

Genetic Markers Associated with Antimalarial Drug Resistance and Haemoglobin Genotypes Among Malaria Patients in Kaduna State, Nigeria

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Abstract

Background Malaria is a disease of public health concern in Nigeria and sub-Saharan Africa. The emergence of drug resistance, particularly among *P. falciparum* strains, has been a major contributor to the global burden of malaria. This research was aimed at detecting genetic markers associated with antimalarial drug resistance and assessing the distribution of haemoglobin genotypes among malaria patients in of Kaduna State, Nigeria.

Methods Three hundred (300) blood samples were collected from consenting individuals attending selected hospitals, in the three senatorial districts of Kaduna State, Nigeria. A structured questionnaire was used to obtain relevant data from the study participants. The samples were screened for malaria parasites by microscopy and malaria rapid diagnostic test kit. Deoxyribonucleic acid was extracted from one third of the malaria positive samples, and Polymerase Chain Reaction (PCR) was used for detection of the drug resistance genes. *Pfcr*, *pfmdr1*, *pfdhfr*, *pfdhps* and *pfatpase6* genes were detected at expected amplicon sizes from the malaria positive samples. The *pfatpase6* PCR amplicons were sequenced and a phylogenetic tree was created using MEGA X to determine their relatedness to published sequences.

Results *Pfcr* (80%) had the highest prevalence, followed by *pfdhfr* (60%), *pfmdr1* (36%) and *pfdhps* (8%). *Pfatpase6* was also detected in 73.3% of the samples. The phylogenetic tree showed that all the *pfatpase6* gene sequences (both the ones from this study and those published in NCBI Genbank) had the same origin and were closely related. However, the sequences from NCBI Genbank were from one clade; arising from a common ancestor (monophyletic) thus they were more closely related to themselves, than to the *pfatpase6* sequences obtained in this study. Of all the malaria positive participants, those with HbAA (73%) haemoglobin genotype had the highest percentage followed by HbAS (23%), HbAC (3%) and HbSS (1).

Conclusion We detected *Plasmodium falciparum* genes associated with drug resistance to commonly used antimalarials in the study area. Expression of these genes could have serious consequences in the treatment of malaria. Persons with HbAS, HbAC and HbSS may enjoy some protection from *falciparum* malaria than those with HbAA.

Introduction

Malaria is caused by intracellular parasites of the genus *Plasmodium*. It is transmitted through the bite of infected female *Anopheles* mosquito. Despite being preventable and treatable, malaria remains one of the major health problems in sub-Saharan Africa [1] even though there were encouraging reports that malaria morbidity and mortality were declining [2, 3].

Nearly half of the world's population was at risk of malaria in 2018 [4]. There was an estimated 228 million cases of malaria worldwide in 2018 which was an increase from the estimated 219 and 217 million cases of malaria in 2017 and 2016 respectively [5, 4]. The estimated number of malaria deaths in 2018 stood at 405 000 [4]. Five countries; Nigeria (25%), the Democratic Republic of the Congo (11%), Mozambique (5%), India (4%) and Uganda (4%) accounted for nearly half of all malaria cases worldwide in 2017 [5]. The 10 highest burden countries in Africa reported increases in cases of malaria in 2017 compared with 2016. Of these, Nigeria, Madagascar, and the Democratic Republic of Congo had the highest reported increases, all greater than half a million cases [5].

Resistance to antimalarial agents arises because of the selection of parasites with genetic mutations or gene amplifications that confer reduced susceptibility [6]. Resistance genes continue to be selected in *Plasmodium* thereby causing resurgences in the disease incidence [7]. One of the main obstacles to malaria control is the ability of the parasites to develop resistance to administered anti-malarial drugs. The development of resistance to drugs poses the greatest threats to malaria control and results in increased malaria morbidity and mortality [8]. The emergence of drug resistance, particularly among *P. falciparum* strains, has been a major contributor to the global burden of malaria in the past three decades [9]. Resistance is the most likely explanation for the doubling of malaria-attributable child mortality in eastern and southern Africa [10, 7, 11].

Artemisinin-based combination therapy is the first-line therapy in Nigeria and most malaria endemic countries [12,11]. In 2005, Nigeria changed its antimalarial drug policy to artemisinin based combination therapy (ACT) [11]. However, recent gains in reducing the burden of malaria are threatened by the emergence of *Plasmodium falciparum* resistance to artemisinins. The current cornerstones in malaria treatment are artemisinin combination therapy for treatment of uncomplicated *Plasmodium falciparum* malaria [13] and sulfadoxine-pyrimethamine (SP) for intermittent preventive treatment of pregnant women. While resistance to SP is already established, there are evidence of decreased susceptibility or resistance to the ACT components; artemisinins and the key ACT partner drugs lumefantrine, amodiaquine and mefloquine [14]. Development of resistance to ACTs and aggravation of SP resistance could be devastating, as there are presently no further well established treatment options.

Malaria is a complex disease that depends on many host genetic factors [15]. Indeed, resistance to *Plasmodium falciparum* is an important adaptive trait of human populations living in endemic areas [16]. Haemoglobin S (HbS) has become a stable polymorphism within malaria endemic regions, associated with a limited life expectancy among homozygous individuals who suffer from sickle cell disease, and an extended life expectancy of heterozygous individuals who are more likely to evade malaria [17]. HbAS is widely known to confer significant protection from severe and uncomplicated malaria [18] although underlying mechanisms are not precisely defined.

The resistance to different anti-malarial drugs is due to single nucleotide polymorphisms (SNPs) in different *P. falciparum* genes, including *pfdhfr*, *pfdhps*, *pfcr*, *pfatcase6*, *pfk13* and *pfmdr1*. The accumulation of SNPs in these parasites can produce *in vivo* resistance [19]. This stresses the importance of understanding the molecular mechanisms of resistance in order to prevent its emergence or spread [20]. This study was aimed at detecting genetic markers associated with antimalarial drug resistance and assessing the distribution of haemoglobin genotypes among malaria patients in of Kaduna State, Nigeria.

Materials And Methods

Study area

The study was conducted in selected general hospitals within the three Senatorial Districts of Kaduna State, Nigeria. Blood samples were collected from patients in Hajiya Gambo Sawaba General Hospital Zaria (Kaduna North Senatorial District), Barau Diko Teaching Hospital (Kaduna Central Senatorial District) and General Hospital Kafanchan (Kaduna South Senatorial district). Kaduna lies at latitude 10°20' north and longitude 7°45' east and covers an area of 45,711.2 km². It has a population of 6,113,503 and a population density of 130 people/km². It accounts for 4.3% of Nigeria's total population. Kaduna lies in the savanna ecological belt. It experiences a rainy (wet) season between April and October and a harmattan (dry and dusty) season between November and March. The area experiences an average annual rainfall of 1099 mm and average daily temperature of 28 °C. Malaria occurs all year round, with peaks during the middle to late rainy season [21].

Study Design

The study was a cross-sectional study that lasted for six months (May to October 2018).

Inclusion Criteria And Exclusion Criteria

All febrile patients presenting symptoms of malaria that were directed to the laboratory for malaria parasite (MP) test and gave consent were included. All patients directed to the laboratory for laboratory tests other than malaria test and those who did not give consent were excluded.

Sample Size

The sample size was determined using a prevalence of 22.4% [21] and the following formula as described by [22]:

$$n = \frac{Z^2 p (1-p)}{d^2}$$

n = number of samples

p = prevalence rate of previous study = 22.4%=0.224

z = standard normal distribution at 95% confidence limit = 1.96

d = absolute desired precision of 5%=0.05

z = 1.96

n = 1.96² *0.224(1-0.224)

0.05²

n = 3.8416 *0.224*0.776

0.0025

n = 267 samples

The sample size was increased to 300; 100 blood samples were collected from each hospital within the three senatorial districts.

Administration Of Consent Forms And Structured Questionnaire

Consent forms and structured questionnaire were administered to consenting individuals who met the inclusion criteria. This was used to obtain bio-data and information relevant to this research.

Sample Collection And Preparation Of Blood Films

Two milliliter (2 ml) of venous blood was collected by a trained laboratory technician and transferred into an EDTA container. Thick and thin blood films were prepared immediately after the samples were collected according to the technique outlined by [23]. A drop of each blood sample was placed in the center of a grease-free clean glass slide, and spread immediately using a smooth edged slide spreader to make a thin film. The thin film was allowed to air dry before being fixed with methanol. The thick film was made by transferring a drop of blood to another clean slide and spread in such a way that it was possible to see (but not read through) newsprint, it was then allowed to dry properly. The blood films were stained using 10% Giemsa working solution for 30 minutes. After staining the blood films, they were allowed to air-dry [23].

Examination Of Stained Blood Film Slides

The stained blood films were examined under the microscope using 100X objective lens after focusing. Presence of ring forms, trophozoites or gametocytes of *Plasmodium falciparum* or other *Plasmodium* sp was recorded as positive results. A blood smear was considered negative if no parasite was seen after 10 minutes of search or examination under 100X high power fields of microscope.

The prevalence of malaria was determined by the number of positives over the number of specimens collected.

Prevalence rate = Number of positives x 100

Total number of samples

Deoxyribonucleic Acid (dna) Extraction

Total DNA was extracted from one third (25 samples) of the 75 malaria positive blood samples using Zymo Research Quick-DNA™ Miniprep Plus Kit, Irvine, California.

Determination Of Haemoglobin Genotype

Cellulose acetate method of haemoglobin electrophoresis was carried out on all malaria positive samples as follows: A drop of blood from all malaria positive blood samples was placed on a clean white tile and mixed with three drops of water to lyse the red blood cells. With the aid of an applicator, the haemolysate was placed on a cellulose acetate paper. This was followed by electrophoresis in Tris buffer solution for 15 minutes at electromotive force of 250v. Haemolysates from blood samples of Hb AS and AC were run as controls [24].

Primers Used For Polymerase Chain Reaction

The genes and primer sequences used for the polymerase chain reaction are shown below in Table 1. The target genes were; *Plasmodium falciparum* resistance transporter (*pfcr1*) gene which is a single copy, 13-exon gene, localised on chromosome 7 and codes for a digestive vacuole trans-membrane protein, which plays a key role in chloroquine resistance. *Plasmodium falciparum* multidrug resistance transporter 1 (*pfmdr1*) gene which codes for a large 12 transmembrane domain ABC-transporter (PfMRP1), located in the parasite plasma membrane [25], *Plasmodium falciparum* dihydrofolate reductase (*pfdhfr*) gene (codes for *P. falciparum* dihydrofolate reductase enzyme), *Plasmodium falciparum* dihydropteroate synthase (*pfdhps*) gene which codes for dihydropteroate synthase enzyme of *P. falciparum* and *P.falciparum* atpase6 gene (*pfatpase6*).

Table 1
Primer sequences used for polymerase chain reaction

S/N	Gene	Primer sequence	Size (bp)	SNPs (codons)	Reference
1	<i>Pfprt</i>	F: GGAGGTTCTTGTCTTGGTAAAT R: ATATTGGTAGGTGGAATAGATTCT	315	391T/A,392G/C, 399G/T 400A/G,402T/A,404A/C (codons C72S, M74I,N75E, K76T)	[26]
2	<i>Pfmdr1</i>	F: TGTGAAAGATGGGTAAAGAGCAGA R: TCGTACCAATTCCTGAACTCACTT	514	256A/T,257A/T (Codon N86Y/F)	
3	<i>Pfdhps</i>	F: GATTCTTTTTTCAGATGGAGG R: TTCCTCATGTAATTCATCTGA	770	1482T/G,1483C/T/G,1486C/G,1794A/G,1918C/G,2013G/T/A, (codon S436A/F/C, A437G, K540E, A581G, A613S/T)	
4	<i>Pfdhfr</i>	F: TGATGGAACAAGTCTGCGACGTT R: CTGGAAAAAATACATCACATTATG	594	148T/C,152A/T,153T/C, 175T/C, 323G/A/C,490A/T (codons C50R,N511, C59R, S108, I164L)	
5	<i>Pfapse6</i>	F: AAAATAAATACCACATCAACACAT R: TCAATAATACCTAATCCACCTAAA	437	2306G/A (codon769N)	

Detection of genetic markers of *Plasmodium falciparum*

The primer sequences in Table 1 were used to amplify *pfprt*, *pfmdr1*, *pfdhfr*, *pfdhps* and *pfatpase6* genes by multiplex PCR as follows: Multiplex PCR master mix cocktail was prepared by adding 3.0 µl of the extracted DNA, 2.5 µl of 10X PCR buffer, 1.5 µl of 50 mM MgCl₂, 1.0 µl of 2.5 Mm DNTPs, 1.0 µl of Taq polymerase (5 µ/ µl), 5 µl of nuclease free water, 1.0 µl each of 5pMol of each of the forward and reverse primers in Table 1 to give a total volume of 25 µl. This was run using the following programme: Initial denaturation at 94 ° C for 5 minutes followed by 9 cycles of denaturation at 94 ° C for 15 seconds, annealing at 65 ° C for 20 seconds, extension at 72 ° C for 30 seconds and another 35 cycles of denaturation at 94 ° C for 15 seconds, annealing at 55 ° C for 20 seconds, extension at 72 ° C for 30 seconds, final extension at 72 ° C for 7 minutes.

The PCR amplicons were separated by electrophoresis on a 1.5% (w/v) agarose gel stained with ethidium bromide. The gel was visualised on UV transilluminator gel imaging system and system photographed, band positions were determined and compared to molecular weight markers.

Amplification and Sequencing of *pfatpase6* Gene

The *pfatpase6* gene was amplified using the *pfatpase6* primers in Table 1 above as follows: The master mix cocktail was prepared by adding 2.0 µl of 100 ng/ul DNA, 2.5 µl of 10x PCR buffer, 1.5 µl of 50 mM MgCl₂, 1.0 µl of DMSO, 2.0 µl of 2.5MmDNTPs, 0.15 µl of Taq Polymerase (5 µ/ µl), 1.0 µl forward primer, 1.0 µl reverse primer and 13.85 µl nuclease free water to make a total of 25 µl. This was run using the following programme: Nine cycles of initial denaturation at 94 ° C for 15 minutes, denaturation at 94 ° C for 40seconds, annealing at 60 ° C for 120 seconds, extension at 72 ° C for 40 seconds. This was followed by another 35 cycles of denaturation at 94 ° C for 40 seconds, annealing at 50 ° C for 120 seconds, extension at 72 ° C for 40 seconds, final extension at 72 ° C for 10 minutes. The PCR amplicons were separated by electrophoresis and visualised on UV transilluminator gel imaging system (gel documentation unit), after which gel pictures were taken from which band positions were determined and compared to molecular weight markers.

Sequencing of *pfatpase6* gene

Amplicon purification was done using QiaQuick DNA gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Five to 10 µl of purified PCR products were used to prepare the sequencing mix using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1. (Applied Biosystem, Foster City, CA, USA).

Phylogenetic Analysis

The multiple sequence alignment programme Clustal W was used to obtain an optimal nucleotide sequence alignment file after comparing with sequences deposited in GenBank. Phylograms were obtained by MEGA X based on aligned nucleotide sequences.

Results

Only *Plasmodium falciparum* was detected in this study. The agarose gel electrograph in Fig. 1 shows the amplification of *pfprt*, *pfmdr1*, *pfdhfr* and *pfdhps* by multiplex PCR. Analysis of the number and percentages of the genes amplified by multiplex PCR (Table 2) showed that out of the 25

DNA samples screened, there were 20 (80%) *pfcr*, 9 (36%) *pfmdr1*, 15 (60%) *pfdhfr* and 2 (8%) *pfdhps* genes amplified by multiplex PCR. The gel electrophoresis of the resultant multiplex PCR products indicated that they were of the expected sizes. Some nonspecific amplifications were observed for the samples. Eleven out of the 15 DNA samples analysed (73.3%) showed amplification of the *pfatpase6* gene (Fig. 2). The phylogenetic tree showing the relatedness between the *pfatpase6* sequences in this study and sequences obtained from the NCBI GenBank is shown in Fig. 3. All the *pfatpase6* sequences showed close relationship as compared to the outgroup (*Plasmodium vivax*_E1-E2_ATPase/hydrolase). Participants with haemoglobin genotype AA had the highest prevalence of malaria (73%), followed by those with haemoglobin genotypes AS (23%), AC (3%) and SS (1%) (Fig. 4).

Key: Lane M = Molecular weight marker (50 bp), Lanes 1–25 = *Plasmodium falciparum* positive samples, bp = base pairs

Table 2
Analysis of the number and percentages of the genes amplified by multiplex PCR

Genes	No. examined	Frequency	% Prevalence
<i>Pfcr</i>	25	20	80
<i>Pfmdr1</i>	25	9	36
<i>Pfdhfr</i>	25	15	60
<i>Pfdhps</i>	25	2	8

Key: *pfcr* = *Plasmodium falciparum* chloroquine resistance transporter, *pfmdr1* = *Plasmodium falciparum* multidrug resistance transporter I, *pfdhfr* = *Plasmodium falciparum* dihydrofolate reductase, *pfdhps* = *Plasmodium falciparum* dihydropteroate synthase

Key: Lane M: Molecular weight marker (50 bp), Lanes 1–15: *Plasmodium falciparum* positive samples, bp = base pairs

Key: *Plasmodium falciparum* ATPase6_1 to 6 = sequences from this study, the other *pfatpase6* sequences were obtained from NCBI, and *Plasmodium vivax*_E1-E2_ATPase/hydrolase = Outgroup.

Discussion

The primers used in this study were designed by Zhang *et al.* [26] to amplify regions containing single nucleotide polymorphisms (SNPs) covering genetic markers reported to be associated with the resistance of *Plasmodium falciparum* to some of the most commonly used antimalarial drugs such as chloroquine, mefloquine, amodiaquine, sulfadoxine-pyrimetamine and artemether. *Pfcr*, *pfmdr1*, *pfdhfr*, *pfdhps* genes were detected at expected amplicon sizes from the malaria positive samples in this study. *Pfcr* (80%) had the highest prevalence, followed by *pfdhfr* (60%), *pfmdr1* (36%) and *pfdhps* (8%). The presence of these genes, if expressed, will pose a significant danger to the management and control of malaria [5].

The wide spread of chloroquine-resistant parasites prompted the WHO to recommend Artemisinin-based Combination Therapy for the treatment of malaria in endemic regions. The first report describing the prevalence of polymorphisms in *pfcr* conferring chloroquine resistance showed that all the *Plasmodium falciparum* isolates were carrying the *pfcr* alleles [27, 28].

The current study successfully amplified segments of *pfcr* as well as *pfmdr1* carrying known mutations associated with chloroquine resistance. The high prevalence of the *pfcr* shows that chloroquine resistance is still very likely in the studied population despite its withdrawal as first line drug for the treatment of uncomplicated malaria in Nigeria in 2005 [29, 30]. In their report, Sidhu *et al.* [31] provided conclusive evidence that *pfcr* mutations increased susceptibility to artemisinin and quinine, and minimally affected amodiaquine activity.

In this study, *pfdhfr* (60%) had a higher prevalence than *pfdhps* (8%) which suggests that there are more *Plasmodium falciparum* parasites carrying the mutant *pfdhfr* gene in the study population than those carrying the *pfdhps* genes. Mutations in the *pfdhfr* and *pfdhps* genes have been associated with resistance to pyrimethamine and sulfadoxine respectively [32]. In Nigeria, sulfadoxine is given in combination with pyrimethamine as sulfadoxine-pyrimethamine (SP) to pregnant women during intermittent preventive treatment (IPT) of pregnant women [33]. The high prevalence of *pfdhfr* gene mutations may be due to wide usage of Pyrimethamine in combination with Sulfadoxine for the treatment of pregnant women in Nigeria. This indicates possible resistance to pyrimethamine and consequently reduce the efficacy of SP as a combination therapy. The possession of more than one drug resistance gene in some of the *Plasmodium falciparum* parasites in this study may increase their resistance to different antimalarials, and probably lead to high rate of treatment failure with these drugs.

In this study, only the portion of *pfatpase6* gene carrying the 2306G/A SNP at codon S769N was amplified. Maslachah *et al.* [34] reported in their study that all the resistant *Plasmodium falciparum* isolates contained *pfatpase6* S769N mutation which is associated with artemisinin resistance. A similar finding was also reported by Jambou *et al.* [35] and Jung *et al.* [36].

The phylogenetic tree revealed the similarities and relatedness between *pfatpase6* sequences in this study and the reference sequences. The phylogenetic tree showed that all the *pfatpase6* gene sequences (both the ones from this study and those from NCBI) had the same origin and

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were closely related. However, the sequences from NCBI were from one clade; arising from a common ancestor (monophyletic) thus they were more closely related than to the *pfatpase6* sequences from this study.

Haemoglobin genotype is known to influence the prevalence of malaria in endemic areas [37]. The results of this study revealed that 73% of the malaria positive participants had the haemoglobin genotype AA (HbAA), which was higher than the percentages in the other genotypes (HbAS [23%] and HbAC [3%]). This is similar to the reports of Albiti and Nsiah [38] and Akanbi [37]. The prevalence of participants with HbAS and HbAC were quite low compared to HbAA. This suggests that individuals with HbAS and HbAC are probably able to resist malaria better than HbAA. This is in agreement with the findings of Aidoo *et al.* [39], Ayi *et al.* [40], Williams *et al.* [41], Albiti and Nsiah [38] and Archer *et al.* [42]. In another study by Williams *et al.* [43], HbAS was 50% protective against mild clinical malaria, 75% protective against admission to the hospital for malaria, and almost 90% protective against severe or complicated malaria. The level of susceptibility to malaria has been reported to be higher in individuals with HbAA when compared with those with HbAS and HbAC, thus, the high frequency of HbAC and HbAS in malaria endemic areas has been attributed to a decrease in malaria morbidity and mortality in malaria endemic areas [44, 42]. The protective role displayed by HbAC and HbAS in malaria infection is as a result of reduced cytoadhesion of infected red blood cell to microvasculature and impaired rosette formation as a result of the presence of abnormal PfEMP1 antigen on HbAC and HbCC [45].

Earlier studies [46, 47] had associated low oxygen tension in HbAS RBCs with impairment in the invasion and growth of *Plasmodium falciparum* parasites in the HbAS red blood cells (RBCs) which causes infected RBCs to sickle under low oxygen tension and lead to their premature destruction in the spleen, thus, reducing parasitaemia and providing protection. In a more recent study by Archer *et al.* [42], they reported that resistance to *Plasmodium falciparum* in sickle cell erythrocytes is driven by oxygen dependent-growth inhibition. Their experiments showed that low oxygen (1% oxygen concentration) indeed stalled the growth of *Plasmodium falciparum* and no DNA replication was evident at that oxygen concentration.

Rosette formation (binding of *Plasmodium falciparum* infected RBCs) which is thought to lead to microcirculatory obstruction in cerebral malaria was found to be impaired in *P. falciparum* infected HbAS RBCs under deoxygenated conditions; this may be due to increased sickling of the RBCs in deoxygenated condition or reduced expression of erythrocyte surface adherence protein [48]. Decreased rosette formation and the resulting decreased circulatory obstruction might contribute to protection against severe malaria in HbAS.

The protective effect of HbC may result from a reduced ability of *Plasmodium falciparum* to grow and multiply in RBCs containing HbC [49]. HbC exerts its protection through a specific effect on cytoadherence, mediated by the altered display of surface expressed parasite proteins [50, 51].

It has been reported that subjects with HbSS appear to be less susceptible than normal to developing malaria but are highly susceptible to the catastrophic consequences of malaria particularly severe anaemia if they do become infected with the parasite [52, 53].

Conclusion

The *Plasmodium falciparum* parasites in the study population have the drug resistance genes; *pfcr1*, *pfmdr1*, *pfdhfr*, *pf dhps* and *pfatpase6*. The expression of these genes will cause resistance to the commonly used antimalarial drugs, which by extension will lead to a significant public health challenge in the treatment and control of malaria within the study area. *Plasmodium falciparum* parasites carrying two or more drug resistance genes may pose a significant danger to malaria chemotherapy. The high frequency of HbAA genotype in malaria positive participants implies that people having the aforementioned haemoglobin type are less protected from malaria than those having HbAS, HbAC and HbSS. This may not be far from the fact that HbAS and HbAC have been reported in many studies to provide protection against malaria, especially *falciparum* malaria.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the Health Research Ethics Committee of Kaduna State Ministry of Health as well as Barau Diko Teaching Hospital Health Research Ethics Committee (BDTH-HREC); HREC Reference number:18-00016.

Consent for publication

All participants gave consent.

Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests.

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None

Author's contributions

GYB designed the study, conducted the laboratory and data analysis, and prepared the manuscript. HII, HIDM and BOO supervised the research, read and approved the manuscript.

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Figures

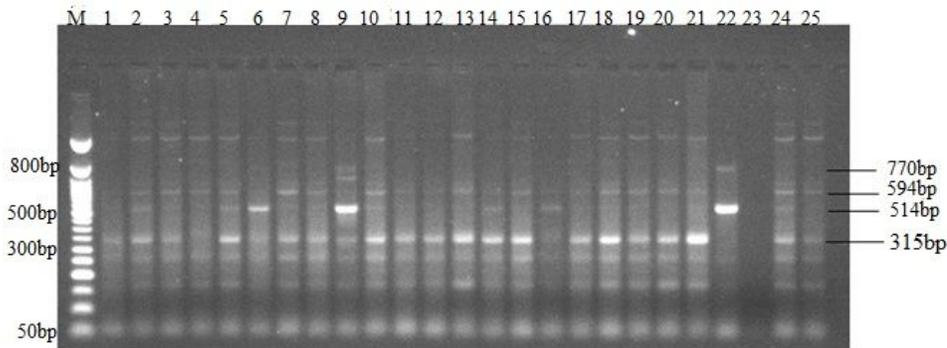


Figure 1

Agarose gel electrograph for the detection of *pfprt* (315bp), *pfmdr1* (514bp), *pfdhfr* (594bp), and *pfdhps* (770bp) genes

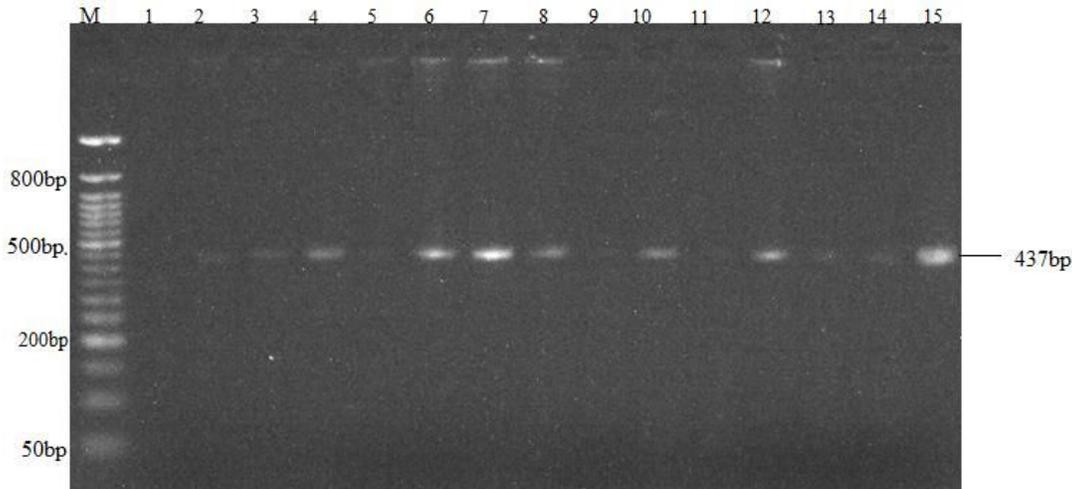


Figure 2

Agarose gel electrograph of amplified *pfatpase6* gene

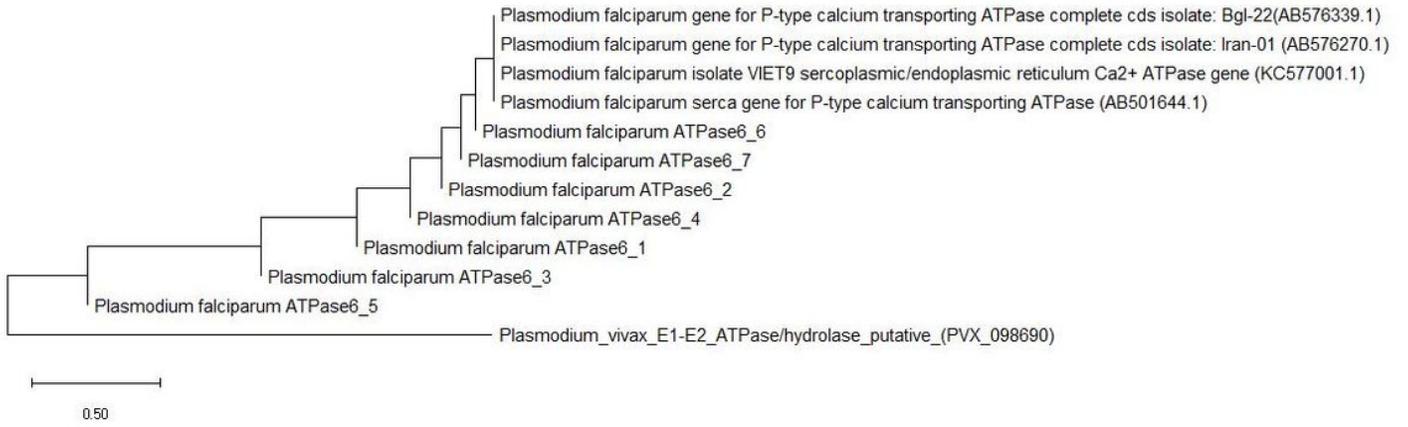


Figure 3

Phylogenetic tree (neighbor joining tree) of pfapase6 sequences

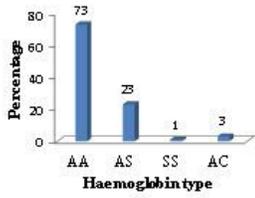


Figure 4

Haemoglobin electrophoresis patterns of malaria positive participants in the study area