the two mutant genes were also

reported by[21] who recorded high prevalence of *Pfcrt* and *Pfmdr1* mutant genes (95% and 45% respectively) in Akure, Nigeria. However, a lower prevalence of *Pfcrt* and *Pfmdr1* mutant genes, 24% and 18.9% respectively in Edo State, Nigeria was reported by[20]. The results obtained from different studies carried out on the mutant genes in different parts of the country indicate that the mutant genes may be spreading in the country. Migration of people from one part of the country to another for trade, job, scholarship and other factors could facilitate the spread of the mutant genes across the country.

There was higher prevalence of the two SNPs of *Pfmdr1* gene compared with *Pfcrt* gene in the study area. Also, there was occurrence of coexistence of the SNPs of the mutant genes in the study area which was also reported in the study of[21]. The two SNPs of *Pfmdr1* gene having higher prevalence than the *Pfcrt* gene could be as a result of the influence of Ministry of Health that discourages the use of chloroquine through regular awareness. Long time abandon use of chloroquine could reduce the prevalence of the *Pfcrt* gene[38,39]. Also, higher prevalence observed for SNPs of *Pfmdr1* gene compared to SNPs of *Pfcrt* gene in the study area could result from continuous exposure of the parasite to Artemisinin-based Combination Therapy (ACTs), the current drug of choice for malaria treatment. which may have caused mutations in the amino acids associated with multidrug resistant phenotypes of the parasite[14].

The prevalence of the mutant genes in relation to sexes of the studied patients showed that the three SNPs of the mutant genes were dominant in the male. Similarly, [21]in their study at Akure, Nigeria also reported higher prevalence of the mutant genes among male patients compared with female patients. In relation to marital status of the patients, the three SNPs of the mutant genes were observed to be more prevalent among patients that are not married. The three mutant genes were distributed at high prevalence across the different age groups, and also the prevalence of the mutant genes among the different ethnic groups revealed that the three SNPs of the mutant genes were present among all the ethnic groups except the Hausas/Fulanis who do not have *Pfmdr1* (codon 86&184) gene among them. This current research presented high prevalence of the three SNPs among the different occupational groups among the patients within the study area. Among the different occupational groups, the SNPs of *Pfmdr1* genes have higher prevalence compared with the SNP of *Pfcrt* gene. Reason for higher prevalence of the SNPs of *Pfmdr1* than *Pfcrt* gene among the groups could also be attributed to drug pressure which could have led to mutations in the amino acid associated with multidrug resistant phenotypes of the parasite[14].

According to attitude of patients towards malaria treatment, the *Pfcrt* gene was present in samples of all patients who used local herbs for malaria treatment presenting 100% prevalence. Also, for the two SNPs of *Pfmdr1* gene, highest prevalence was recorded among patients who used local herbs for malaria treatment. Since quinine (active ingredient in chloroquine) is known to be present in most antimalarial herbs[40], presence of *Pfcrt* gene in all samples from patients who engaged in the use of antimalarial herbs for malaria treatment confirms mutations associated with *Pfcrt* gene in the parasite[13]. Quinine has been reported to be linked with multidrug resistance[1], and may also be complicit in the high prevalence observed among patients who used herbs for malaria treatment causing mutations associated with multiple drug resistance in the parasite.

Conclusion

It is evident that there is occurrence of the *Pfcrt* and *Pfmdr1* mutant genes which are responsible for resistance of *Plasmodium* to antimalarial regimes in the study area. This could lead to failure of some malaria drugs used in malaria therapy, therefore contributing greatly to high prevalence of malaria infection in the study population. Chloroquine was not the mainstay in malaria therapy in the study area, and this could have led to the reduced prevalence of *Pfcrt* compared with *Pfmdr1*. It is important that more research on resistant genes of malaria parasite be conducted to advise on development and adoption of new therapies to combat malaria infection aside ACTs which is widely in use in the country. This will go a long way in the management of the disease and even reducing the prevalence of *Plasmodium* mutant genes responsible for antimalarial drug resistance.

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Figures

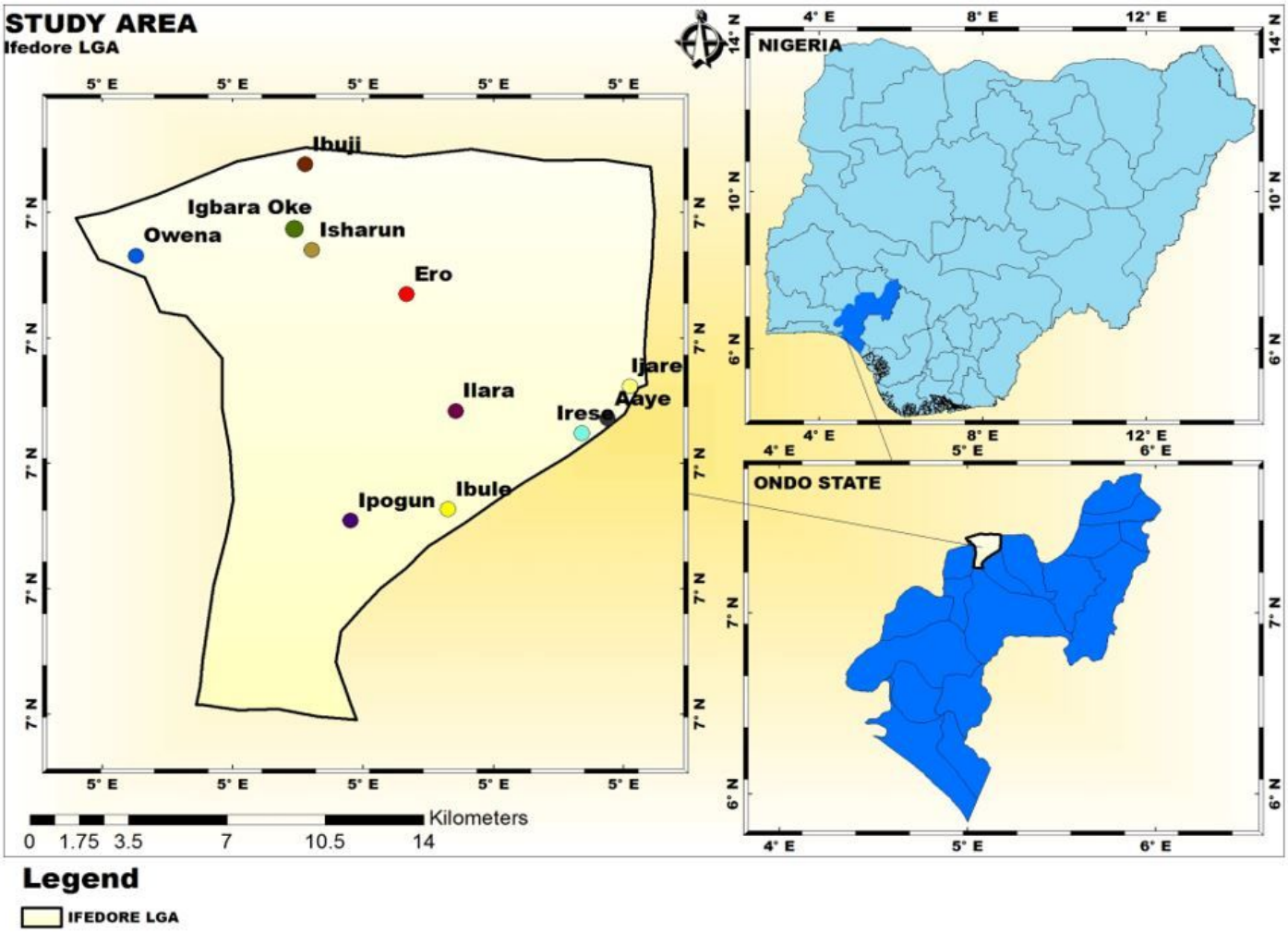


Figure 1

Map of Ifedore LGA Showing the eleven sampled Communities.

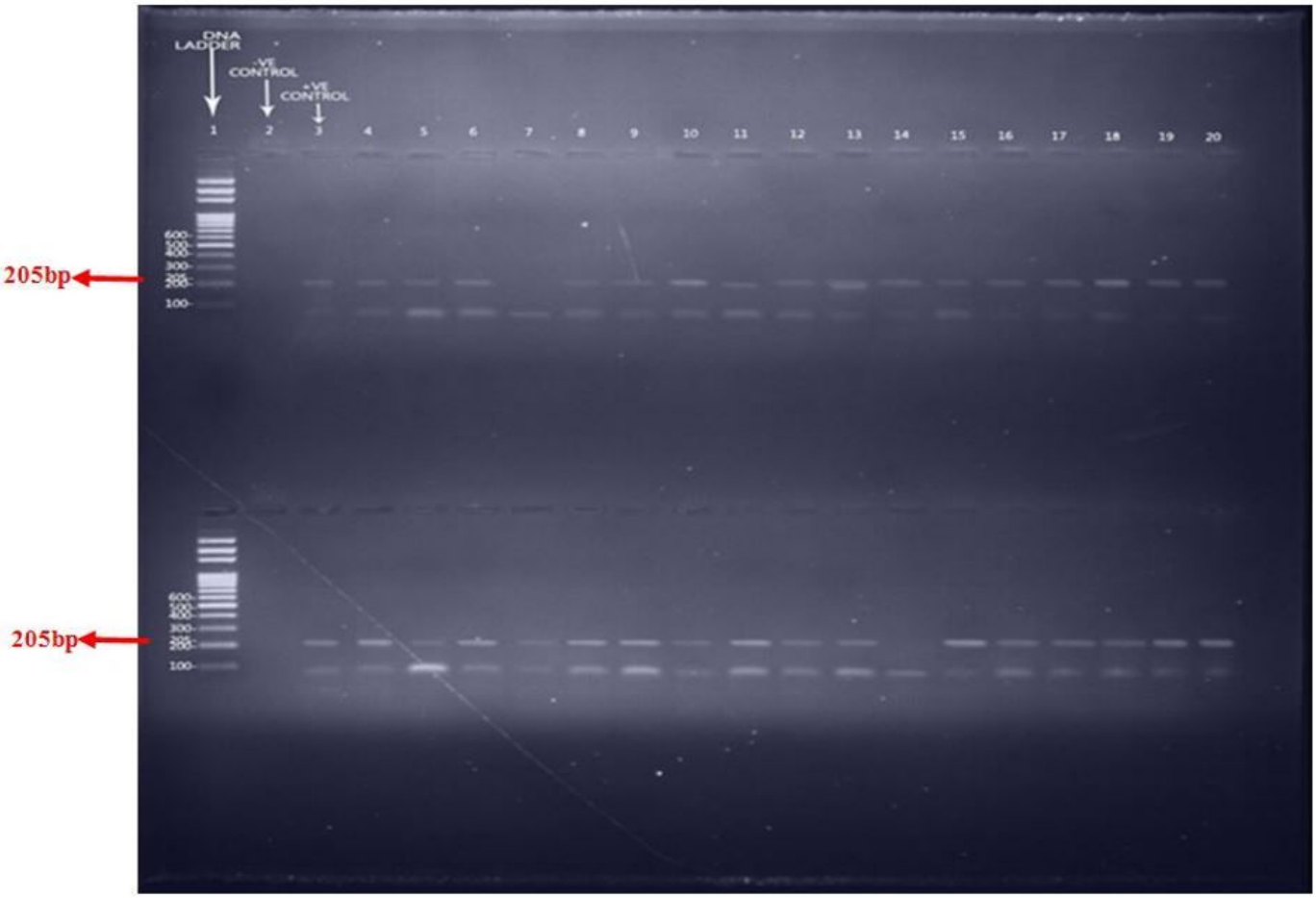


Figure 2

2% Agarose gel electrophoresis analysis of *Plasmodium falciparum* nested-PCRs from blood samples of malaria positive patients under UV Transilluminator.

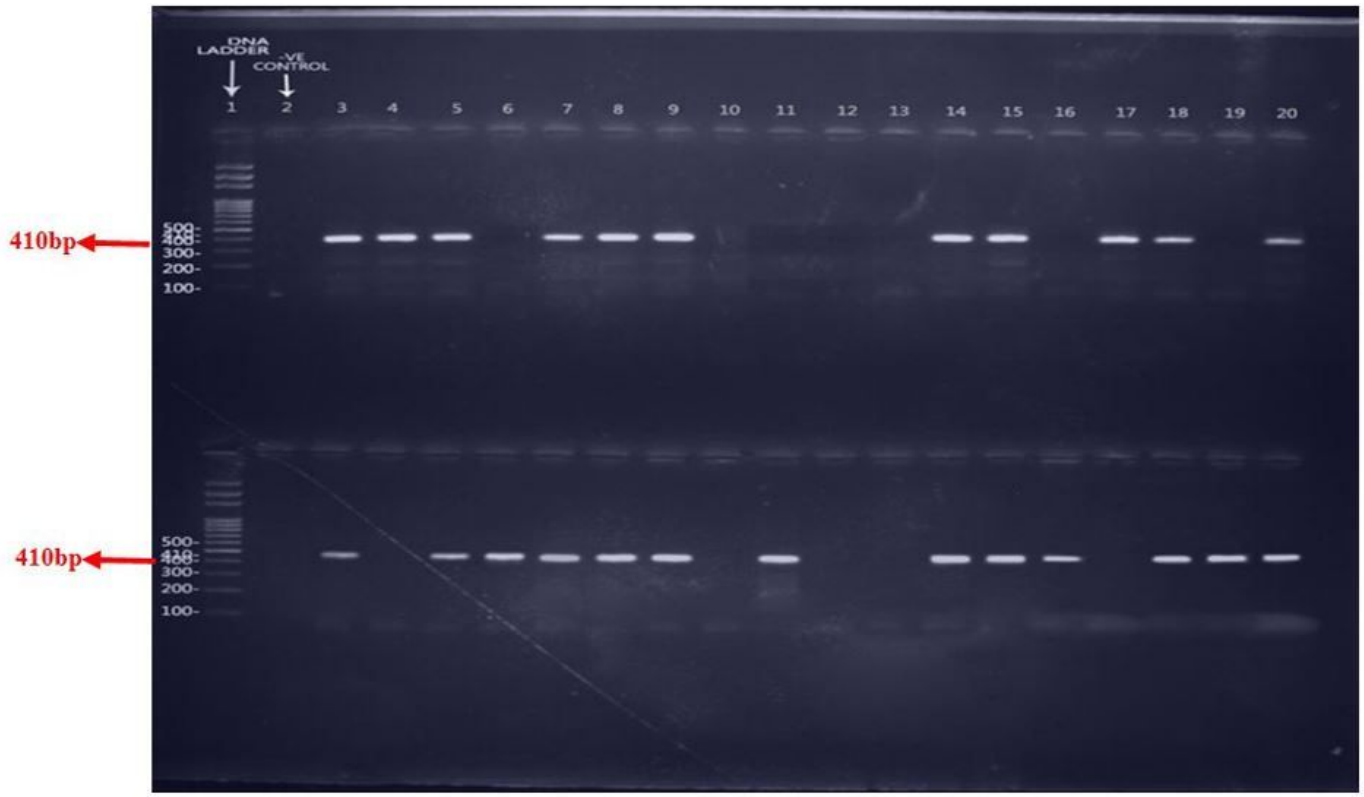


Figure 3

2% Agarose gel electrophoresis analysis of *Pfcrt* gene at SNPs codon 72&76 nested-PCRs from blood samples of malaria positive patients under UV Transilluminator.2% Agarose gel electrophoresis analysis of *Pfcrt* gene at SNPs codon 72&76 nested-PCRs from blood samples of malaria positive patients under UV Transilluminator.

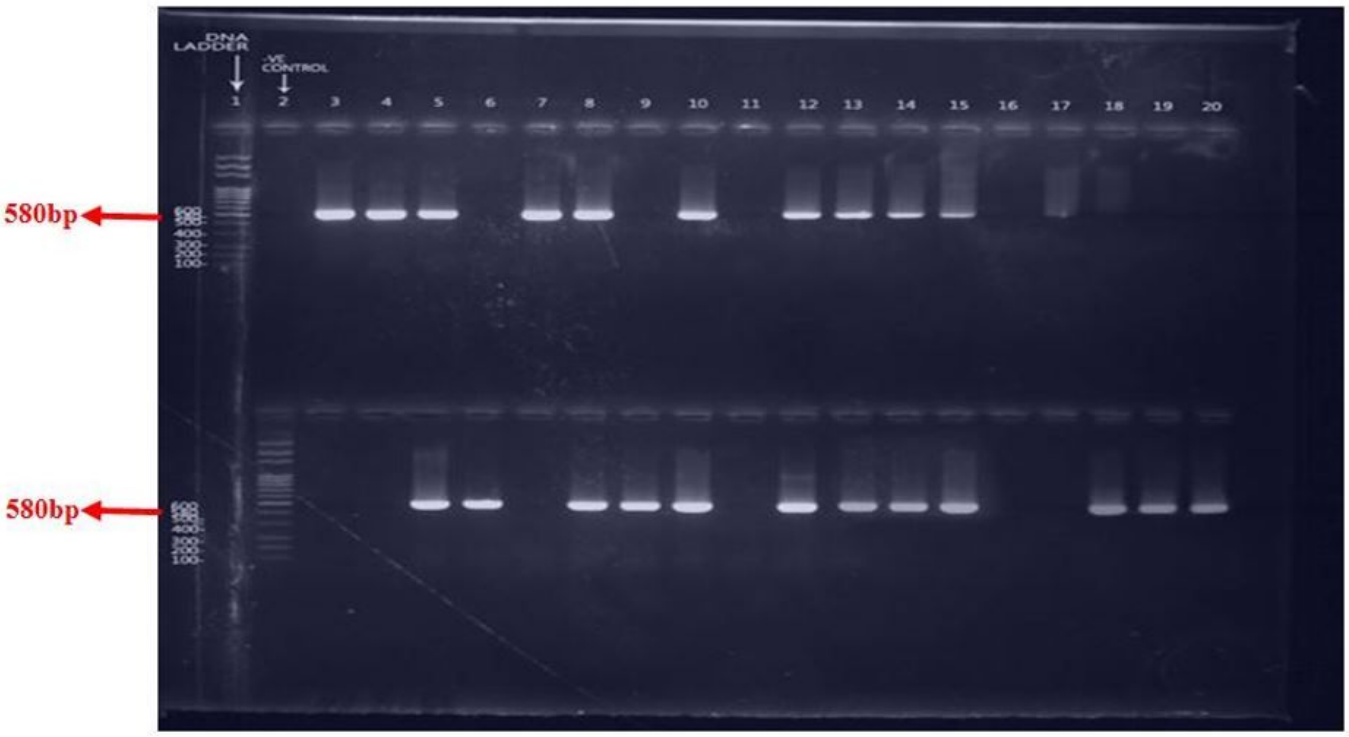


Figure 4

2% Agarose gel electrophoresis analysis of *Pfmdr1* gene SNPs at codon 86&184 nested-PCRs from blood samples of malaria positive patients under UV Transilluminator.

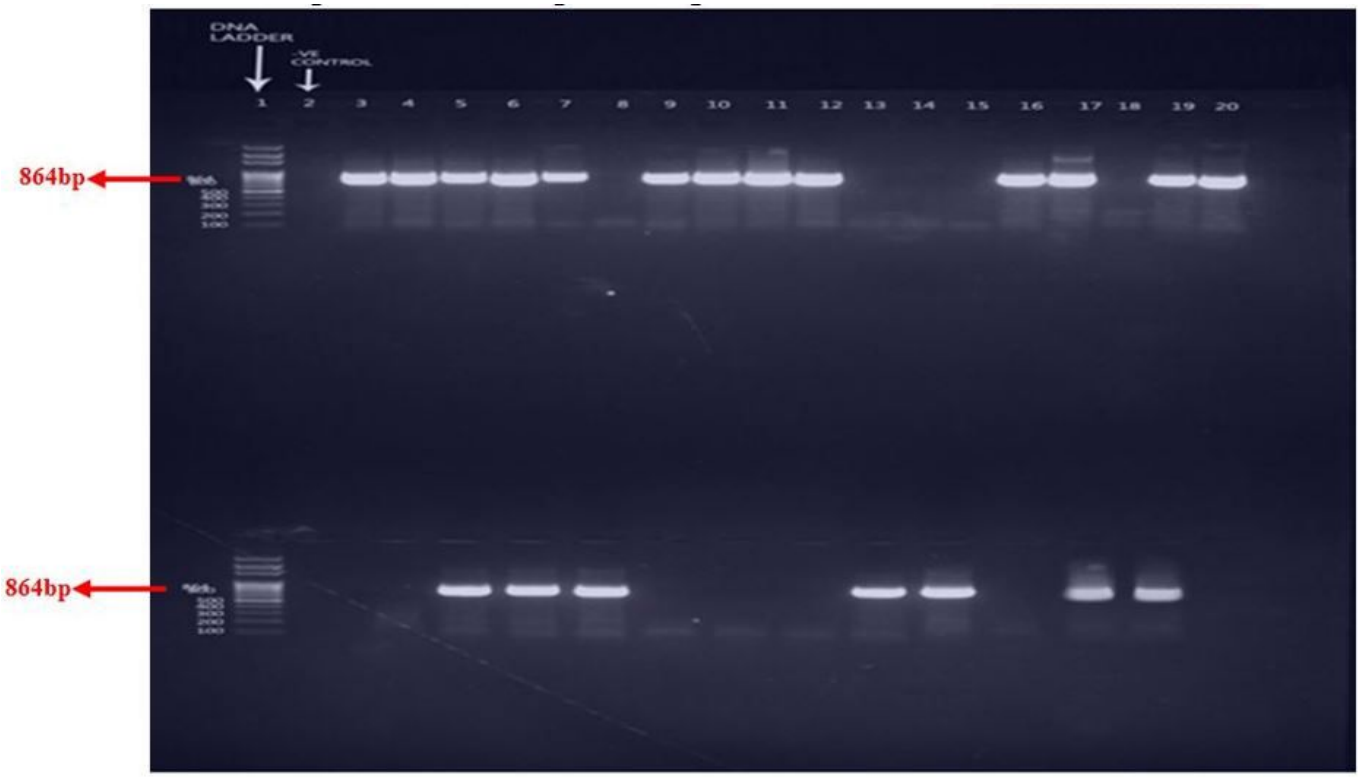
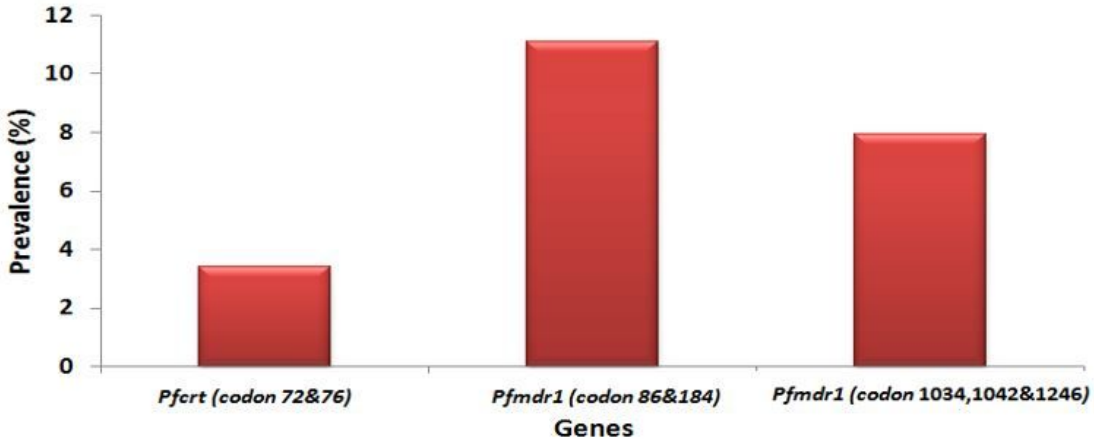


Figure 5

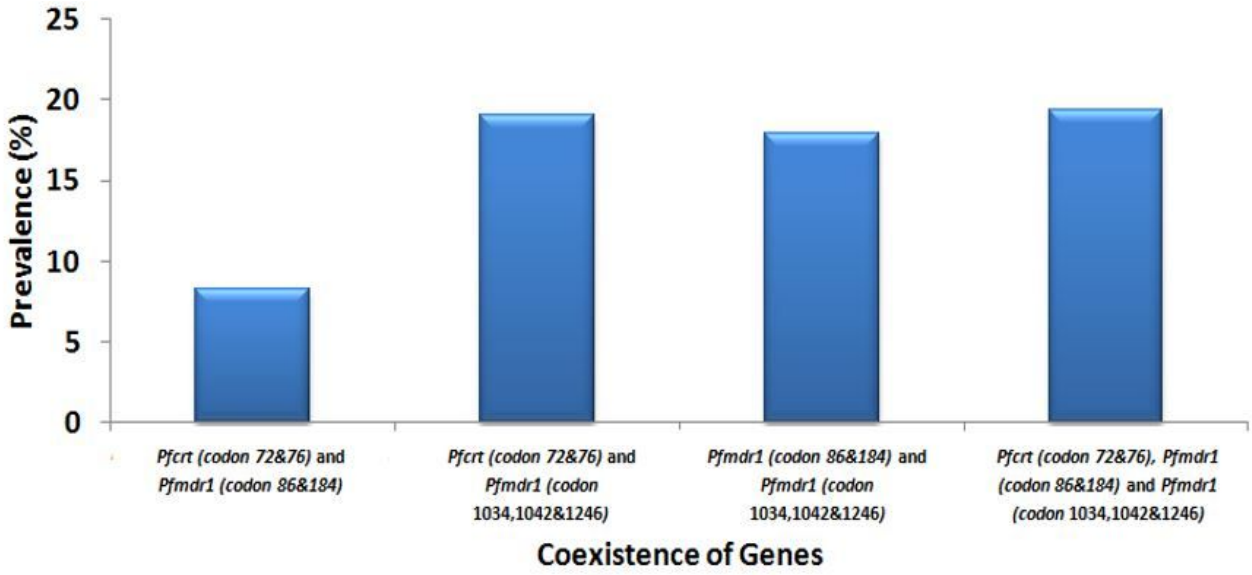
2% Agarose gel electrophoresis analysis of *Pfmdr1* gene SNPs at codon 1034,1042&1246 nested-PCRs from blood samples of malaria positive patients under UV Transilluminator.



Pfcrt (codon 72&76): χ^2 =26.205, df = 10, P = 0.003, Phi = 0.003, Cramer's V= 0.003; *Pfmdr1* (codon 86&184): χ^2 = 42.073, df = 10, P = 0.001, Phi= 0.001, Cramer's V= 0.001; *Pfmdr1* (codon 1034,1042&1246): χ^2 = 77.774, df = 10, P = 0.001, Phi= 0.001, Cramer's V= 0.001.

Figure 6

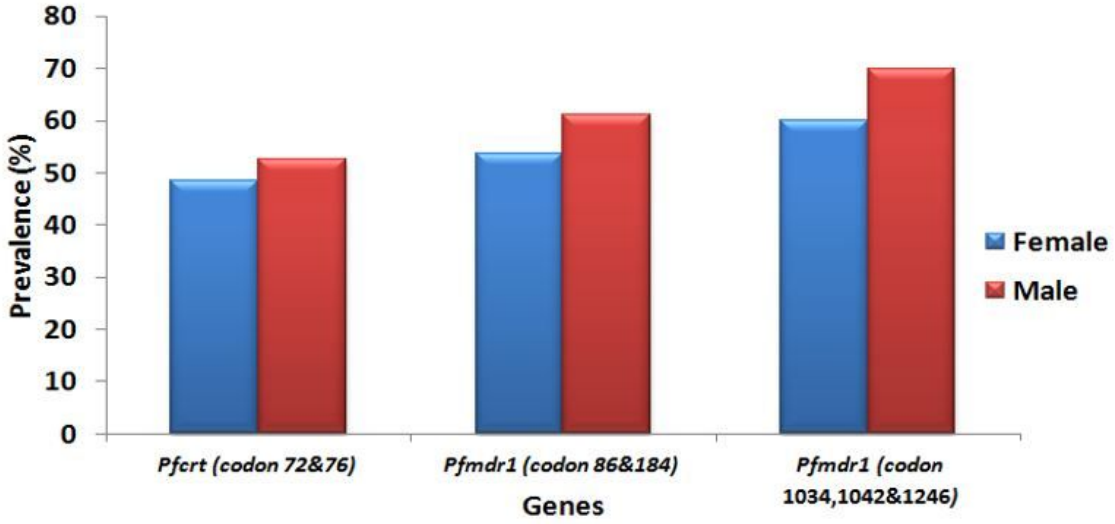
Prevalence of mutant genes among the Patients



Pfcrt (codon 72&76): χ^2 =26.205, df = 10, P = 0.003, Phi = 0.003, Cramer's V= 0.003; *Pfmdr1* (codon 86&184): χ^2 = 42.073, df = 10, P = 0.001, Phi= 0.001, Cramer's V= 0.001; *Pfmdr1* (codon 1034,1042&1246): χ^2 = 77.774, df = 10, P = 0.001, Phi= 0.001, Cramer's V= 0.001.

Figure 7

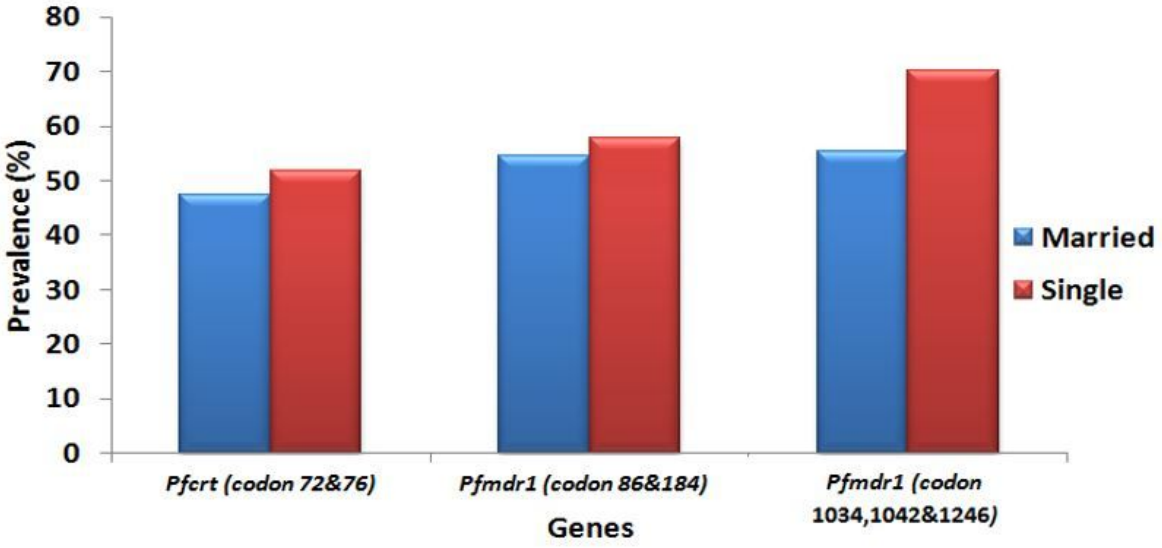
Prevalence of coexistence of the three mutant genes among the Patients



Pfcrt (codon 72&76): χ^2 =0.600, df = 1, P = 0.439, Phi = 0.439, Cramer's V= 0.439; *Pfmdr1* (codon 86&184): χ^2 = 1.835, df = 1, P = 0.176, Phi= 0.176, Cramer's V= 0.176; *Pfmdr1* (codon 1034,1042&1246): χ^2 = 2.742, df = 1, P = 0.098, Phi= 0.098, Cramer's V= 0.098.

Figure 8

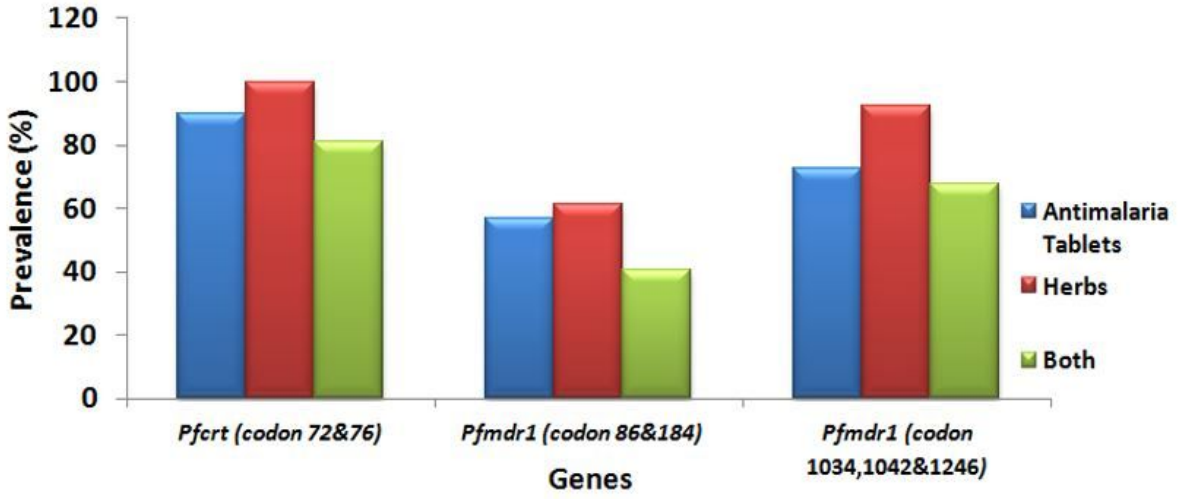
Prevalence of Mutant genes in relation to sexes of patients in the study area



Pfcrt (codon 72&76): $\chi^2 = 0.580$, df = 1, P = 0.446, Phi = 0.446, Cramer's V= 0.446; *Pfmdr1* (codon 86&184): $\chi^2 = 0.354$, df = 1, P = 0.552, Phi= 0.552, Cramer's V= 0.552; *Pfmdr1* (codon 1034,1042&1246): $\chi^2 = 8.081$, df = 1, P = 0.004, Phi= 0.004, Cramer's V= 0.004

Figure 9

Prevalence of Mutant genes in relation to marital status of patients



Pfcrt: $\chi^2 = 3.591$, df = 2, P = 0.166, Phi = 0.166, Cramer's V= 0.166; *Pfmdr1* (codon 86&184): $\chi^2 = 2.976$, df = 2, P = 0.226, Phi= 0.226, Cramer's V= 0.226; *Pfmdr1* (codon 1034,1042&1246): $\chi^2 = 3.032$, df = 2, P = 0.220, Phi= 0.220, Cramer's V= 0.220

Figure 10

Prevalence of Mutant Genes in relation to how malaria is being treated by patients in the study Area