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EXPRESSION OF SULPHADOXINE-PYRIMETHAMINE (SP) RESISTANCE MARKERS IN *Plasmodium falciparum* AMONG PREGNANT WOMEN IN OYO STATE: A POTENTIAL THREAT TO MALARIA PREVENTION IN PREGNANCY

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Abstract

Background: The interrelationship between pregnancy, *Plasmodium falciparum* infection and antimalarial drug resistance serve as recipe for adverse outcomes that impact on the mother and the foetus, hence the adoption of intermittent preventive treatment in pregnancy (IPT_P) using sulphadoxine-pyrimethamine (SP). Antifolate drugs have been in wide use in Nigeria and other African countries where high level resistance (through dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) gene mutations) to the drugs has been observed. This study was conducted to determine the expression of genetic markers of resistance to SP in *Plasmodium falciparum* infection among pregnant women in Oyo State.

Materials and Methods: Eighty-two (82) dry blood spots of malaria positive samples on Whatman no 1 filter paper were used for parasite DNA extraction and subsequent detection of

SP-resistance genetic markers using restriction fragment length polymorphism (RFLP) with specific restriction enzymes for *Pfdhfr* and *Pfdhps* mutant genes.

Results: The expression of *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) mutant gene was 92.68% at codon 51 and 59 respectively. Similarly, *Pfdhps* mutant gene was expressed in 95.12% of *P. falciparum* studied at codons 437G and 540E of the parasite studied.

Conclusion: The high prevalence of these 2 major markers of SP-resistance in this study, portends a serious threat to the continued usage of SP in the intermittent preventive treatment in pregnancy (*IPT$_P$*) especially in the study area. It is therefore imperative to intensify efforts at finding more effective alternatives while also ensuring testing before drug use and avoiding self-medication.

Key words: sulphadoxine-pyrimethamine, resistance markers, *Pfdhfr*, *Pfdhps*, *Plasmodium falciparum*

Introduction

It is estimated that, in sub-Saharan Africa, twenty-five million pregnant women are at risk for malaria and according to the World Health Organization (WHO), malaria accounts for 10,000 maternal and 200,000 neonatal deaths per year (1). The most vulnerable groups are incontrovertibly children of age 0 – 5 years and pregnant women in their first and second pregnancy (2). Pregnant women are 3 times more likely to suffer from severe disease as a result of malarial infection compared with their non-pregnant counterparts and have a mortality rate from severe disease that approaches 50% (3, 4). Pregnant women become more susceptible to the infection because their immune response is suppressed by Human Chorionic Gonadotropin (HCG) and Prolactin levels which are high in pregnancy (5). The second trimester appears to bring the highest rate of infection, thereby supporting the need for antepartum care as part of malarial prevention and treatment efforts (6). This was supported by Frank *et al.* (7) who observed in their study that women in their first trimester had 40% parasitaemia compared with 66.7% in the second trimester and 79.2% in the third trimester. In contrast to the foregoing, findings from other studies indicated that the prevalence of

parasitaemia was highest in the first trimester and decreased steadily in the second and third trimesters (8, 9).

Antifolate drugs have been in wide use in Nigeria and other African countries like Equatorial Guinea where high level resistance (through dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) gene mutations) to the drugs has been observed (10). As molecular markers, Pfdhfr and Pfdhps genes are widely used for SP resistance (SPR) surveillance (10). Regular surveillance of molecular markers of SP resistance will provide the required data for an evidenced-based decision in formulating or revising antimalarial treatment or malaria prevention protocols especially in pregnant women to avoid adverse outcomes. Hence, the conception of this study with the aim of determining the expression of sulphadoxine-pyrimethamine (SP) resistance markers in *Plasmodium falciparum* among pregnant women in Oyo State.

Materials and Methods

Study area

This study was conducted at 3 selected hospitals, namely: Adeoyo Maternity Hospital, Ibadan, State Hospital, Oyo and State Hospital, Ogbomoso, in Oyo State, Nigeria. The climatic condition of this state is typically warm with a temperature range of 30-37 °C and has two seasons namely- dry and rainy. The dry season is between October and April whereas the rainy season lasts between May and September. Oyo State is an inland state in south-western Nigeria, with its capital city at Ibadan. It is bounded in the North by Kwara State, East by Osun State, South by Ogun State, West partly by Ogun State and Republic of Benin. The state covers 28,454 km^2 with an estimated population of 5,591,589 (11)

Study Population

This study was conducted among pregnant women attending antenatal clinics in three selected hospitals in Oyo State namely- Adeoyo Maternity Hospital, Ibadan (Oyo South); State Hospital, Oyo (Oyo Central); State Hospital, Ogbomoso (Oyo North). The age of the participants ranged from 16 to 45 years. Pregnant women attending antenatal clinic and those that consented to participate were included in this study. Participants that were non-pregnant, those that refused consent, as well as antenatal patients with underlining illnesses were excluded from participation. A well-structured questionnaire bothering on biodata, socio

demographic characteristics and history of antimalarial treatment/prophylaxis was administered to participants prior to collection of specimen.

Ethical approval

The protocol for this study was approved by the Ethics and Research Committee of the Ministry of Health, Ibadan, Oyo State with Reference number AD 13/479/1106. A written informed consent was endorsed by participants following the detailed explanation about the study.

Specimen collection

Approximately, 4.5 ml of venous blood specimen was collected into ethylene diamine tetraacetic acid (EDTA) container and mixed.

Specimen processing

Initial screening

The initial screening for malaria parasite infection was carried out using Standard Diagnostic Bioline Malaria Antigen P.f./P.v RDT test kit (05FK80) for *Plasmodium falciparum* and *P. vivax* at the three study sites' laboratories according to the manufacturer's instruction (SD Standard Diagnostics, Inc, 2015).

Microscopy

Thick and thin films were made from the blood samples on the same slide (clean grease-free frosted-end) and stained with 10% Giemsa stain as previously described (12). The stained slides were examined microscopically using the oil immersion lens for parasite identification and speciation.

Dry spot sampling

Few drops from each *Plasmodium falciparum* positive blood sample were placed on Whatman's No 1 filter paper, allowed to air-dry and refrigerated in a tight-seal container containing desiccants until used for the expression of SP resistance genetic markers.

Molecular analysis (*pfdhfr* and *pfdhps* resistant genes detection)

DNA extraction, nested PCR and restriction fragment length polymorphism (RFLP) were all carried out at the Biotechnology Research Laboratory of the Biochemistry Department, Federal University of Technology (FUTA), Akure, Ondo State.

DNA extraction:

The *Plasmodium falciparum* trophozoite genomic DNA was extracted from the blotted drops of blood on the Whatman no 1 filter paper using the Quick-DNA^{TM} Miniprep Plus Kit from Zymo Research Corporation (Catalog Nos D4068 & D4069) according to the Manufacturer's instructions. Briefly, the blood sample-spot on each filter paper was cut into small pieces into 1.0 ml eppendorf micro tube and into each tube was added: 95 µl of Nuclease-free water, 95µl of Solid Tissue Buffer and 10µl of Proteinase K. The mixture was properly mixed on vortex mixer for 10 – 15 sec and the micro tubes incubated at 55°C using water bath DK 420 for 3 hr. The mixture was thereafter thoroughly mixed. The tubes were centrifuged using Kendro centrifuge at 12,000 x g for 1 min to remove the insoluble debris while the aqueous supernatant from each tube was transferred into a new clean micro tube. Two volumes of Genomic Binding Buffer were added to the supernatant and mixed thoroughly. The mixture in each tube was transferred to a Zymo-Spin^{TM} IIC-XLR Column in a collection tube and centrifuged 12,000 x g for 1 min. The collection tube was thereafter discarded with the flow through. To the column was added 400µl DNA Pre-Wash Buffer in a new collection tube and centrifuged for 1 min at 12,000 x g. The collection tube was thereafter emptied. Approximately, 700µl g-DNA wash buffer was added and centrifuged again for 1 min at 12,000 x g. The collection tube was emptied and about, 200µl g-DNA wash buffer was added and centrifuged again for 1 min. The collection tube was discarded with the flow through. To elute the DNA, the column was transferred to a new micro tube, 50µl DNA elution buffer added, incubated for 5 min at room temperature and then centrifuged at maximum speed for 1 min. The eluted DNA in each column was quantified to ascertain the purity and concentration of the DNA extracted and then stored at -20^{0}C until used for the PCR.

Primers design and synthesis

The primers were designed and synthesized by Inqaba Biotech of South Africa

***Pfdhfr* gene amplification and digest**

The amplification of the *Pfdhfr* genes was done using Eppendorf Mastercycler AG 22331 Hamburg PCR Machine as previously described (13). The PCR amplification was performed using an optimization template DNA 5µL, 10µM primers (Table 1) each of *Pfdhfr* F (5' – TTAATTTCCCAAGTAAAACTATTAGAGCTTC -3') (Inqaba Biotech) and reverse (5' – AAATTCTTGATAAAACAACGGAACCTTTTA -3') and Taq polymerase PCR master mix

(NEB). The mixture was topped-up to a volume of 30µL with DNAse water. PCR programme was set at 95^{0}C for 5 min, 92^{0}C for 30 sec, 45^{0}C for 45 sec, 72^{0}C for 45 sec, 45 cycles, final extension at 72^{0}C for 3 min, then held at 4^{0}C to halt reaction. In the outer PCR, the primers above were used to amplify the region consisting of 326 base pairs containing cys 59 arg and ser 108 thr.

Nested PCR: Each reaction tube consisted of 10ul of outer PCR product thawed on ice, and Taq polymerase PCR master mix (NEB), 10µM primers (Table 1) each of *Pfdhfr* F1 (5' - GAATGTAATTCCCTAGATATGGAATATT -3') (Inqaba Biotech SA) and *Pfdhfr* M3 (5' – TTAATTTCCCAAGTAAAACTATTAGAGCTTC -3') (Inqaba Biotech, SA) forward and reverse respectively each to a final volume of 30µL. The PCR programme was set at 95^{0}C for 5 min, 92^0 C for 30 sec, 45^{0}C for 30 sec, 72^0 C for 45 sec, 45 cycles, final extension at 72^0 C for 3 min, then held at 4^{0}C to halt reaction. The first set of nested reaction used the primers F-M4 (forward and reverse respectively) amplifying the region of 326 base pairs containing Cys 69 arg and Ser 108 thr while the second set of nested reaction also used primers M3-F1 to amplify the region that consisted of 522 base pairs containing Asn 51 ile and Ser 108 ile (12).

Restriction Fragment Length Polymorphism (RFLP) digests of the *Pfdhfr* gene

The restriction fragment length polymorphism (RFLP) was done as previously described (13) using restriction endonuclease Tsp5091 (New England Biolabs, Beverly MA) for codons 51 and 59 of *Pfdhfr*. Briefly, in 15µL, 1.5µL of 10x buffer 3, 0.15µL of bovine serum antigen, 8µL of amplified DNA, 0.5µL of Tsp5091 restriction endonuclease was added to 9.85µL of nuclease-free PCR water. This was followed by incubation for 14 hr at 50^{0}C with no agitation.

Gel eletrophoresis

Each sample was mixed with 2µL of loading dye and loaded onto a 2.0% agarose gel in TBE buffer. The gel was subsequently run for 35 min at a voltage of 80 volts on gel electrophoresis tank from Biorad and was viewed on the gel/photo-documentation system for analysis of the results.

***Pfdhps* gene amplification and digest**

For the outer PCR reaction, the amplification of the *Pfdhps* gene was done using R1 and R2 (Inqaba Biotech) as forward and reverse primers respectively (Table 2). The PCR amplification was performed using an optimization template DNA 5µL, 10µM of each primers of *Pfdhps* R1R2 (5' –AATTGTGTGATTTGTCCACAA -3') and R2 reverse (5' – AACCTAAACGTGCTGTTCAA -3') (Inqaba, Biotech SA) and Taq polymerase PCR master

mix (NEB). The mixture was topped-up to a volume of 30µL with DNAse water and the samples were then loaded onto the thermocycler set at 95^{0}C for initial denaturation for 3 min. This was followed by denaturation at 92^{0}C for 30 sec, annealing temperature of 50^{0}C for 45 sec, 72^{0}C extension for 1 min. These conditions were repeated for 30 cycles followed by a final extension for 3 min at 72^{0}C, then halted at 4^{0}C. Nested PCR was done using primers K and K1 (Inqaba Biotech) as forward and reverse primers respectively. Each reaction tube consisted of Taq polymerase master mix, 10µL of emplate DNA, 10µM each of the nested primers K (5' –TGCTAGTGTTATAGATATAGGATGAGCATC -3') and K1 (5' – CTATAACGAGGTATTGCATTTAATGCAAGAA -3') to final volume of 30 µL. PCR was the run with the initial denaturation being set at 94^{0}C for 3 min, followed by a denaturation temperature of 94^{0}C for 30 sec, the annealing temperature of 45^{0}C for 1 min and extension at 72^{0}C for 1 min. The steps were repeated for 40 cycles and then followed by a final extension at 72^{0}C for 3 min before halting the reaction at 4^{0}C. The nested PCR products (K-K1 and R1-R2) were digested using AvaII (New England Biolabs, Beverly MA) for *Pfdhps* 437 and 540 respectively following the procedure described above (13).

Restriction Fragment Length Polymorphism of *Pfdhps*

The products of amplification of *Pfdhps* were subjected to enzyme digestion to detect which of these samples had mutations. The digestion was done according to the instructions of the enzyme's supplier. For the target codons 437 and 540, enzymes *Ava II* and *Fokl* were respectively used. For this reaction, 1.5 µL of the NEB buffer, 0.4 µL of the restricted enzyme Ava II, 1.7 µL of nuclease-free water and 11 µL of the PCR product were used. The product was then incubated at 37°C for 10 hr followed by, another incubation at 80°C for 20 min (13).

Gel eletrophoresis

Each sample was mixed with 2µL of loading dye and loaded onto a 2.0% agarose gel in TBE buffer. The gel was subsequently run for 35 min at a voltage of 80 volts on gel electrophoresis tank from Biorad and was viewed on the gel/photo-documentation system for analysis of the results.

Table 1. PCR Primer sequences for amplification of *P. falciparum* dihydrofolate reductase (*pfdhfr*) gene (13)

Primer pairs	Sequence	Cycling conditions

Pfdhfr 1(forward)	5’-TTAATTTCCCAAGTAAAACTATTAGAGCTTC-3’	95^0c for 5 min
Pfdhfr 2(reverse)	5’-AAATTCTTGATAAAACAACGGAACCTTTTA-3’	92^0c for 30 sec
Pfdhfr 3(forward)	5’-GAATGTAATTCCCTAGATATGGAATATT-3’	45^0c for 45 sec
Pfdhfr 4(reverse)	5’-TTAATTTCCCAAGTAAAACTATTAGAGCTTC-3’	72^0c for 45 sec
		72^0c for 3 min
		Hold at 4^0c

Forty-five (45) cycles were done

Table 2 PCR Primer sequences for amplification of *P. falciparum* dihydropteroate synthase (*pfdhps*) gene (13)

Primer pairs	Sequence	Cycling conditions
Pfdhps 1(forward)	5’-AATTGTGTGATTTGTCCACAA-3’	95^0c for 3 min
Pfdhps 2 (reverse)	5’-AACCTAAACGTGCTGTTCAA-3’	92^0c for 30 sec
Pfdhps 3(forward)	5’-TGCTAGTGTTATAGATATAGGATGAGCATC-3’	50^0c for 45 sec
*Pfdhps*4 (reverse)	5’-CTATAACGAGGTATTGCATTTAATGCAAGAA-3’	72^0c for 1 min
		72^0c for 3 min
		Hold at 4^0c

Forty-five (45) cycles were carried out.

Statistical analysis

Mutant gene expression frequency was calculated using a simple percentage. All charts were created using Microsoft Excel**.**

Results

A total of 76 samples across the study sites expressed mutant gene of *Pfdhfr*, with 38, 28 and 10 respectively from Ibadan, Oyo and Ogbomoso while 78 expressed the *Pfdhps* mutant gene with 37 from Ibadan, 33 from Oyo and 8 from Ogbomoso study sites (Table 1). The individual and co-expression of the mutant genes of *Pfdhfr* and *Pfdhps* are as indicated in Figure 1, where 37 co-expressed the mutant genes of *Pfdhfr* and *Pfdhps* at Ibadan study site while the co-expression was 28 and 8 for Oyo and Ogbomoso study sites respectively.

Table 1. Frequency of expression of *Pfdhfr* and *Pfdhps* mutant genes across study sites

	Pfdhfr mutant gene		*Pfdhps* mutant gene	
	E	NE	E	NE
Ibadan	38	2	37	2
Oyo	28	4	33	0
Ogbomoso	10	0	8	2
Total	76	6	78	4

Key

E – Mutant gene expressed

NE – Mutant gene not expressed

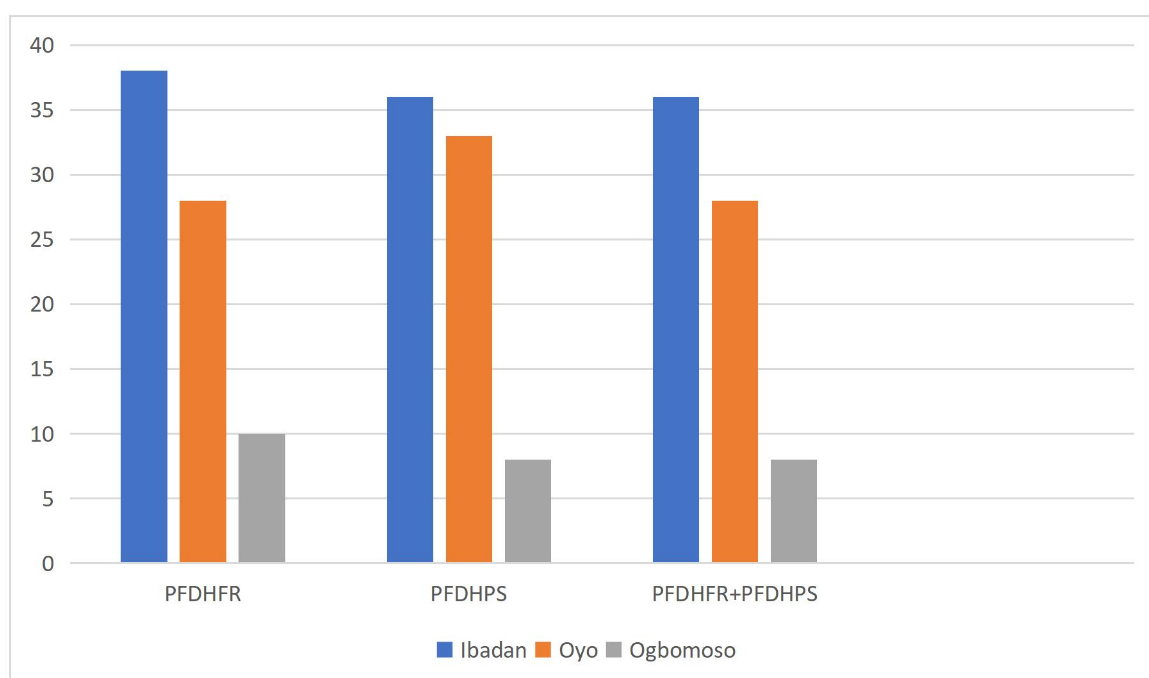


Fig 1. Expression and distribution of *Pfdhfr* and *Pfdhps* mutant genes across study sites

The gel electrophoresis results are as indicated on plates 1-6

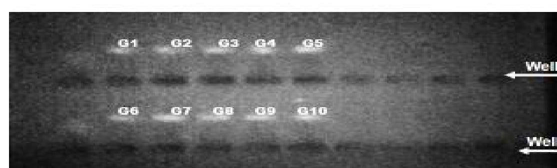
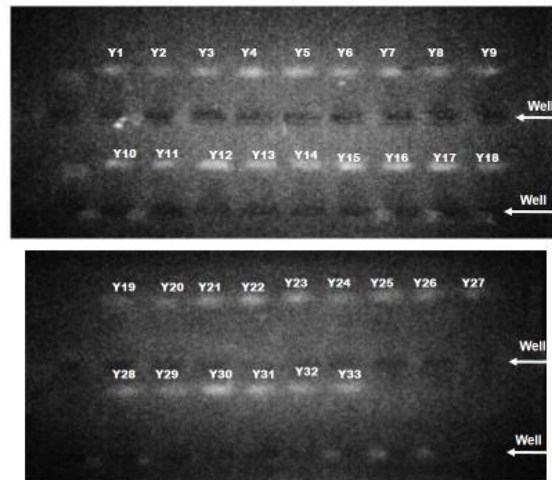
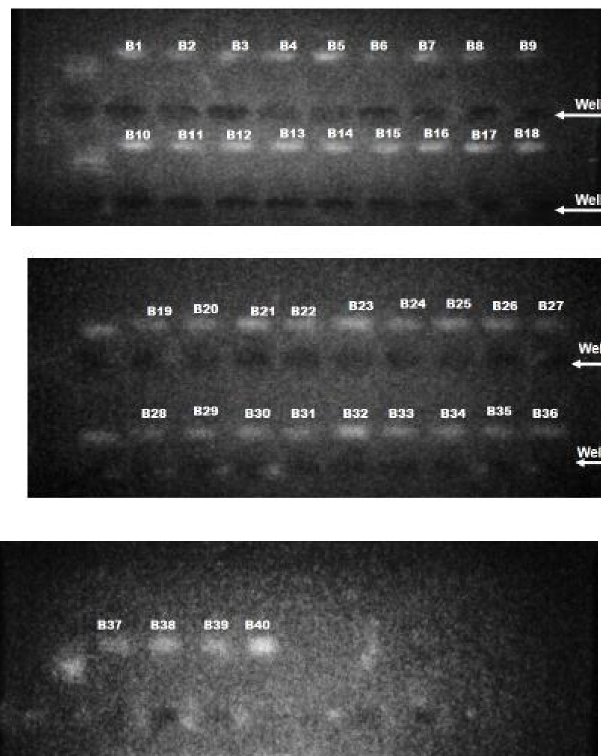


Plate 1: Electrophoresis for *Pfdhfr* mutant gene from Ogbomoso samples coded G1 – G10.



Plates 2-3: Agarose Gel Electrophoresis for *Pfdhfr* mutant gene from Oyo samples coded Y1 – Y33.



Plates 4 – 6: Agarose Gel Electrophoresis for *Pfdhfr* mutant gene from Ibadan samples coded B1 – B40.

Samples B6, B9 (from Ibadan study site), Y2, Y19, Y20 and Y27 (from Oyo study sites) showed none or poor expression of the *Pfdhfr* mutant gene based on the lack of visible bands on the Agar rose gel electrophoresis.

The Gel electrophoresis results for *Pfdhps* mutant gene are shown on plates 7-12. The bands in G5 and G8 (Plate 7) are not visible, which is an indication of non-expression of the mutant gene from Ogbomoso study site.

In the Ibadan study site, the *Pfdhps* gene was not expressed by *P. falciparum* sample with ID B1, B2 and B9 as shown in Plate 8 without any visible bands.

All the 33 *Plasmodium falciparum* samples from pregnant women at the Oyo Study site expressed the *Pfdhps* mutant gene (Plates 11-12)

OGBOMOSO 1-10

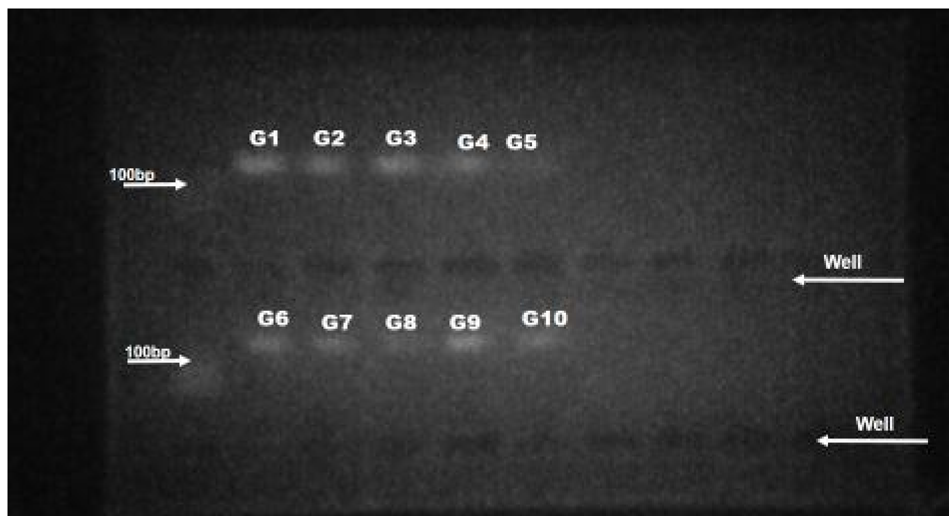
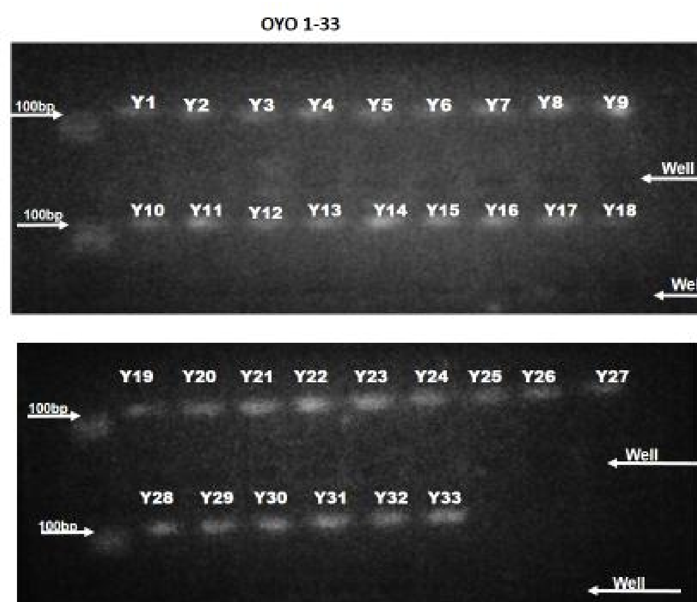


Plate 7: Agarose Gel Electrophoresis for *Pfdhps* mutant gene from Ogbomoso samples G1 – G10.

IBADAN 1-30

IBADAN 31-40

Plates 8 – 10: Agarose Gel Electrophoresis for *Pfdhps* gene from Ibadan samples coded B1 – B40.



Plates 11–12: Agarose Gel Electrophoresis for *Pfdhps* gene from Oyo samples coded Y1 – Y33.

Discussion:

Antifolate drugs has been in wide use in Nigeria and other African countries where high level resistance (through dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) gene mutations) to the drugs has been observed (14, 15, 16). A recent study in Equatorial Guinea revealed that, high level resistance (through dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) gene mutations) to the drugs has been observed (10).

The prevalence of 92.68% of mutant gene expression in *Pfdhfr* observed in our study is substantially in concordance with the report of Ojurongbe *et al.* (17) that observed 90.4% and 98.7% mutation at codon 51 and 59 respectively. Similarly, *Pfdhps* mutant gene was observed

in 95.12% of *P. falciparum* studied. This high frequency of mutations observed in this study is in tandem with the findings of Asua *et al.* (18) that reported high mutations at codons 437G and 540E of the parasite studied in Uganda and Quan *et al.* (19) with a reported prevalence of 95.8%. Our findings in this study are also in concordance with the report of Fagbemi *et al*. (24) where 98% mutation rate was observed in a study conducted in Ogun State, another State in Western Nigeria. This high prevalence of mutation in *Pfdhfr* and *Pfdhps* are a reflection of widespread resistance of *P. falciparum* to antifolate malaria drugs (20) particularly sulphadoxine-pyrimethamine (SP) leading to its earlier abandonment for malaria treatment (21). However, SP remains the standard of care for IPT_p in areas of Africa where malaria is endemic (22) and is equally increasingly being used for seasonal malaria chemoprophylaxis whereby treatment courses of SP plus amodiaquine is provided monthly during the rainy season in parts of west and central Africa (23). However, these high prevalence of resistance markers may be a worrisome concern if the trend continues as it may render the use of sulphadoxine-pyrimethamine (SP) in the intermittent preventive treatment of malaria in pregnancy a nugatory. Concerted efforts are therefore advocated to stem the tide. It is therefore imperative to intensify efforts at finding more effective alternatives while also ensuring testing before drug use and avoiding self-medication. Continuous monitoring and surveillance of molecular markers of SP drug resistant *P. falciparum* is imperative and is hereby advocated.

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