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# Frequency of Merozoite Surface Protein 2 (MSP2) Allelic Families and Sulphadoxine-Pyrimethamine (SP) Resistance Markers Among Pregnant Women in Delta State, Nigeria)

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**Abstract:** Malaria in pregnancy is a public health problem and requires prophylactic treatment with Sulphadoxine–Pyrimethamine (SP) drug. However, the emergence and spread of SP-resistance *P. falciparum* parasite across Nigeria poses serious threat to the efficacy and effectiveness of this preventive intervention. This study aimed to determine the frequency of MSP2 allelic families and SP resistance molecular markers of *P. falciparum* among asymptomatic pregnant women in Asaba, Delta State. Rapid Diagnostic Test (RDT) and Microscopy were used to detect malaria parasite infection among the study participants. Polymerase Chain Reaction (PCR) was used to confirm parasitaemia using *P. falciparum* MSP2 as a marker while Restriction Fragment Length Polymorphism (RFLP) was used to identify *P. falciparum* SP-resistance molecular markers at codons 51, 59, 108, 164 of dihydrofolate reductase (dhfr), and codons 437, 540, 581 and 431 of dihydropteorate synthetase (dhps) genes. The prevalence of malaria from the 410 pregnant women examined at first antenatal registration were 8.29% and 17.07% using RDT and microscopy, respectively ($P < 0.05$). MSP2 alleles of *P. falciparum* were confirmed in 43 (61.43%) cases of the microscopy result. Fourteen different MSP2 fragments of the two major allelic families: 3D7 and FC27 were obtained. The allelic frequencies were 52.9% and 15.7% for 3D7 and FC27, respectively, with overlap in 5 cases ($P < 0.05$). Multiplicity of infection was 1.31. Using RFLP, 47 (67.12%) samples showed polymorphism in at least one codon of *Pf*dhfr and *Pf*dhps genes. In the *Pf*dhfr gene, C59R and N51I mutations showed the highest (78.72%) and the least (23.4%) prevalence, respectively ($P < 0.05$). In the *Pf*dhps gene, A437G and K540E mutations showed the highest (44.68%) and the least (36.17%) prevalence respectively ($P > 0.05$). Single mutation of C59R in 10 (21.28%) cases was the most prevalent haplotype in the *Pf*dhfr/*Pf*dhps genes ($P < 0.05$). Mutation variations of *Pf*dhfr/*Pf*dhps identified were 15 (31.91%) single, 14 (29.79%) double, 13 (27.66%) triple and 5 (10.64%) quadruple with 5, 8, 6 and 3 unique haplotypes, respectively. The study recorded the presence of malaria parasites among the study participants. *P. falciparum SP* resistant genes were also detected and this may present a challenge in the usefulness of Intermittent Preventive Treatment (IPTp-SP) in pregnancy.

**Keywords:** Intermittent Preventive Treatment, Sulphadoxine-Pyrimethamine, *Plasmodium falciparum*, Pregnant Women, Resistance Molecular Markers

# 1. Introduction

Malaria in pregnancy (MIP) is a major health concern in Sub-Saharan African countries. The most vulnerable to malaria are persons with no or little immunity against the disease, as seen in pregnant women. Malaria in pregnancy (MIP) is one of the major causes of maternal anemia, intrauterine growth retardation, preterm birth, low birth weight, as well as maternal and foetal morbidity and mortality. Malaria caused by *P. falciparum* is the most deadly and highly prevalent in Africa [1]. In malaria-endemic areas, *P. falciparum* infections during pregnancy is rarely symptomatic and so undetected and untreated [2]. These resulted to the adoption of intermittent preventive chemotherapy by the World Health Organization (WHO) for all pregnant women in malaria endemic areas regardless of being infected with malaria. Till date, Sulphadoxine–Pyrimethamine (SP) is the drug of choice for intermittent preventive treatment of malaria in pregnancy (IPTp) because of its safety, tolerability, ease of use, ability to clear asymptomatic malaria; and prevents re-infection in pregnant women. Sulphadoxine–Pyrimethamine (SP), an antimalarial agent works by blocking the folate biosynthetic pathway of the parasite, and consequently kills it. Currently, the efficacy of IPTp has been reduced due to the emergence of *P. falciparum* parasites with SP- resistance genetic mutations. Malaria parasites develop resistance to SP through single nucleotide polymorphism (mutation) in dihydrofolate reductase (dhfr) and dihydropteroate synthetase (dhps) genes whose enzymes are targets for Pyrimethamine and Sulphadoxine or other sulpha drugs, respectively [3]. A single-nucleotide polymorphism refers to variation at a specific base position of adjacent nucleotides of a codon which differs from the usual base among members of a species. Malaria parasite gene mutation varies, where a parasite could have single or combination of two (double), three (triple), four (quadruple) or five (quintuple) mutations even more at different sites of the two genes. Since SP is a combination of Sulphadoxine and Pyrimethamine, its resistance is measured by detecting mutations in both dhfr and dhps genes. High SP resistance and treatment failure in West, East and South Africa has been attributable to quintuple mutation i.e. triple mutations at codons 108, 51 and 59 of the dihydrofolate reductase gene (serine at codon 108 being replaced with asparagines (S108N), asparagine at codon 51 by isoleucine (N51I), cysteine at codon 59 by arginine (C59R)) combined with the double mutations at codons 540 and 437 of dihydropteroate synthase gene (Lysine at codon 540 to Glutamic acid (K540E), Alanine at codon 437 to Glycine (A437G) [4]. Molecular monitoring of parasites in humans is a valuable tool for checking the spread of antimalarial resistance and identifying high risk group among pregnant women for possible alternative preventive intervention during pregnancy. In this study, the prevalence of malaria and SP-resistance markers were assessed among pregnant women in Asaba, Delta state.

# 2. Materials and Methods

### *2.1. Study Area*

The study was carried out in Asaba (6.2059° N latitude, 6.6959° E longitude), Oshimili South LGA, Delta State, Southern Nigeria. Asaba, the capital of Delta State, is also the headquarters of Oshimili South LGA; one of the twenty-five Local Government Areas in Delta State. Asaba is located at the Northern end of the State, and has an estimated population of 150,032 (2006 census) with an estimated area of 762 square kilometres. The city of Asaba is strategically located on a hill at the Western edge of the River Niger. The Niger Bridge links Asaba to Onitsha in Anambra State, Nigeria.

### *2.2. Ethical Consideration*

The study protocol was reviewed and approved by the Ethical Committee of Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH) Amaku, Awka. Approval was also obtained from Delta State Hospitals' Board with an introduction letter stating the aim of the study. The Head of Maternity Unit as well as the nurses in charge of the unit were informed. The participants were enlightened on the aim and objectives of the study and their verbal consent obtained.

### *2.3. Study Population and Blood Sample Collection*

The study population included pregnant women that attended General Hospital, Okwe, one of the communities in Asaba, Delta State; from November 2017 to December 2018. The pregnant women were clinically examined on their first antenatal visit for fever before blood collection, with the intent of excluding ones having fever. Fever was defined as an axillary temperature $\geq$ 37.5°C. *P. falciparum* Rapid Diagnostic Test kit and microscopy were employed for determination of malaria parasite. Blood samples of asymptomatic patients were spotted in triplicates on Whatmann 3mm filter papers, air dried and persevered in plastic envelopes for rDNA-PCR and RFLP analysis.

### *2.4. Molecular Analysis for Extraction of Genomic DNA, Genotyping of Malaria Parasites and Assessment of Polymorphism*

Extraction of genomic DNA from individual dried blood spots was carried out using Qiagen Mini Kit (Qiagen, UK). The quality as well as quantity of each DNA sample was determined using nanodrop (Thermo Fisher Scientific, United States of America). The extracted DNA samples were stored at -20°C until further molecular analyses were carried out.

The extracted *P. falciparum* DNA samples were subjected to Polymerase Chain Reaction (PCR) for amplification and detection of Merozoite Surface Protein 2 (MSP2) of the parasite, if present. Presence of *P. falciparum* MSP-2 was confirmed using Agarose Gel electrophoresis for visualizing bands (100bp -500bp) from fragments of the MSP 2 allelic family. The absence of *P. falciparum* DNA indicated a negative result or failed DNA extraction from the dot blood

sample. Results were recorded following the formular by [5].

Also, blood samples positive for malaria parasite by microscopy were subjected to RFLP for detection of mutations on the *Pf*dhfr and *Pf*dhps genes. Primers used were: M1 (5'TTTATGATGGAACAAGTCTGC3') and M5 (5'AGTATATACATCGCTAACAGA3') as forward and reverse primers respectively in primary (nest I) PCR reaction. In the secondary (nest II) PCR reaction, primer pair M3 (5'TTTATGATGGAACAAGTCTGCGACGTT3') and F/ were used as forward and reverse primers respectively to amplify the region around the genes where the point mutation is anticipated to occur.

For the *Pf*dhfr gene, DNA fragments were amplified at codons 51, 59, 108 and 164 while for the *Pf*dhps gene, the DNA fragments were amplified at codons 437, 540, 581 and 431 using codon-specific primers. A DNA sample of the *P. falciparum* clone *Dd2* was used as a control. These genes were digested with a recommended polymorphism-specific restriction enzyme to identify molecular markers of SP-resistance at the codons of *P. falciparum* as described by [6]. Restriction enzymes used were AluI (S108N/T), TSP509I (N51I), Xmnl (C59R) for *Pf*dhfr and AvaII (A437G), FokI (K540E) for *Pf*dhps genes. Digested *P. falciparum* DNA were electrophoresed on 2% agarose gel pre-stained with ethidium bromide and visualized under UV light to size the DNA bands relative to a 100bp DNA ladder and control DNA fragments. Electrophoretic band patterns were categorized as wild type, mixed, or mutant genotypes. The presence of the double, triple, to quintuple mutants was noted. The quintuple mutant is defined as an isolate containing *Pf*dhfr mutations N51I, C59R, S108N, and *Pf*dhps mutations A437G and K540E as reported by [7]. PCR product pairs of *Pf*dhfr and *Pf*dhps were randomly selected and the analyses re-run in order to further confirm the results.

### *2.5. Data Summary and Statistical Analysis*

Data obtained from the study were summarized using a chart and Tables. Test of statistical significance was done at 5% level using SPSS version 25.0. Chi square analysis was used to test the relationship between malaria parasite prevalence and the diagnostic tool used, compare the occurrence and number of mutation at different codons of *Pf*dhfr and *Pf*dhps genes. McNemar test was used to compare the level of occurrence of the allelic family of Merozoite Surface Protein 2 (3D7 and FC27).

## 3. Results

Out of the 410 pregnant women examined using rapid diagnostic test and microscopy, 34 (8.29%) and 70 (17.07%), respectively were positive (Table 1). There was significant difference in the prevalence of malaria using the two diagnostic tools (P = 0.00).

***Table 1.*** *Prevalence of malaria infection amongst pregnant women.*

| Technique | No examined | Positive samples | Negative samples | Prevalence (%) |
|---|---|---|---|---|
| RDT | 410 | 34 | 376 | 8.29 |
| Microscopy | 410 | 70 | 340 | 17.07 |

Out of the 70 asymptomatic pregnant women that recorded positive with microscopy, Merozoite Surface Protein 2 (MSP2) genes of *P. falciparum* were confirmed in 43 (61.43%) cases using PCR-genotyping. Malaria parasites in blood samples genotyped fell within two major allelic families of MSP2: 3D7 and FC27. The 3D7 allelic family was detected in 37 (52.9%) while FC27 was detected in 11 (15.7%) of the microscopy positive samples (P = 0.00). Out of 43 samples with MSP2 gene detected, 5 (11.6%) had both 3D7 and FC27 genes, 6 (14%) had only FC27 gene and 32 (74.4%) had only 3D7 gene (Figure 1). The number of fragments (alleles) detected for 3D7 and FC27 were 6 and 8 respectively while the fragment range fell between 125-400bp and 110-475bp respectively (Table 2). Monoclonal infection was in 69.77% (30/43) of pregnant mothers positive for MSP2 alleles while 30.23% (13/43) were polyclonal. Multiplicity of infection (MOI) is defined as the number of genetically distinct parasite strains co-infecting a single host. MOI calculated from Table 2 was 1.31.

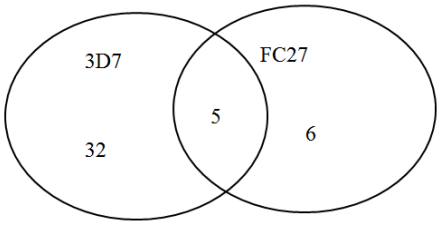


***Figure 1.*** *Distribution of MSP2 families in the microscopy positive samples.*

***Table 2.*** *MSP2 families detected from the blood samples and multiplicity of the infection.*

| Variable | Categories | Number of fragments (alleles) | Fragment range | No of sample positive |
|---|---|---|---|---|
| MSP2 family | 3D7 | 6 | 125-400 | 32 |
| | FC27 | 8 | 110-475 | 6 |
| | 3D7/FC27 | 14 | 110-475 | 5 |
| Multiplicity | Monoclonal | 5 | 125-410 | 30 |
| | Polyclonal | 8 | 125-475 | 13 |

Multiplicity of infection calculated = 1.31.

Restriction Fragment Length Polymorphism (RFLP) detected the *Pf*dhfr and *Pf*dhps genes on 53 (75.71%) out of 70 pregnant women. Polymorphisms were investigated at eight codons: 108, 51, 59 and 164 of *Pf*dhfr genes and 431, 437, 581 and 540 of *Pf*dhps genes of 70 pregnant women. Out of 53 samples, 47 had mutants while 6 had wild type of malaria parasite. Among the 47 samples with mutants, *Pf*dhfr gene had 37 (78.72%) samples with C59R mutation, 16 (34.04%)

samples with S108N mutation and 11 (23.4%) samples with N51I mutation (Table 3). Only two pregnant mothers had mixed mutation at codon C59R while 1 had one mixed mutation at codon N51I (mixed =wild + mutant at a particular codon). Investigations of the *Pf*dhps gene showed that 21 (44.68%) samples had A437G mutation while 17 (36.17%) samples had K540E mutation (Table 3). Five pregnant women had mixed mutation at codon K540E while one had one mixed mutation at codon A437G.

***Table 3.*** *Distribution of different Single Nucleotide Polymorphism (SNP) in the Pfdhfr and Pfdhps genes of P. falciparum found in the blood samples of the pregnant women.*

| Gene | Codon | SNP | Genotype | Frequency |
|---|---|---|---|---|
| *Pf*dhps | 540 | K540E | Wild | 36 |
| | | | Mutant | 12 |
| | | | Mixed | 5 |
| | 437 | A437G | Wild | 32 |
| | | | Mutant | 20 |
| | | | Mixed | 1 |
| *Pf*dhfr | 108 | S108N | Wild | 37 |
| | | | Mutant | 16 |
| | | | Mixed | 0 |
| | 59 | C59R | Wild | 16 |
| | | | Mutant | 35 |
| | | | Mixed | 2 |
| | 51 | N51I | Wild | 42 |
| | | | Mutant | 10 |
| | | | Mixed | 1 |

Table 4 shows the different haplotypes or variations in the mutant genes. In *Pf*dhfr gene, no mutant genotype was detected in five pregnant women. Single mutation at codon N51I, S108N and C59R was found in 2, 3 and 20 pregnant women respectively. There were 2 haplotypes of double mutation in the *Pf*dhfr gene: N51I and C59R occurring in four pregnant women and S108N/T and C59R occurring in 8 pregnant women. Triple mutation involving N51I, S108N/T and C59R were detected in five pregnant women.

In *Pfdhps* gene, no mutant genotype was detected in eighteen pregnant women. The prevalence of double mutant genotype (437+540) was 19.15%. Single mutant at codon 437 was higher, 25.53% than its combination as double mutants genotypes (437 +540) (Table 4). Only twenty four pregnant mothers had mutants for both genes.

***Table 4.*** *Variations in SP-resistance mutation.*

| Gene | Mutation | (SNP) | Frequency |
|---|---|---|---|
| *Pf*dhfr | Single- | N51I | 2 |
| | | S108N/T | 3 |
| | | C59R | 20 |
| | Double- | N51I and S108N/T | 0 |
| | | N51I and C59R | 4 |
| | | S108N/T and C59R | 8 |
| | Triple- | N51I, S108N/T and C59R | 5 |
| *Pf*dhps | Single- | K540E | 8 |
| | | A437G | 12 |
| | Double- | K540E and A437G | 9 |

Combining *Pf*dhfr and *Pf*dhps genes, eight double mutant haplotypes were observed (Table 5); S108N/T & C59R in 3 (6.38%) pregnant mothers, N51I & C59R in 1 (2.13%) pregnant mother of dhfr genes and K540E & A437G in 1 (2.13%) pregnant mother of dhps gene. Then, A437G &C59R (dhps + dhfr in 5 (10.64%) pregnant mothers, K540E & C59R (dhps + dhfr in 1 (2.13%) pregnant mother), K540E & N51I (dhps + dhfr in 1 (2.13%) pregnant mother), A437G & S108N/T (dhps + dhfr in 1 (2.13%) pregnant mother) and A437G & N51I (dhps + dhfr in 1 (2.13%) pregnant mother).

Six triple mutant haplotypes; double mutant in dhfr and single mutant in dhps genes: K540E, S108N/T & C59R in 3 (6.38%) pregnant mothers, A437G, N51I & C59R in 1 (2.13%) pregnant mother, K540E, N51I & C59R in 1 (2.13%) pregnant mother; 2 double mutant in dhps and single mutant in dhfr i.e K540E, A437G & S108N/T in 1 (2.13%) pregnant mother, K540E, A437G& C59R in 4 (8.51%) pregnant mothers were observed and N51I, S108N/T & C59R in 3 (6.38%) pregnant mothers from dhfr gene only. Three quadruple mutant haplotypes were detected (A437G, N51I, S108N/T & C59R in 2 (4.26%) pregnant mothers, K540E, A437G, S108N/T & C59R in 2 (4.26%) pregnant mothers and K540E, A437G, N51I & C59R in 1 (2.13%) pregnant mother. No quintuple Pfdhfr–Pfdhps mutant was detected.

***Table 5.*** *Variation of Pfdhfr-Pfdhps mutations among pregnant women.*

| Mutation | SNP | Gene | Frequency |
|---|---|---|---|
| Single- | N51I | Dhfr | 0 |
| | S108N/T | Dhfr | 1 |
| | C59R | Dhfr | 10 |
| | K540E | Dhps | 2 |
| | A437G | Dhps | 2 |
| Double- | S108N/T & C59R | Dhfr | 3 |
| | N51I & C59R | Dhfr | 1 |
| | K540E & A437G | Dhps | 1 |
| | A437G & C59R | Dhps + Dhfr | 5 |
| | K540E & C59R | Dhps + Dhfr | 1 |
| | K540E & N51I | Dhps + Dhfr | 1 |
| | A437G & S108N/T | Dhps + Dhfr | 1 |
| | A437G & N51I | Dhps + Dhfr | 1 |

| Mutation | SNP | Gene | Frequency |
|---|---|---|---|
| Triple- | N51I, S108N/T & C59R | Dhfr | 3 |
| | K540E, S108N/T & C59R | Dhps+Dhfr+Dhfr | 3 |
| | A437G, N51I & C59R | Dhps+Dhfr+Dh fr | 1 |
| | K540E, A437G&S108N/T | Dhps+Dhps+D hfr | 1 |
| | K540E, N51I & C59R | Dhps+Dhfr+Dhfr | 1 |
| | K540E, A437G& C59R | Dhps+Dhfr+Dhfr | 4 |
| Quardruplet | A437G, N51I, S108N/T & C59R | Dhps+Dhfr+Dhfr +Dhfr | 2 |
| | K540E, A437G, S108N/T & C59R | Dhps+Dhps+Dhfr+Dhfr | 2 |
| | K540E, A437G, N51I & C59R | Dhps+Dhps+Dhfr+Dhfr | 1 |
| Quintuplet | Nil | Nil | - |
| Total | | | 47 |

# 4. Discussion

The study has shown that there could be asymptomatic cases of malaria parasite infection during pregnancy and therefore provides enough justification for administration of IPTp-SP. However, malaria during pregnancy is a problem of major concern due to its effect on the mother and the unborn child [8]. The prevalence of 8.29% and 17.07% were obtained for malaria parasites by rapid diagnostic test and microscopy respectively in consistent with the low prevalence of 6.6% observed in Kwale, Delta state [9] among pregnant mothers. The low prevalence may be attributed to the fact that some of the pregnant women in the study area might have been taking measures to prevent malaria parasite infection. This is in line with the Delta State vision 2020; aimed at providing standard and adequate facilities, infrastructure and human resources to achieve the highest quality of healthcare [10]. It is therefore a good indication of a substantial progress in meeting the vision. Nevertheless, the low prevalence rates are higher than those obtained in Port Harcourt, 4.8% by RDT and 6.2% by microscopy [11]; and 2% recorded in Lagos [12]. It was evident in the study that the prevalence of malaria obtained through microscopy was higher than RDT, and this is in conformity with the results of comparative assessment of microscopy and rapid diagnostic test (RDT) by [13, 14]. Accurate malaria diagnosis has a major role to play in reducing over or under diagnosis, excessive use of anti-malarial drugs, non-compliance to treatment and development of resistant strains of the parasites [15]. Thus microscopy result was used as a yardstick for determining malaria positive cases and should always be adopted for malaria diagnosis in all conditions.

With respect to *P. falciparum* PCR based genotyping using MSP2 as a marker, 3D7 and FC27 were the allele types detected among the 70 asymptomatic pregnant women before the administration of IPTp-SP. The 3D7 allelic family showed significantly higher level of occurrence than the FC27 allelic family. This is similar to the results of previous studies in Burkina Faso, South-East and Western Myanmar and Congo where 3D7 allelic family was the most frequently detected compared to FC27 allelic family [16–19]. From the two allelic families, 14 genotypes or alleles were detected and it shows the level of genetic diversity in *P. falciparum* population within the study area. This study therefore shows lower genetic diversity in *P. falciparum* population compared to the reported in Tanzania with 32 alleles detected [20] and in Equatorial Guinea with 25 alleles detected [21]. Thus there is high effectiveness of control measures and reduced malaria transmission intensity in the study area. This is because, genetic diversity in a given region may reflect the transmission intensity, effectiveness of malaria control measures, and potential emergence of resistant parasites [16, 22, 23]. Individuals with FC27 alleles of malaria parasite had high parasite densities and some studies have suggested that infection with malaria parasites that possess FC27 alleles may be associated with severity of disease [18, 20]. So in the present study, some of the pregnant women might have come up with severe cases if not treated, especially those with dimorphic infections i. e. infection with malaria parasite possessing both 3D7 and FC27 allele types. This may be the reason for administering IPTp-SP whether the pregnant woman shows symptoms of malaria or not.

Monoclonal infection was reported in 69.77% (30/43) of pregnant mothers positive for MSP-2 alleles while 30.23% (13/43) were polyclonal which is in line with the previous studies where monoclonal infection was higher than polyclonal [5, 24]. High proportion of monogenetic infections suggests that genetically diverse parasite strains have been imported from highly endemic regions via extensive human migration events [25]. Polyclonal infection can be the result of independent bites of infected mosquitoes, or a single mosquito bite transmitting a genetically diverse sporozoite inoculum [26]. Moreover, a research in Congo showed there was a significant association between the complexity of infection and polyclonal infections [27]. Multiplicity of infection (MOI) was 1.31 in consistent with the previous studies in South- West Nigeria (1.2), [28]. Parasite genetic diversity and MOI studies have also been found to be useful indicator for monitoring malaria transmission level in the endemic areas [29].

Out of the 70 pregnant mothers infected with *P. falciparum*, RLFP analysis detected the dhfr and dhps genes of malaria parasite in 53 cases, more than the 43 malaria-positive pregnant mothers detected by PCR-genotyping of MSP2 genes. The difference could be attributed to poor loading of the extracted DNA into the well of the electrophoretic tank during MSP2 determination and not necessarily insufficient DNA extraction that could give a negative PCR result [30]. Also, it could be related to the different targets of the two analyses. rDNA-PCR genotyping targets the merozoite surface protein 2 (MSP 2) while RFLP targets the dihydrofolate reductase (dhfr) and dihydropteroate synthase

(dhps) genes of *P. falciparum* parasite. It is also possible the ten samples with dhfr and dhps genes might have *Plasmodium* species other than *P. falciparum*. The detection of parasitaemia and drug resistance markers by RFLP could be for another parasite related to *P. falciparum* with similar resistant markers for SP. Out of the 53 cases, RFLP identified 47 pregnant women with mutant type of malaria parasite.

Competitive facilitation could be the reason for the high frequency of *P. falciparum* with SP resistance molecular markers among the pregnant women. This is because the resistant alleles selected by the use of SP in the large population for treatment of malaria, subsequently remain in the infected pregnant women [31] after the clearance of wild type strains of the parasite by chemotherapy [32]. Nevertheless, the prevalence of mutant alleles (except at codon 59) is lower than that observed in Lagos, Nigeria by [33]. Similar studies in Lagos, Nigeria and Uganda had a higher prevalence than this study [34, 35]. C59R mutation was the most prevalent in the study area, in line with the findings in Burkina Faso [36] and Northern Borno, Nigeria [37] but in contrast with the findings in Equitorial Guinea, Lagos and Yemen where mutation at codon S108N was predominant [33, 38, 39]. There was no polymorphism detected at codon I164V unlike in Uganda, Senegal and Comoros Island where it was detected [35, 40]. The present result therefore is in line with the report that I164L mutation is hardly seen in Africa, and consequently its association with resistance in this continent is doubtful [41].

Accumulation of mutations in *Pf*dhfr gene starts at codon 108, resulting in low levels of pyrimethamine resistance followed by mutations 51 and 59, then, codon 164 which is related to high level of resistance [42]. The reason for higher prevalence of C59R mutation in this study than S108N could be due to an interplay between IPTp-SP and host immunity which plays a role in shaping the parasite drug resistant profile in pregnant women [43]. In other words, *P. falciparum* with C59R mutation has a survival advantage under drug pressure over those with mutations at codons 108 and 51. This might have led to the higher frequency of the C59R mutation [32].

Mutation at codon 437 of *Pf*dhps gene was the most prevalent though with lower frequency of occurrence compared to the reports in Cameroon [44] and in Equitorial Guinea [39]. However it was higher than that found in rural area of Burkina Faso [36] and in Lagos, Nigeria [33]. The frequency of K540E mutation on the other hand was far lower than the rate in Malawi [45] and Uganda [34] but higher than the rate in Lagos [33]. WHO considers the discontinuation of IPTp with SP in case of *P. falciparum* population prevalence of *Pf*dhps 540E >95% [46] with the option of long-lasting insecticide treated nets, prompt diagnosis and effective treatment as replacement. However, the threshold was not met in the study. No mutation was found at codon I431V and it contradicts the report that I431V mutation was widespread throughout Nigeria [47].

Double mutation occurred in *Pf*dhfr gene, with S108N/T and C59R as the most prevalent haplotype. This is the same with studies in Iran [48] and Burkina Faso [36] that recorded double mutations occurring at the same location. However, another study in Uganda showed that double mutation involving codons 51 and 108 was more prevalent than the one involving codons 59 and 108 [34].

The only haplotype responsible for triple mutation in *Pf*dhfr gene was N51I, S108N/T and C59R. Its frequency of occurrence was lesser than that reported in Lagos [33], Cameroon [44], Uganda [34], Kenya [49], Benin [43], and Zambia [50]; higher than the rate in Northern Benin [51]. *Pf*dhfr triple mutation is known to confer intense pyrimethamine resistance in vitro and was found only in five samples, hence, its resistance might not have much impact on the overall prevalence after IPTp-SP.

The only haplotype recorded in *Pf*dhps double mutation was K540E and A437G. Its frequency of occurrence was much lower than in Uganda [34] and Kenya [49]; consistent with data from Zambia [50] and higher than the rate in another study in Zambia [52].

Considering the relationship among the different variations of mutation when the two genes were combined, single mutation was revealed to occur more than double, triple and quadruple mutation. The codon affected most was codon 59. Six variants or haplotype of triple mutation in the *Pf*dhfr and *Pf*dhps genes combined were: N51I, S108N/T & C59R; K540E, S108N/T & C59R; A437G, N51I & C59R; K540E, A437G & S108N/T; K540E, N51I & C59R and K540E, A437G& C59R. According to [53], the combination of triple mutant of *Pf*dhfr- N51I, C59R, S108N and A437G of *Pf*dhps, confers partial resistance; the combination of triple mutant of *Pf*dhfr- N51I, C59R, S108N and double mutant of *Pf*dhps- A437G, K540E confers full resistance to SP drugs, combination of triple mutant, *Pf*dhfr N51I, C59R, S108N and triple mutant, *Pf*dhps A437G, K540E, A581G, confers super resistance. Three variants or haplotype of quadruple mutation in the *Pf*dhfr and *Pf*dhps genes combined were: A437G, N51I, S108N/T & C59R.; K540E, A437G, S108N/T & C59R; K540E, A437G, N51I & C59R. Quadruple mutation of different variants has also been reported; 51+ 59+108 +437 in Uganda [34], 51+59+108N+437G in Ghana [54]; and 436/437+51+59+108 in Gabon [55]. In this study, the predominant quadruplets were 437+ 51 +108+ 59 and 540+ 437 +108+ 59 and therefore categorized as partial resistance.

This study recorded no quintuple mutation even though there are earlier reports of such in some parts of Nigeria and other West African countries at large [33, 34, 49, 50, 52]. The absence of quintuple mutation which might have contributed to reduced SP resistance as the extent of resistance is likely related to the number of mutant genes.

# 5. Conclusion

In conclusion, malaria parasites were found among the asymptomatic pregnant women studied. The prevalence is low yet it is still a problem of major concern because of its effect on the mother and the unborn child. This is because of the high rate of SP-resistance molecular markers detected in the study population. The 3D7 parasite strain was the common genotype circulating in the study area. Based on

classification, the study area had partial SP-resistant genotype of mutants. This might have insignificant or no negative effect on the efficacy of SP. However, the presence of double, triple and quadruple mutations with different haplotypes showed a progressive expansion of SP resistance markers and may develop to full or super resistance in the parasite strains that affect the pregnant women. Thus there is need to monitor the emergence of new genotypes with mutations at codon 436, 431, 581 and 164 which subsequently may present a challenge for the future usefulness of IPTp-SP intervention in pregnancy.

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