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Research Article

Molecular Epidemiology of Single Nucleotide Polymorphisms (SNPs) in *Plasmodium falciparum* Dihydropteroate Synthase and *P. falciparum* Dihydrofolate Reductase Genes among Pregnant Women in Sokoto Metropolis, Sokoto, Nigeria

***Abubakar, H.[1], Bala, A. Y. [2], Bandiya, H. M. [2] and Imam, M. U. [3]**

[1]Department of Biological Sciences, Federal University Birnin-Kebbi, Kebbi, Nigeria
[2]Department of Zoology, Usmanu Danfodiyo University Sokoto, Nigeria
[3]Centre for Advanced Medical Research and Training, Usmanu Danfodiyo University Sokoto, Nigeria
****Corresponding Authors' email*: abubakarhafiz95@gmail.com; Phone:** +2348030510042

ABSTRACT
Malaria continues to cause significant morbidity and mortality in pregnant women despite the implementation of various interventions. Currently, the World Health Organization (WHO) recommends Sulfadoxine/Pyrimethamine (SP) for intermittent preventive treatment during pregnancy (SP-IPTp) in the second and third trimesters. However, the efficacy of SP is threatened by the emergence of *Plasmodium falciparum* dihydropteroate synthase (Pfdhps)and *Plasmodium falciparum* dihydrofolate reductase (Pfdhfr) genes that confer resistance against sulfadoxine and pyrimethamine respectively. This research was aimed to determine the prevalence of Pfdhps and Pfdhfr genes among pregnant women attending antenatal care (ANC) in the Sokoto metropolis. Thirty-two (32) blood samples of malaria-infected pregnant women who came for ANC between April and July 2024 were evaluated for single nucleotide polymorphisms of Pfdhps and Pfdhfr genes via polymerase chain reaction (PCR) protocol. It was interesting to note that, none of the antimalarial drug-resistant genes was detected from the isolates tested. This finding has validated the efficacy of Sulfadoxine/Pyrimethamine for intermittent preventive treatment of malaria during pregnancy in Sokoto metropolis. It is therefore recommended that health facilities should continue to issue SP for IPTp in line with WHO guidelines.

Keywords: Malaria; Pregnant women; PFDHPS gene; PFDHFR gene; Pyrimethamine; Sulfadoxine

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INTRODUCTION

Malaria continues to cause significant morbidity and mortality across Africa, despite the intensification of control interventions (WHO, 2018). A worldwide decline in its burden was reported in 2015, with rebound and age shift in morbidity as control and interventions are scaled up (Trape*et al.,* 2011; Griffin *et al.,* 2014; Apinjoh *et al.,* 2015; WHO, 2016). Reports suggest that malaria incidence increased between 2014 and 2017 in several regions across sub-Saharan Africa (SSA), where most global investments in control and elimination are spent (WHO, 2018). Malaria remains the most prevalent parasitic disease in tropical and subtropical regions (WHO, 2018).

Sulfadoxine-Pyrimethamine is given to pregnant mothers and children as a prophylaxis strategy to prevent malaria in malaria-endemic areas and was recommended for use as a combination therapy with artemisinin derivatives. (Karolin *et al.,* 2014; Jovel *et al.,* 2017). However, soon after its introduction, resistance to SP gradually emerged and spread widely from Asia to Africa where it was replaced with artemisinin-based combination therapy (ACT). Reports of resistance to the currently recommended artemisinin-based combination therapy are consequently of major

concern and highlight the importance of the evolution of drug resistance (Jovel *et al.,* 2017).

Previous studies have reported SP resistance in Nigeria (Happi *et al.,* 2005; Oguike *et al.,* 2016) and other countries where strategies are deployed (Mita *et al.,* 2014; Jiang *et al.,* 2019) thus, the need for periodic monitoring of SP efficacy to ensure early detection of drug resistance. Single nucleotide polymorphisms (SNPs) in *P. falciparum* dihydrofolate reductase (Pfdhfr) and *P. falciparum* dihydropteroate synthase (Pfdhps) are strong predictors of SP resistance (Happi *et al.,* 2005; Mita *et al.,* 2014; Jiang *et al.,* 2019; Basuki *et al.,* 2018). Pyrimethamine resistance is associated with point mutations at codons N51I, C59R, S108N, and I164L of Pfdhfr (Sulyman*et al.,* 2021), while sulfadoxine resistance is associated with point mutations at codons S436A/F, A437G, K540E, A581G, and A613S/T of Pfdhps (Sulyman *et al.,* 2021).

Abubakar *et al.* (2022) recommended the need for molecular survey to check the status of *Plasmodium falciparum* dehydroaptorase synthase (Pfdhps) and *Plasmodium falciparum* dehydrofolatereductase (Pfdhfr) genes in Sokoto. Therefore, this study is aimed at determining the molecular epidemiology of *Plasmodiumfalciparum* drug resistant genes among pregnant women in Sokoto metropolis, Sokoto State, Nigeria.

MATERIALS AND METHODS

Study Area

Sokoto city is located in the extreme northwest of Nigeria, near the confluence of the Sokoto River and the River Rima. As of 2006, it has a population of 427,760. Sokoto town is the capital of Sokoto State (C-GIDD, 2008).

Sokoto experiences a hot semi-arid climate It is located in the dry Sahel surrounded by sandy Savannah and isolated hills with an annual average temperature of 28.3 °C (82.9 °F) and maximum day time temperatures are generally under 40 °C (104.0 °F) for most of the year. The warmest months are February to April, where daytime temperatures can exceed 45 °C (113.0 °F). The highest recorded temperature is 47.2 °C (117.0 °F), which is also the highest recorded temperature in Nigeria. The rainy season is from June to October. From late October to February is the cold season where the climate is dominated by the harmattan wind blowing Sahara dust over the land (C-GIDD, 2008).

Sokoto State is located in the savannah zone with land area of 28,232.37 sq kilometer. It's located between latitude 11^{o} 30" to 13^{o} 50" North and longitudes 4^{o} to 6^{o} East. It's bordered in the north by Niger Republic, Zamfara state to the North and Kebbi state to the south and west (C-GIDD, 2008).

Women and Children Welfare Clinic Sokoto (WCWC) is located in Kanwuri area, Sokoto North Local Government Area behind Sultan Palace, Sokoto State. It lies within the coordinates: Latitude: 13° 4' 6"N and Longitude: 5° 14' 52"E. the clinic was established to cater for medical, health and welfare services for mothers and children. Its medical aspect includes pre-natal (ante-natal) and postpartum (post-natal) services as well as paediatric care in infancy, childhood and adolescence.

Primary Health Centre Arkilla (Arkilla PHC) is situated in ArkillaGwiwa Ward, Wamakko Local Government Area, Sokoto State along Kalambaina road behind NYSC office Sokoto. It lies within the coordinates Latitude 13° 2' 1" North and Longitude 5° 11' 57''E. medical services in the health facility includes medical care, Maternal-child health including family planning, prevention and control of locally endemic diseases, collection and reporting of vital statistics and health education.

Primary Health Centre Gagi (PHC Gagi)is located inGagi C Ward, Along Durbawa Road, Sokoto South Local Government Area, Sokoto State. It lies within the coordinates Latitude 13° 2' 47"N Longitude 5° 16' 42"E. It was established to provide medical care, Maternal-child health including family planning, prevention and control of locally endemic diseases, collection and reporting of vital statistics and health education.

Primary Health Centre More (PHC More) is located in More town, Kware Local Government Area, Sokoto State. It lies within the coordinates: Latitude 12^{o} 58' 51" North and Longitude 5^{o} 16' 0" East. Medical services in the health facility include: medical care, Maternal-child health including family planning, antenatal and postnatal care, prevention and control of locally endemic diseases.

Primary Health Centre Kwannawa (PHC Kwannawa) is located along DangeShuni road, DangeShuni Local Government Area, Sokoto State. It is located between Latitude 12^{o} 58' 51" North and Longitude 5^{o} 16' 0". It was established to cater for medical care, Maternal-child health including family planning, antenatal and postnatal care, prevention and control of locally endemic diseases.

Ethical Clearance

Prior to the commencement of the research, Introduction letter from the Head of Biology department, Usmanu Danfodiyo University Sokoto was obtained. The letter was addressed to Usmanu Danfodiyo University Sokoto (UDUS) research ethics committee and Sokoto State ministry of health seeking them to grant ethical approval for the conduct of the research. The ethical clearance/approval was granted by the research and ethics committee of the UDUS and

State Ministry of Health with ethical clearance number SKHREC/009/2024.

Informed Consent

Blood samples were collected from consenting patients who were clearly informed on the aim and objectives of the research. Consent form was given to the participating pregnant women.

Molecular Screening

Malaria positive samples obtained via microscopy and RDT were subjected to molecular screening comprising of deoxyribonucleic acid (DNA) extraction and quantification, Polymerase Chain Reaction (PCR) for the detection of *Plasmodium* specific gene (small subunits of ribosomal DNA), House-keepers (Human DNA), PCR for Single Nucleotide Polymorphisms (SNP's) genotyping in Pfdhfr, Pfdhps, Pfmdr1 and Pfcrt genes, agarose gel electrophoresis and sanger sequencing.

Deoxyribonucleic acid (DNA) Extraction

DNA extraction was carried out using Qiagen DNA easy (Qiagen, Krefeld, Germany) extraction kit following manufacturer's instructions (Olasehinde *et al.,* 2014).

Multiplex PCR (Duplex PCR)

To detect *Plasmodium* genus and house-keeping genes (Human DNA) from the 32 samples, a set of primers (PLS and REV primers) were used to amplify the small subunit ribosomal DNA gene while another primer (Huf primer) was used for the detection of house-keeping genes. Appearance of a 787 bp and 231 bp bands revealed pregnant women infection with *Plasmodium* spp. and presence of house-keeping genes (human DNA) respectively (Rubio *et al.*, 2002; Seyedeh*et al.,* 2022). Duplex PCR was carried out in 13 µl reaction mix with the following components; 0.5 µl of 0.30 µM primers - PLF, REV and Huf, 3.25 µl of Nuclease free water (Qiagen, Hilden, Germany), 6.25 µl of Top Taq master mix (Qiagen, Hilden, Germany) and 2 µl DNA template. The tubes were transferred to Applied Biosystem (ABS) 9700 thermocycler and the cycling conditions were: an initial denaturation at 94 °C for 10 min, followed by 39 cycles of final denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec. followed by an initial extension at 72 °C for 30 sec. and final extension at 72 °C for 5 min and post hold at 8 °C. The PCR amplicons were visualized in 1.5 % agarose gel electrophoresis. The list of primers used in the PCR amplification is provided in Table 1.

Table 1: Nucleotide sequences of the primers used in Multiplex PCR

Name of primer	Oligonucleotide sequences (5'- 3')	Expected band size
PLS	AGTGTGTATCAATCGAGTTTC	787 bp
REV	GACGGTATCTGATCGTCTTC	
Huf	GAGCCGCCTGGATACCGC	231 bp

Source: Seyedeh *et al*. (2022)

SNP's genotyping in Pfdhfr and Pfdhps genes

Plasmodium falciparum positive samples were used for SNP's genotyping in Pfdhfr and Pfdhps genes via PCR protocol to detect Single Nucleotide Polymorphism (SNPs) correlated with sulfadoxine and pyrimethamine resistance using the following protocols.

PCR amplification to detect SNP's in pfdhfr gene

720 base pair fragment of Pfdhfr gene was amplified as described earlier (Ahmed *et al.,* 2004; Sharma *et al.,* 2015) and nested PCR was performed to amplify 648-bp fragment covering various single nucleotide polymorphism (SNP) A16V, N51I, C59R, S108N and I164L correlated with pyrimethamine resistance. Primary PCR was carried out in a 0.2 ml nuclease free microfuge tube containing 25 µl reaction mix with following components; 12.5 µl of Top Taq master mix (Qiagen, Hilden, Germany), 0.5 µl of each 20 µM AMP-1 F and AMP-2R primers, 6.5 µl of Nuclease free water (Qiagen, Hilden, Germany), 5 µl of DNA template. The tubes were transferred to Applied Biosystem (ABS) 9700 thermocycler. The cycling conditions are as follows: an initial denaturation at 94 °C for 3 min, followed by 45 cycles of final denaturation at 94 °C for 30 s, annealing at 45 °C for 45 s, initial extension at 72 °C for 45 s followed by final extension at 72 °C for 5 min and post hold at 8 °C. Nested PCR was performed using 2.5 µl templates from first round PCR product, 0.5 µl of 0.30 µM primers M1 and M5, 2.5 µl of coral load, 6.5 µl of Nuclease free water and 12.5 µl of Top Taq master mix in a 25 µl reaction. The tubes were transferred to Applied Biosystem (ABS) 9700 thermocycler. The cycling conditions were: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of final denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min followed by an initial extension at 72 °C for 1 min and final extension at 72 °C for 10 min and post hold at 8 °C. The PCR amplicons were visualized in 1.5 % agarose gel electrophoresis. The list of primers used in the PCR amplification is provided in Table 2.

Table 2: Primers to be use in the study

Name of Primer	Oligonucleotide Sequences (5'- 3')	Expected band size
AMP-1 F	TTTATATTTTCTCCTTTTTA	720 bp
AMP-2 R	CATTTTATTATTCGTTTTCT	
M1	TTTATGATGGAACAAGTCTGC	648 bp
M5	AGTATATACATCGCTAACAGA	
M3717F	CCATTCCTCATGTGTATACAACAC	1287 bp
186R	GTTTAATCACATGTTTGCACTTTC	
Rc	GGTATTTTTGTTGAACCTAAACG	728bp
Rd	ATCCAATTGTGTGATTTGTCCAC	

Source: Sharma *et al.* (2015)

PCR amplification to detect SNP's inPfdhps gene

Nested PCR assay will be performed to amplify 728-bp fragment of the Pfdhps gene covering SNP's S436A, A437G, K540E, A581G and A613S known to be associated with sulfadoxine resistance as described earlier (Ahmed *et al.,* 2004; Sharma *et al.,* 2015). Primary PCR reaction of 25 µl was prepared consisting of 12.5 µl of Top Taq master mix (Qiagen, Hilden, Germany), 0.5 µl of each 20 µMM3717F and 186R primers, 6.5 µl of Nuclease free water (qiagen, USA), 5 µl of DNA template. The tubes were transferred to Applied Biosystem (ABS) 9700 thermocycler. The cycling conditions were as follows; an initial denaturation at 94 °C for 5 min followed by 45 cycles of final denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s and initial extension at 72 °C for 90 s followed by final extension at 72 °C for 10 min and post hold at 8 °C. Nested PCR was performed using 2.5 µl templates from first round PCR product, 0.5 µl 0.30 µM of Rc (forward) and Rd (reverse) primers, 2.5 µl of coral load, 6.5 µl of Nuclease free water and 12.5 µl of Top Taq master mix in a 25 µl reaction. The tubes were transferred to Applied Biosystem (ABS) 9700 thermocycler. The cycling conditions were as follows; an initial denaturation at 94 °C for 4 min, followed by 30 cycles of final denaturation at 94 °C for 30 s, annealing at 50 °C for 40 s, initial extension at 72 °C for 1 min followed by final extension at 72 °C for 10 min and post hold at 8 °C. The PCR amplicons were visualize in 1.5 % agarose gel electrophoresis. The list of primers used for PCR-amplification is provided in Table 2.

Agarose Gel Electrophoresis

The amplified products were subjected to 1.5% agarose gel electrophoresis pre-stained with ethidium bromide with a 100 bp ladder used as a standard. The electrophoresis was carried out at 100 volts for 35 min. using a BioRadagarose gel electrophoresis unit. The gel was visualized using a U-V trans-eliminator in a BioRad XRS gel documentation device.

RESULTS

In this study, four antimalarial drugs resistant genes were screened via polymerase chain reaction (PCR) protocol and the result is shown in Table 3. It could be seen from the table that *Plasmodium falciparum* multidrug resistant gene (Pfmdr 1) was the highest (43.8%) detected among the pregnant women followed by *Plasmodium falciparum* chloroquine resistant transporter gene (Pfcrt) with 28.1% prevalence. It was evident that *Plasmodium falciparum* dihydrofolate reductase (Pfdhfr) and *Plasmodium falciparum* dihydropteroate synthase (Pfdhps) were not detected in this study.

Table 3: Prevalence of antimalaria drug resistant genes among the pregnant women

Genes	Number Examined	Number Positive	Percentage Positive (%)
Pfdhfr	32	-	-
Pfdhps	32	-	-
Pfcrt	32	9	28.1
Pfmdr 1	32	14	43.8

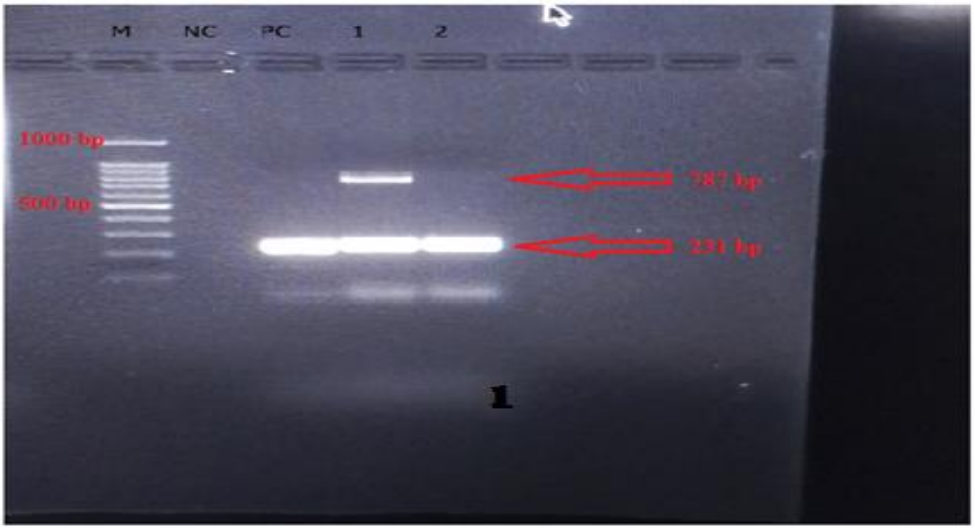

Fig 1: Agarose gel electrophoresis for the detection of *Plasmodium* spp. and House-Keeping genes.
Key:
M: Molecular Ladder, NC: Negative control, PC: Positive control, Lane 1 and Lane 2: *Plasmodium* spp at 787 bp and House-Keeping genes at 237 bp.

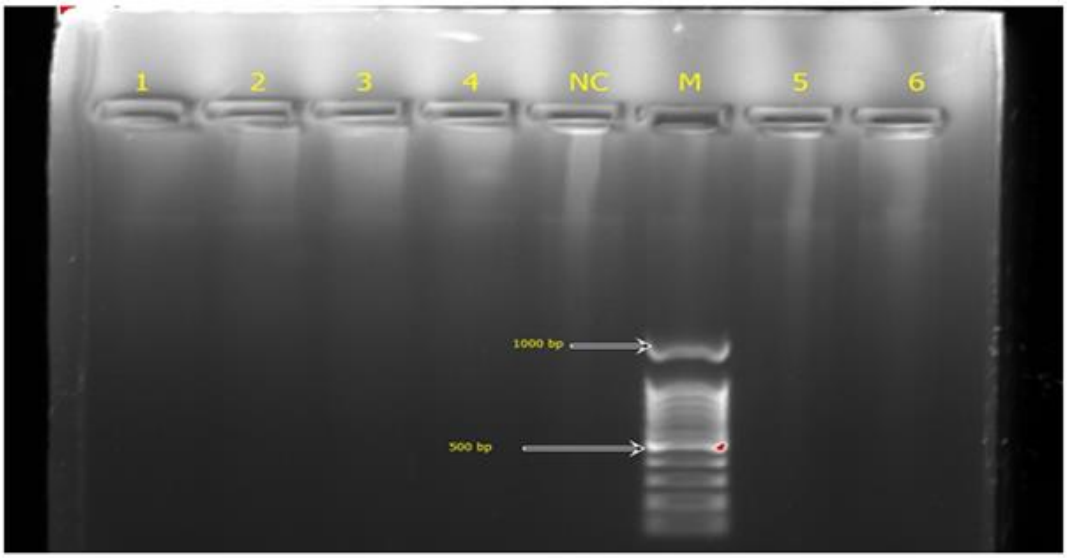

Fig 2: Agarose gel electrophoresis for the detection of Pfdhps gene (Sample 1-6).
Key:
M: Molecular Ladder
NC: Negative Control
Negative Samples: Sample 1-6

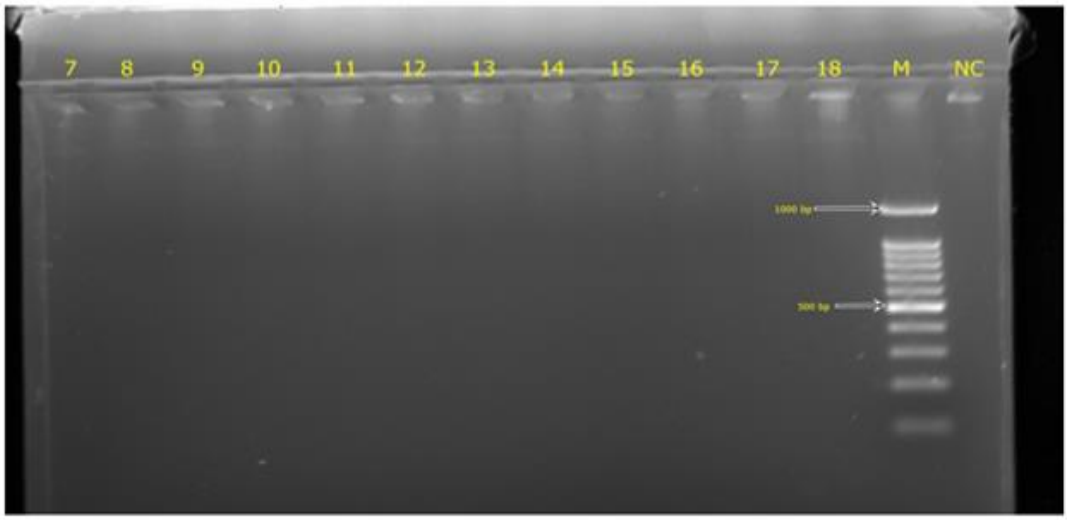

Fig. 3: Agarose gel electrophoresis for the detection of Pfdhps gene (Sample 7-18).
Key:
M: Molecular Ladder
NC: Negative Control
Negative Samples: Sample 7-18

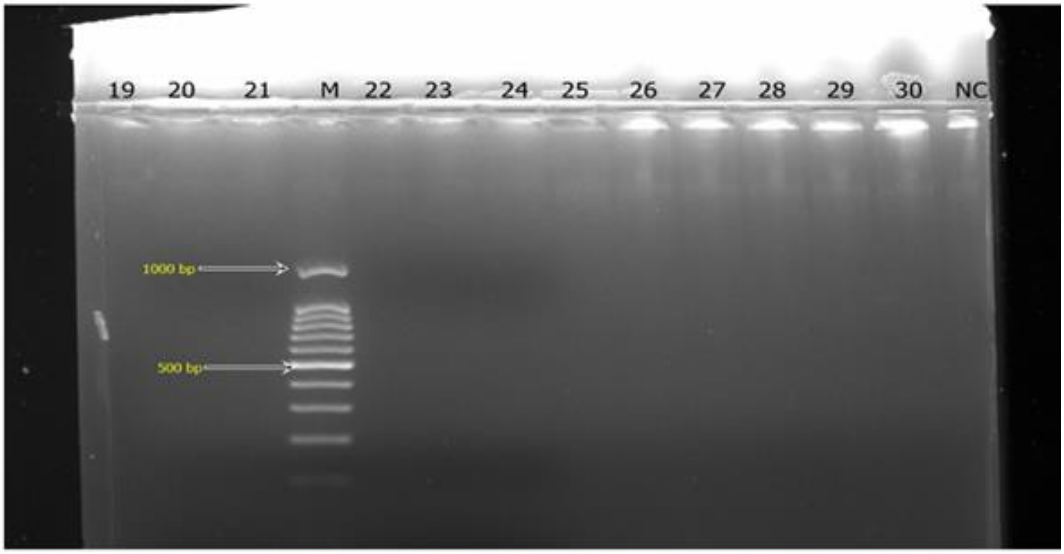

Fig. 4: Agarose gel electrophoresis for the detection of Pfdhps gene (Sample 19-30).
Key:
M: Molecular Ladder
NC: Negative Control
Negative Samples: Sample 19-30

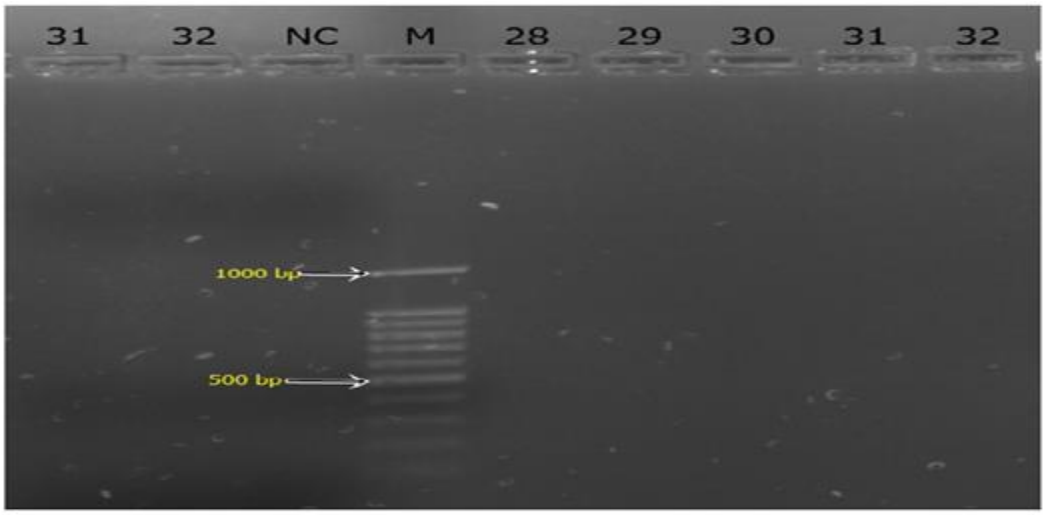

Fig 5: Agarose gel electrophoresis for the detection of Pfdhps and Pfdhfr gene.
Key:
M: Molecular Ladder
NC: Negative Control
Negative Samples: Pfdhps (Sample 31-32) and Pfdhfr (Sample 28-32)

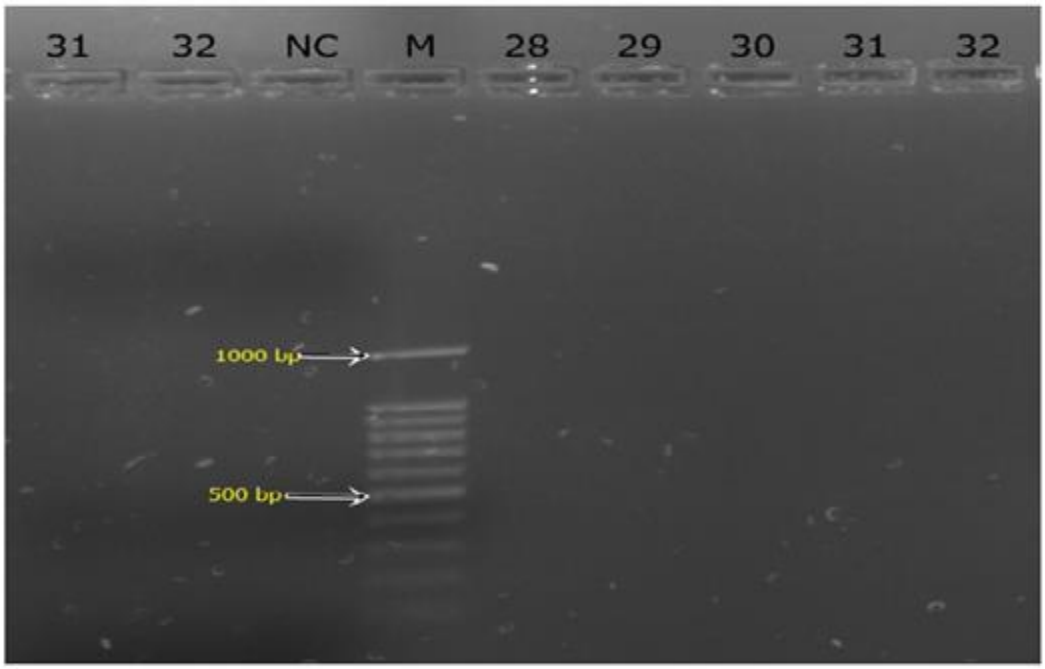

Fig 6: Agarose gel electrophoresis for the detection of Pfdhps and Pfdhfr gene.
Key:
M: Molecular Ladder
NC: Negative Control
Negative Samples: Pfdhps (Sample 31-32) and Pfdhfr (Sample 28-32)

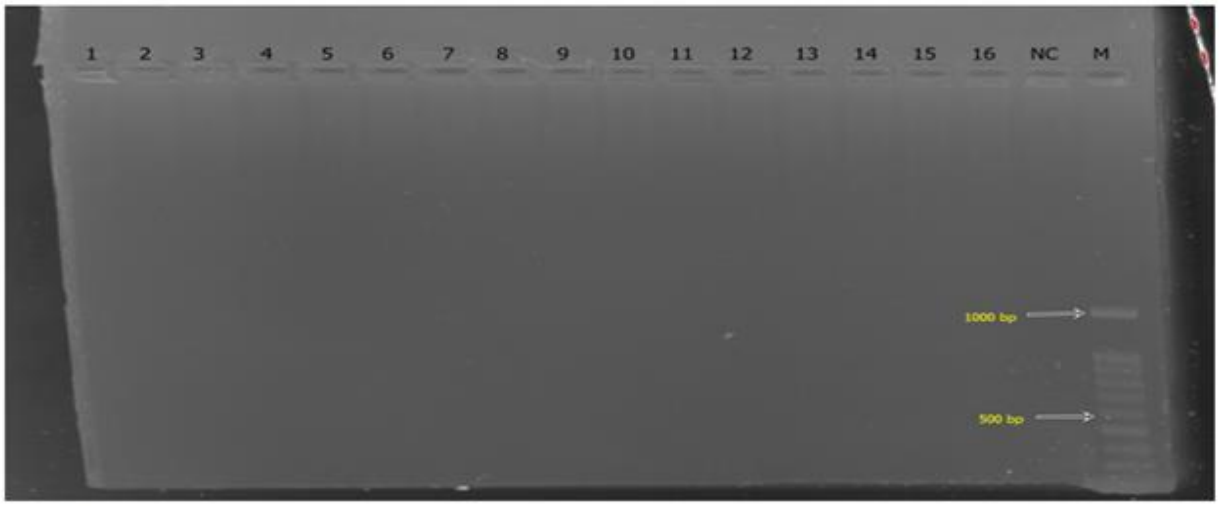

Fig. 7: Agarose gel electrophoresis for the detection of Pfdhfr gene (Sample 1-16).
Key:
M: Molecular Ladder
NC: Negative Control
Negative Samples: Sample 1-16

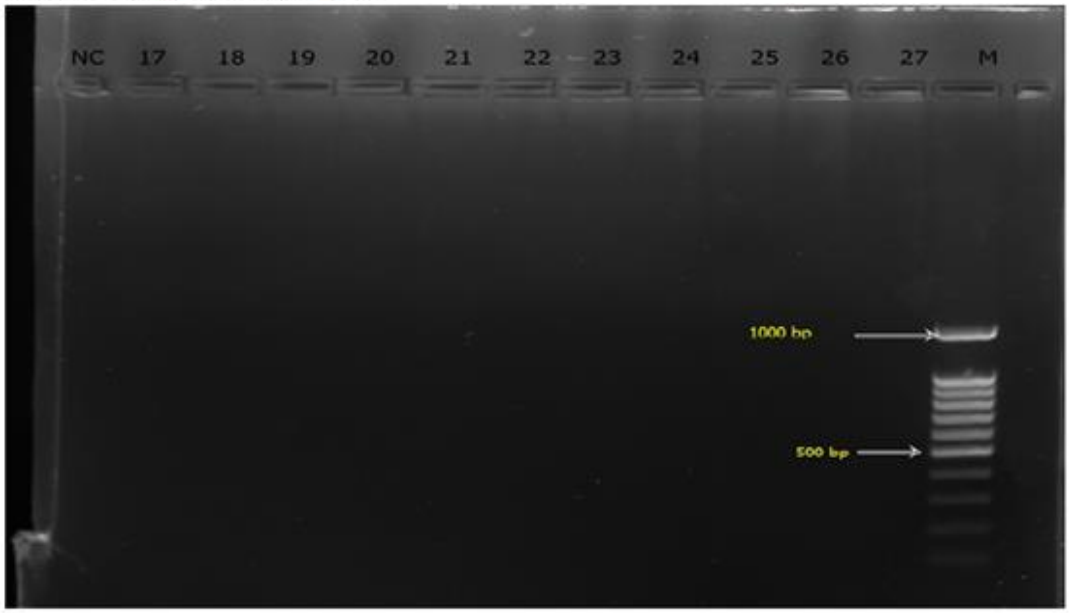

Fig. 8: Agarose gel electrophoresis for the detection of Pfdhfr gene (Sample 17-27).
Key:
M: Molecular Ladder
NC: Negative Control
Negative Samples: Sample 17-27

DISCUSSION

In this study, Pfdhps and Pfdhfr genes were not detected from all the isolates tested. This is the first attempt to detect these genes in the study area. No occurrence of Pfdhps and Pfdhfr genes observed could be attributed to the fact that the pregnant women did not abuse the drugs (sulfadoxine/pyrimethamine) as the drug was not used indiscriminately. The drug was issued to the subjects in the health facilities during antenatal visits. However, 10.3% Pfdhps gene was reported by Fagbemi*et al*. (2020) in Ogun State, Nigeria, and 42.9%Pfdhps gene was reported by Ibekpobaoku*et al*. (2024) in Lagos, Southwestern Nigeria. In the same studies, 28.6% Pfdhfr gene was reported by Ibekpobaoku*et al*. (2024) and 12.8%Pfdhfr gene by Fagbemi*et al*. (2020). Single nucleotide polymorphisms in the *P. falciparum* dihydrofolatereductase (Pfdhfr) and

the *P. falciparum* dihydropteroate synthase (Pfdhps) genes have been associated with resistance to pyrimethamine and sulfadoxine respectively (Ibekpobaoku*et al*., 2024. Detection of the mutant genes (Pfdhps and Pfdhfr) will affect the efficacy of the antimalarial drugs and will therefore have to be withdrawn. On the other hand, absence of the mutant genes will validate the efficacy of the medication in the treatment of malaria.

The study has also provided evidence of absence of *Plasmodium falciparum* dihydropteroate synthase and *Plasmodium falciparum*dihydrofolatereductase gene that confer resistance against sulfadoxine and pyrimethamine respectively. This study has therefore validated the efficacy of sulfadoxine and pyrimethamine in the treatment of malaria among pregnant women in Sokoto metropolis.

CONCLUSION

This research has provided evidence of the absence of single nucleotide polymorphisms in Pfdhps and Pfdhfr genes that confer resistance against sulfadoxine and pyrimethamine respectively. This finding has therefore validated the efficacy of the antimalarial drugs in the treatment of malaria among pregnant women in Sokoto metropolis.

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