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Research Paper

# Analysis of sulphadoxine–pyrimethamine resistance-associated mutations in *Plasmodium falciparum* isolates obtained from asymptomatic pregnant women in Ogun State, Southwest Nigeria

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ABSTRACT

Intermittent preventive treatment in pregnancy with sulphadoxine – pyrimethamine (IPTp-SP) is one of the main strategies for protecting pregnant women, fetus, and their new-born against adverse effects of *P. falciparum* infection. The development of the drug resistance linked to mutations in *P. falciparum* dihydrofolate reductase gene (*pfdhfr*) and *P. falciparum* dihydropteroate synthase gene (*pfdhps)*, is currently threatening the IPTp-SP approach. This study determined the prevalence of *pfdhfr* and *pfdhps* mutations in isolates obtained from pregnant women with asymptomatic *P. falciparum* infection in Nigerian. Additionally, *P. falciparum* genetic diversity and multiplicity of infection (MOI) was assessed by genotyping the *P. falciparum* merozoite surface Protein 1 and 2 (*pfmsp-1* and *pfmsp-2*) genes. The *pfdhfr* and *pfdhps* were genotyped by direct sequencing, and the *pfmsp-1* and *pfmsp-2* fragment analysis by polymerase chain reaction was used to determine *P. falciparum* genetic diversity. Of the 406 pregnant women recruited, 123 had *P. falciparum* infection by PCR, and of these, 52 were successfully genotyped for *pfdhfr* and 42 for *pfdhps* genes. The *pfdhfr* triple-mutant parasites (N51I, C59R, and S108N) or the IRN haplotype were predominant (98%), whereas *pfdhfr* mutations C50R and I164L did not occur. For *pfdhps* gene, the prevalence of A437G, A581G, A436A, and A613S mutations were 98, 71, 55, and 36%, respectively. Nineteen (44%) isolates with quintuple mutations (C**IRN**I- S**G**K**G**A) had the highest combined *pfdhfr-pfdhps* haplotype. Isolates with sextuple mutants; C**IRN**I- **AG**KA**S** and C**IRN**I- **AG**K**G**A had a prevalence of 29 and 14%, respectively. High genetic diversity (7 *pfmsp-1* alleles and 10 *pfmsp-2* alleles) and monoclonal infection rate (76%) was observed. This study demonstrated a continuous high prevalence of *pfdhfr* mutation and an increase in *pfdhps* mutations associated with SP-resistance in southwest Nigeria. Continuous surveillance of IPTp-SP effectiveness and consideration of alternative IPTp strategies is recommended.

## 1. Introduction

Malaria in pregnancy (MiP) is a major global public health burden. In 2018 alone, the World Health Organization (WHO) estimated 11 million MiP cases, and 0.9 million MiP-associated low birthweight deliveries (< 2500 g) globally (WHO, 2019). Africa bears the brunt of this health burden, with most MiP cases reported in West African countries, particularly Nigeria, which accounts for about 25% of the global burden of malaria (WHO, 2019). MiP is largely caused by *Plasmodium falciparum* infections and is usually characterized by

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submicroscopic parasitemias (Walker-Abbey et al., 2005). Manifestation and consequences of MiP vary with transmission intensity and parasite densities, defined by a multiplicity of infection (MOI) (Beck et al., 2001). The clinical consequences include maternal illness and anemia, premature birth, low birth weight delivery, spontaneous abortion, and an increased risk of maternal or infant mortality (Guyatt and Snow, 2004; Takem and D'Alessandro, 2013). The MiP related effects decrease with successive pregnancies, and primigravidae are the most vulnerable group (Beck et al., 2001; Walker-Abbey et al., 2005).

Intermittent preventive treatment in pregnancy with sulphadoxine−pyrimethamine (IPTp-SP) is recommended for the prevention of MiP (WHO, 2019). The IPTp-SP treatment is provided from the early second trimester during a hospital antenatal care visit. It comprises at least three doses of SP given one dose at least a month apart under directly observed therapy (WHO, 2015). SP act by inhibiting two enzymes of *P. falciparum* folate biosynthesis pathway; sulphadoxine inhibits *P. falciparum* dihydrofolate reductase (PfDHFR) and pyrimethamine inhibit *P. falciparum* dihydropteroate synthase gene (PfDHPS) (Nzila et al., 2000). Prior to SP use for IPTp, it was used as a first-line treatment for uncomplicated *P. falciparum* malaria (Ekanem et al., 1990; Nkhoma, 2007). However, its usefulness was short-lived due to widespread clinical failure associated with mutations in the *pfdhfr* and *pfdhps* genes (Basco et al., 1998; Black et al., 1981; Rumans et al., 1979) and was replaced by artemisinin-based combination therapy for uncomplicated malaria in the mid-2000s (Dondorp et al., 2009). Resistance to pyrimethamine and sulphadoxine is associated with mutations in *pfdhfr* S108N and *pfdhps* A437G positions, respectively. The resistance increases with the accumulation of additional mutations, namely C50R, N51I, C59R and I164L in the *pfdhfr* locus, and S436A, K540E, A581G and A613S/T in *pfdhfs* locus (Gregson and Plowe, 2005; Peterson et al., 1988). Despite the high prevalence of SP-resistance, the positive outcome on improved MiP and birth outcome led to its adoption as chemoprophylaxis in MiP across Africa (White, 2005). Outside malaria, IPTp-SP has also been reported to be protective against sexually transmitted infections and reproductive tract infections (Chico et al., 2017). High prevalence of sextuple *P. falciparum* mutants with C**IRN**I-S**GEG**A haplotype has been reported in East Africa, but yet to be reported in West Africa, raising the alarm on the future usefulness of IPTp-SP (Minja et al., 2013; Naidoo and Roper, 2013). Currently, the threshold for continued IPTp-SP use is set at a prevalence of < 95% for *pfdhps* K540E and < 10% for *pfdhps* A581G alongside surveillance of *pfdhps* A437G and *pfdhfr* mutations (WHO, 2013).

Nigeria adopted IPTp-SP as a national strategy in 2005 following the 2002 recommendation of the WHO strategic framework for the control of malaria during pregnancy in Africa (FMOH, 2005). The IPTp-SP intervention is based on the strategy that SP can clear existing parasites (treatment) and as well as prevent new infection (prophylaxis) in pregnant women. The positive impact of IPTp-SP in Nigeria has been reported by many studies to include reduced risk of maternal anemia, prevention of spontaneous abortion, reduced placental parasitemia and improved fetal birth weight (Falade et al., 2007; Igboeli et al., 2017). Currently, a major challenge of this strategy is the rising levels of parasite resistance to SP, making the constant surveillance of SP resistance by monitoring molecular markers imperative. This study, therefore, investigated the prevalence of *pfdhfr* and *pfdhps* mutations in *P. falciparum* isolates obtained from pregnant women presenting for antenatal care in Ogun State, Southwest Nigeria. Also, *P. falciparum* genetic diversity and MOI was assessed.

## 2. Materials and methods

### 2.1. Study site and sample collection

This study was conducted in four hospitals (State hospital Ijebu-Ode, State hospital Abeokuta, General hospital Ijebu-Igbo and General hospital Ifo) in Ogun State, Southwest Nigeria. Ogun State lies in Southwestern Nigeria within the latitudes 6°N and 8°N and longitudes 3°E and 5°E. The State is within the tropical humid climatic zone of Nigeria, which is characterized by high rainfall and high relative humidity (Solanke, 2015). The State experiences malaria transmission all-year-round with peak transmission during the rainy season (March – October) owing to the optimal mosquito breeding environment offered by a tropical rain forest. Since 2005, IPTp-SP is provided during antenatal care in Nigeria from the second trimester, at least three SP doses one month apart, following the national and WHO guidelines for malaria control in pregnancy (FMOH, 2005). The sample size was obtained, using the formula for a cross-sectional study (Charan and Biswas, 2013). Using a prior prevalence of 41% for the proportion of pregnant women positive for *P. falciparum* (Babalola et al., 2017), a marginal error of 5% and a type 1 error of 5%, a minimum sample of 372 pregnant women was required. In all, a total of 406 afebrile pregnant women (age range 18–45 years) under IPTp-SP at the state government hospitals in four communities in Ogun State Nigeria were recruited between June 2018 and August 2019. The women showed no clinical signs of malaria, as they are just in the clinic for a routine antenatal visit.

Questions on gravidae, insecticide-treated bednet usage, and general health were asked, and responses recorded. About 2 ml of blood was drawn aseptically from the antecubital vein into EDTA bottle from the consenting afebrile pregnant women. The blood sample were utilized for malaria rapid diagnostic test (RDT; SD Bioline™, Standard Diagnostics Inc., Korea) and measurement of hemoglobin (Hb) level (Automated hemoglobin analyzer, Sysmex, USA). Additionally, dried blood spots on Whatman 3MM filter paper were prepared for molecular analyses by spotting about 100 µl whole blood.

### 2.2. Molecular analyses

Total genomic DNA (gDNA) was extracted from dried blood spots using the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Nested PCR was used to amplify four distinct *Plasmodium* genes, namely 18S ribosomal RNA (rRNA) for malaria diagnosis, *P. falciparum* merozoite surface proteins (*pfmsp-1* and *pfmsp-2*) for determining *P. falciparum* MOI, and *P. falciparum pfdhfr* and *pfdhps* for genotyping *P. falciparum* resistance to SP. For the *Plasmodium* genus PCR, the primer sequences were as previously described (Snounou et al., 1993). The reaction, master mix was prepared as follows: 5 µl of total genomic DNA was added to PCR master mix containing 1× PCR buffer, 0.2 mM dNTPs, 100 nM of each primer, 1 unit of *Taq* DNA polymerase in a volume of 20 µl. The nested PCR (*Plasmodium* species−specific PCR) was conducted using 1 µl of outer PCR amplicon as a template under the same conditions as the outer PCR except for the annealing temperature (58 °C). Amplicons were separated using 2% agarose gel electrophoresis.

The genetic diversity of *P. falciparum* was determined by amplifying *pfmsp-1* (K1, RO33 and MAD20) and *pfmsp-2* (FC27 and 3D7*)* allelic families using a nested PCR with the previously described primers (Snounou et al., 1999) and condition (Funwei et al., 2018). The master mix for the outer PCR was prepared as follows; 3 µl of genomic DNA was added into a master mix containing 1× PCR buffer, 0.2 mM dNTPs, 100 nM of each primer, 1 unit of *Taq* DNA polymerase in a volume of 20 µl. Secondary PCR was performed using 1µl of the outer PCR product as a template under the same master mix concentrations. The outer *pfmsp-1* PCR conditions were: 94 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The family-specific nested PCR followed the outer PCR conditions except for the annealing temperature, which was 61 °C for 30 s. For *pfmsp-2* the outer PCR was performed using the following cycling condition: initial denaturation step at 94 °C for 5 min followed by 30 cycles of 30 s at 94 °C, annealing step was carried out for1min at 48 °C and the extension step for 1 min at 72 °C. The final extension step was carried out 72 °C for 3 min. The family-specific nested PCR was

carried out using the same PCR conditions as the outer PCR except for the annealing temperature that was carried out for 1 min at 61 °C. The sizes of the amplicons were determined relative to the 100 bp DNA ladder in a 2% agarose gel. The MOI was defined as the largest number of alleles at each locus, and a single infection was defined by the amplification with only one allele for all of the genotyped loci (Mohd Abd Razak et al., 2016).

Sanger sequencing was employed for the genotyping of *pfdhfr* (C50R, N51I, C59R, S108N and I164L) and *pfdhps* (S436A, A437G, K540E, A581G and A613S/T) mutations. Distinct PCR amplicons were synthesized using previously published nested PCR primers for *pfdhps* (Wang et al., 1995) and semi-nested PCR for *pfdhfr* (Ojurongbe et al., 2011). For the outer PCR, 5 µl of genomic DNA was added into a master mix containing 1× PCR buffer, 0.2 mM dNTPs, 10 µM of each primer, 1 unit of *Taq* DNA polymerase in a total volume of 20 µl. Three microliters of the first PCR product was used as a template for the nested PCR reaction using the same PCR master mix concentration. Thermocycling conditions for the outer and the nested PCR for *pfdhps* are as follows: 95 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, annealing at 52 °C for 1 min and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 10 mins. The same thermocycling conditions were used for *pfdhfr,* except the annealing temperature was set at 61 °C. Amplification was confirmed using 2% agarose gel electrophoresis. The *pfdhps* nested PCR products were sequenced in both forward and reversed direction with primers 153_F and 165_R (Wang et al., 1995), respectively, while the *pfdhfr* nested PCR products were sequenced with Dhfr_F2 primer (Ojurongbe et al., 2011) in the forward direction only. Nucleic acid base calling was done using Geneious software (https://www.geneious.com/), with *P. falciparum* 3D7 *Pfdhps* (PF3D7_0810800) and *Pfdhfr* (PF3D7_0417200) serving as a reference.

### 2.3. Statistical analyses

Data were entered and analyzed using Microsoft Excel 2010 and IBM SPSS statistics 21 (Chicago USA). Descriptive statistics was used to summarize data (frequencies, mean, and median), while chi square ($\chi^2$) was utilized for comparison of proportions. Two-tailed statistical significance was set at $p$ − value < 0.05. The detection of one *pfmsp-1* and *pfmsp-2* allele was considered as one parasite genotype. Monoclonal infections were defined as the amplification of only one allele in all loci (*pfmsp-1* and *pfmsp-2*) while polyclonal infections were defined by amplification of > 1 allele for at least one loci.

### 2.4. Ethics

The human protocol described in this study was carried out following the 1964 Helsinki declaration. Ethical approval for the study was obtained from the ethical review committees of Ogun State Ministry of Health Hospital Management Board, Abeokuta, Nigeria (SHB/2427/45). Written informed consent was obtained from every participant before being recruited into the study.

## 3. Results

### 3.1. Participant baseline characteristics

A total of 406 afebrile pregnant women were recruited into this study, with 202 from Ijebu-Ode, 103 from Abeokuta, 59 from Ijebu Igbo and 42 from Ifo. Table 1 shows the breakdown of the general characteristics and *P. falciparum* positivity of the study population. The mean age of participants from Ijebu-Ode (29.1 ± 5.3) was the highest, but the difference was not statistically significant. The majority of participants (61%) were multigravid (246/406), but the proportion was not statistically significant ($p$ = 0.057*)* among the study sites. The mean hemoglobin (Hb) level was significantly different among the study sites ($p$ < 0.0001), with participants from Abeokuta having a mean Hb level (10.3 ± 1.5) below the threshold (Hb ≤10.9 g/dl) defining anemia in pregnancy. The use of insecticide-treated bednets was significantly different among the study sites ($p$ = 0.0001) with Abeokuta (58%) recording the lowest usage. The highest prevalence of *P. falciparum* infection was observed in Abeokuta for both PCR (34%) and RDT (19%; $p$ = 0.0001) detection methods (Table 1). A total of 148 (36%) (*P. falciparum* 123; *P. ovale* 18; *P. malariae* 7) study participants were infected by a single parasite species whereas 16 (4%) (*P. falciparum* − *P. malariae* 3; *P. falciparum* − *P. ovale* 13) participants had co-infections (Fig. 1).

### 3.2. P. falciparum multiplicity of infections

A subset of *P. falciparum* parasites isolated from *P. falciparum* mono-infections identified in this study was successfully genotyped using two genes; *pfmsp-1* (37/123; 30.1%) and *pfmsp-2* (47/123; 38%). For *pfmsp-1*, K1 allelic family was the predominant and most diverse in all the study sites except in Ifo where MAD20 was predominant (Table 2). The R033 allelic family did not occur in Ifo. Among *Pfmsp-1* allelic families. K1 family had the highest number of alleles (4 alleles; 150–300 bp), followed by RO33 family (2 alleles; 150 and 200 bp) and MAD20 (1 allele; 200 bp). Low polyclonal infection of *pfmsp-1* was observed in Ijebu-ode and Abeokuta and none in Ijebu-Igbo and Ifo. The mean MOI ranged from 1.0 to 1.3. For *pfmsp-2*, the 3D7 was the predominant and most polymorphic allelic family with a total of 8 distinct alleles (300–700 bp) in Abeokuta (73%) and Ijebu-Igbo (82%), and there was a significant difference in the distribution ($p$ = 0.009). On the other hand, the FC27 was predominantly more and more polymorphic in Ijebu-ode (38%) and Ifo (80%) with 3 distinct alleles (400–600 bp) and the distribution was significantly different ($p$ = 0.002) (Table 2). High polyclonal *pfmsp-2* infection was recorded in Abeokuta (31%). All of the participants from Ifo region had *P. falciparum* monoclonal infections.

### 3.3. Pfdhfr and Pfdhps genotypes

A high prevalence of SP resistance alleles were observed in this study among the 52 *pfdhfr* and 42 *pfdhps* genotyped isolates. The *pfdhfr* mutation was characterized by *the* presence of three mutations (> 98%) and the complete absence of two mutations (C50R and I164L) in all isolates analyzed (Fig. 2). The prevalence of *pfdhfr* mutations observed were 100% for both N51I, S108N, and 98% for C59R. For *pfdhps*, four distinct mutations were observed, and these include the *Pfdhps* A437G (98%) with the highest occurrence, A581G (71%), S436A (55%) and A613S/T (36%). K540E was not found in the study population (Fig. 2).

### 3.4. Pfdhfr and Pfdhps haplotypes

Overall, a total of fifteen haplotypes were identified in this study. Two haplotypes were present in *pfdhfr*, five haplotypes occurred in *Pfdhps* and the remaining six occurred in *pfdhfr-pfdhps* combination (Table 3). Interestingly, the *pfdhfr* and the *pfdhps* wild type haplotypes were not detected in this population. The *pfdhfr* triple mutations (C**IRN**I positions 51, 59 and 108) was detected in the 98% of the samples. The S**G**K**G**A double mutation was the predominant (44%) *pfdhps* haplotype followed by the triple mutants **AG**KA**S** (28%) and **AG**K**G**A (14%). The quadruple *pfdhps* mutant (**AG**K**GS**) was observed at a frequency of 9% in the parasite population. The *pfdhfr-pfdhps* combined haplotypes occurred as quadruple, quintuple, sextuple, and septuple mutants at frequencies ranging from 44% to 2% (Table 3).

## 4. Discussion

The IPTp-SP is one of the main strategies in the war against malaria in pregnancy and improving birth outcomes, particularly in sub-Saharan Africa. Nevertheless, persistent circulation of SP-resistant (C**IRN**I-S**GEG**A) *P. falciparum* isolates and the imminent emergence of

**Table 1**
Characteristics and malaria positivity of the pregnant women enrolled in the study.

| Characteristics | Ijebu-Ode | Abeokuta | Ijebu-Igbo | Ifo | Total | *p*-value |
|---|---|---|---|---|---|---|
| Number enrolled | 202 | 103 | 59 | 42 | 406 | |
| Mean Age ± SD (years) | 29.1 ± 5.3 | 28.6 ± 5.8 | 28.2 ± 4.5 | 28.8 ± 5.9 | | 0.690 |
| Mean Hb ± SD (g/dl) | 11.0 ± 1.2 | 10.3 ± 1.5 | 11.0 ± 1.0 | 11.1 ± 1.2 | | 0.0001* |
| Primigravid | 92 (46%) | 37 (36%) | 20 (34%) | 11 (26%) | 160 (39%) | |
| Multigravid | 110 (54%) | 66 (64%) | 39 (66%) | 31 (74%) | 246 (61%) | 0. 057 |
| Bednet Use | 171 (85%) | 60 (58%) | 44 (75%) | 34 (81%) | 309 (76%) | 0.0001* |
| PCR Positive | 58 (29%) | 35 (34%) | 15 (25%) | 15 (36%) | 123 (30%) | 0.54 |
| RDT Positive | 13 (6%) | 20 (19%) | 1 (2%) | 2 (5%) | 36 (9%) | 0.0001* |

* Statistical significance at $p < 0.05$ and df = 3.

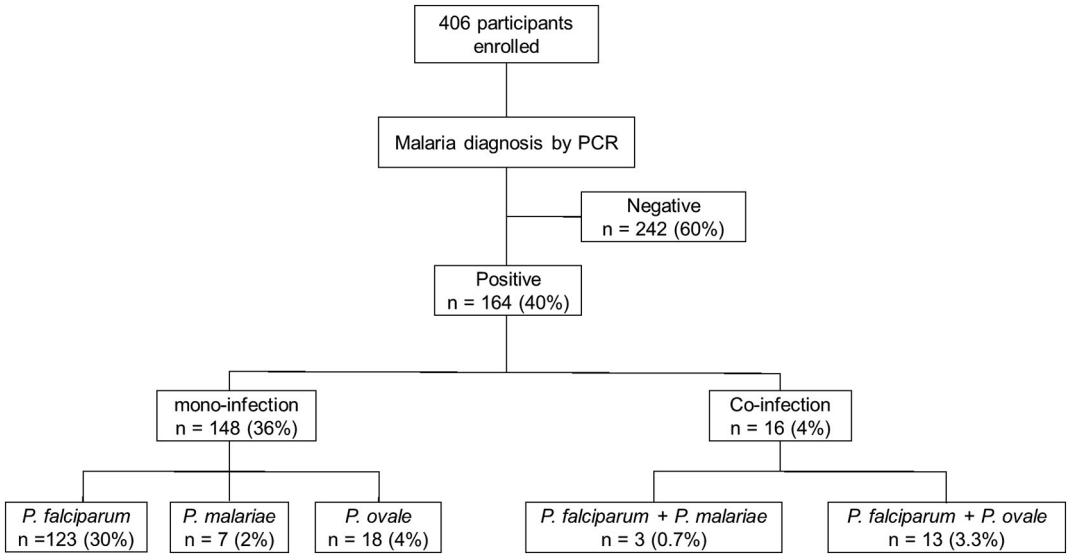


**Fig. 1.** Malaria diagnosis by nested PCR targeting the *Plasmodium* 18S ribosomal RNA gene.

**Table 2**
Distribution of merozoite surface proteins (*msp*-1 and 2) allelic families among *P. falciparum* isolates from Nigeria.

| | Study site | | | | | | |
|---|---|---|---|---|---|---|---|
| Genotype | Ijebu-Ode n (%) | Abeokuta n (%) | Ijebu-Igbo n (%) | Ifo n (%) | Allelic size (bp) | $\chi 2$ | *p-value* |
| *MSP-1* | | | | | | | |
| K1 | 4 (50) | 9 (53) | 5 (56) | 1 (33) | 150–300 | 0.17 | 0.982 |
| MAD20 | 1 (12.5) | 3 (18) | 2 (22) | 2 (67) | 200 | NC | NC |
| RO33 | 1 (12.5) | 3 (18) | 2.(22) | 0 | 150–200 | NC | NC |
| K1 + MAD20 | 1 (12.5) | 1 (6) | 0 | 0 | | NC | NC |
| K1 + R033 | 0 | 1 (6) | 0 | 0 | | NC | NC |
| K1 + MAD20 + R033 | 1 (12.5) | 0 | 0 | 0 | | NC | NC |
| *Mean MOI ± SD* | *1.3 ± 0.65* | *1.1 ± 0.35* | *1* | *1* | | NC | NC |
| *MSP-2* | | | | | | | |
| 3D7 | 5 (31) | 11 (73) | 9 (82) | 1 (20) | 300–700 | 11.37 | 0.009* |
| FC27 | 6 (38) | 3 (20) | 1 (9) | 4 (80) | 400–600 | 9.42 | 0.002* |
| 3D7 + FC27 | 5 (31) | 1 (7) | 1 (9) | 0 | | | |
| *Mean MOI ± SD* | *2.1 ± 1.8* | *1.3 ± 0.45* | *1.1 ± 0.30* | *1* | | | |

Number of samples genotyped for *msp-1* per study site: Ijebu-Ode ($n = 8$); Abeokuta ($n = 17$); Ijebu-Igbo ($n = 9$); and Ifon ($n = 3$).
Number of samples genotyped for *msp-2* per study site: Ijebu-Ode ($n = 16$); Abeokuta ($n = 15$); Ijebu-Igbo $n = 11$); and Ifon (n = 5).
MOI: multiplicity of infections. *Statistical significance at $p < 0.05$ and df = 3; NC: Not calculated because the values in all the cells are < 5.

and/or spread of super-SP resistant isolates threaten the gains attained thus far (Naidoo and Roper, 2013). This has underscored the need for continuous monitoring of molecular markers of antimalarial drug resistance in malaria endemic countries with the view of tracking the distribution pattern of the resistant genes and haplotypes that may compromise the drug-dependent interventions with the view of initiating prompt identification and control. This study determined the prevalence of SP resistance markers and genetic diversity of *P. falciparum* isolates among pregnant women with asymptomatic malaria who presented for antenatal care in Ogun State, Southwest Nigeria.

The distribution of *pfdhfr* and *pfdhps* mutations across Africa is quite heterogeneous with some mutations being confined in some regions (Pearce et al., 2009; Xu et al., 2019). High prevalence of the three distinct *pfdhfr* mutations (N51I, C59R, and S108N) observed in this study concurs with previous reports from Nigeria (Oguike et al., 2016; Ojurongbe et al., 2011). Of the three *pfdhfr* mutations commonly

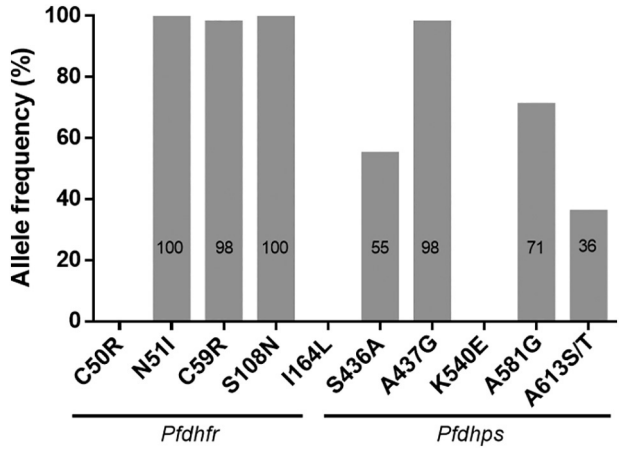


**Fig. 2.** Occurrence of *Pfdhfr* and *Pfdhps* mutations among *P. falciparum* isolates from Ogun State, Nigeria.

**Table 3**
Occurrence of *Pfdhfr* and *Pfdhps* haplotypes among *P. falciparum* isolates from Nigeria.

| Gene | Genotype | Haplotype | Occurrence n (%) |
|---|---|---|---|
| *Pfdhfr* (*n* = 52) | Wild type | CNCSI | 0 |
| | Double Mutant | C**I**C**N**I | 1 (2) |
| | Triple mutant | C**IRN**I | 51 (98) |
| *Pfdhps* (*n* = 43) | Wild type | SAKAA | 0 |
| | Single Mutant | S**G**KAA | 1(2) |
| | Double mutant | S**G**K**G**A | 19 (44) |
| | | **A**AK**G**A | 1(2) |
| | Triple mutant | **AG**KA**S** | 12 (28) |
| | | **AG**K**G**A | 6 (14) |
| | Quadruple mutant | **AG**K**GS** | 4 (9) |
| *Pfdhfr-Pfdhps* (n = 43) | Quadruple mutant | C**IRN**I- S**G**KAA | 1 (2) |
| | Quintuple mutant | C**IRN**I- S**G**K**G**A | 19 (44) |
| | | C**IRN**I-**A**AK**G**A | 1(2) |
| | Sextuple mutant | C**IRN**I- **AG**KA**S** | 12 (29) |
| | | C**IRN**I- **AG**K**G**A | 6 (14) |
| | Septuple mutant | C**IRN**I- **AG**K**GS** | 4 (9) |

Bold underline: mutant *Pfdhfr* or *Pfdhps* mutation.

referred to as *pfdhfr* triple mutants, S108N is the key mutation that initiates the low-level pyrimethamine resistance (Cowman et al., 1988; Peterson et al., 1988) and the co-occurrence of N51I and C59R synergistically increases the resistance (Reeder et al., 1996). Our study showed high *pfdhfr* triple mutation prevalence (> 98%) similar to the previous observation in southern Nigeria, where the prevalence of these mutations has been reported to be high (Oguike et al., 2016; Ojurongbe et al., 2018). The near fixation of the triple mutation (N51I, C59R, and S108N or IRN haplotype) in this study suggests persistent selection pressure of pyrimethamine-resistant parasites, possibly due to continued use of SP in the frame of IPTp or treatment of uncomplicated malaria. Also, the widespread use of daily cotrimoxazole prophylaxis, an antifolate drug in HIV-infected individuals in the country may contribute to the observed high tripple *pfdhfr* mutation (Flateau et al., 2011). The complete absence of I164L in this study though offers some reprieve since the addition of I164L to the *pfdhfr* triple mutation creates a quadruple-mutant allele that is known to aggravate pyrimethamine resistance that originated from Southeast Asia and South America (Plowe et al., 1997; Roper et al., 2004). The I164L mutation has now been reported in many East African countries (Lynch et al., 2017; Ménard et al., 2008; van Lenthe et al., 2019) and imported cases from West and Central Africa in China (Zhao et al., 2020), but till date not in Nigeria.

The five *pfdhps* mutations investigated exhibited relatively low prevalence except for A437G, A581G and S436A, in addition to the complete absence of K540E. The A437G was identified in 98% of the isolates analyzed in-line with previous reports showing high A437G prevalence in most African countries (Naidoo and Roper, 2013; Nkoli Mandoko et al., 2018; Osarfo et al., 2018; Pearce et al., 2009; Triglia et al., 1998) with few reporting otherwise (Apinjoh et al., 2017). This mutation is the principal mutation responsible for sulphadoxine resistance, and as such sequential accumulation of other mutation(s) at other *pfdhps* positions exacerbate the resistance (Triglia et al., 1998, 1997; Wernsdorfer and Noedl, 2003). The A437G mutation is believed to have emerged first in Southeast Asia and spread to other malaria-endemic regions (Wernsdorfer and Noedl, 2003), and this explains its predominance in this and other settings. Double *pfdhps* mutation at positions A437G and K540E reduce *P. falciparum* susceptibility to sulphadoxine by up to 200 folds relative to A437G, whereas A437G alone increases the resistance by 10 fold relative to wildtype parasites (Triglia et al., 1997). The occurrence of both A437G and K540E plus A581G further increases sulphadoxine resistance (Minja et al., 2013). In light of this, WHO recommends the replacement of IPTp-SP when the prevalence of K540E is > 95% and A581G is 10% (WHO, 2013). Since K540E was completely absent and A581G was 71% in this study, our finding suggests that sulphadoxine may remain a useful partner drug for IPTp − SP in Nigeria, a finding supported by a recent report (Oguike et al., 2016). Of note, the high prevalence of A437G (98%) and A581G (71%) suggests an imminent emergence of K540E in this setting, akin to *P. falciparum* isolates from East Africa, where both mutations are well established (Minja et al., 2013). Previous studies in Nigeria have reported the emergence of the K540E mutation in samples collected from pregnant women in Lagos (Iwalokun et al., 2015; Quan et al., 2020), indicating that the future use of sulphadoxine in Nigeria may be under serious threat.

The combined *pfdhfr* − *pfdhps* haplotypes synergistically modulate *P. falciparum* susceptibility to SP (Sicuri et al., 2015). The complete absence of the super − resistant sextuple *P. falciparum* mutants, carrying a C**IRN**I-S**GEG**A haplotype, further support IPTp-SP efficacy in this setting. Nevertheless, the occurrence of a quintuple mutant (C**IRN**I-S**G**K**G**A) and a different sextuple mutant (C**IRN**I-**AG**KA**S**) at a prevalence of 44% and 29%, respectively underscore the importance of routine surveillance, particularly of the emergence of the highly-resistant sextuple mutant C**IRN**I-S**GEG**A in Nigeria. Additionally, SP induction of *P. falciparum* gametogenesis, particularly among SP-resistant parasites (Gonçalves et al., 2017; Kone et al., 2010), the association of pregnant women with asymptomatic infections and both high gametocytemias and MOI (Fehintola et al., 2012; Lamptey et al., 2018; Walker-Abbey et al., 2005) depict pregnant women as possible reservoirs of infection. A recent study suggested that continued IPTp-SP could increase malaria transmission (Jafari-Guemouri et al., 2018).

Analyses of *P. falciparum* genetic diversity and MOI by genotyping two distinct genes, encoding *pfmsp-1* and*pfmsp-2* revealed high genetic diversity and high *P. falciparum* monoclonal infection rates (> 76%). Considering that MOI and genetic diversity are proxies of malaria transmission, high genetic diversity observed in our study is consistent with high malaria transmission in sub-Saharan Africa countries, including Nigeria (Funwei et al., 2018; Nguetse et al., 2017). Although not surprising, the low MOI in this study could be attributed to reduced exposure of the pregnant women to malaria possibly due to the high reported use of insecticide bed nets in this study population, despite previous reports showing low insecticide bed nets (Onyeneho et al., 2014) and long-lasting insecticidal net (LLIN) (Musa et al., 2009; Onyiah et al., 2018) use among pregnant women in Nigeria. An alternative explanation for the low MOI and high proportion of mono-infection could be due to parasite immunity developed during previous pregnancies since the majority of our study participants are multigravidae (Beck et al., 2001). High prevalence of asymptomatic infections by SP-resistant *P. falciparum* isolates at low MOI in this study

imply that mosquitoes drawing blood from these pregnant women acquire and transmit parasites less susceptible to IPTp-SP. This observation threatens IPT for malaria in pregnancy and infant malaria prophylaxis (IPTi), as well as promote the selection of SP-resistant *P. falciparum* in Nigeria.

In conclusion, this study shows high prevalence of SP resistant markers in southwest Nigeria. Observation of high prevalence of quintuple mutant (C**IRN**I- S**G**K**G**A) and sextuple-mutant (C**IRN**I-**AG**K**A**S**)** parasites in Nigeria suggests that the emergence of the highly resistant sextuple haplotype (C**IRN**I-S**GEG**A) reported in East and South Africa is imminent. Critical surveillance of IPTp-SP effectiveness and consideration of alternative IPT strategies is therefore recommended.

## Data statement

The data that support the findings of this study are available from the corresponding author, [OO], upon reasonable request.

## Author contributions

KAF, DND, SAA and SRP carried out molecular genotyping and analysis; SAAdebusuyi recruited patients, obtained informed consent and collected samples; KAF, DND, drafted the manuscript; BNT and OO reviewed the manuscript and contributed to the discussion and the overall scientific content; TPV Provided molecular study materials and contributed to the discussion and the overall scientific content; AOJA and OO conceived, designed and provided oversight and leadership responsibility;. All authors read and approved the final version of the manuscript.

## Declaration of Competing Interest

None.

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