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High prevalence of dihydrofolate reductase gene mutations in *Plasmodium falciparum* parasites among pregnant women in Nigeria after reported use of sulfadoxine-pyrimethamine

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ABSTRACT

This study assesses the prevalence of asymptomatic *Plasmodium falciparum* parasitemia positivity and *P. falciparum dihydrofolate reductase* (*pfdhfr*) mutations in parasite isolates among pregnant women in Southwest Nigeria. *Plasmodium falciparum* parasitemia was confirmed by microscopy and nested PCR in 200 pregnant women attending antenatal care. The prevalence of *pfdhfr* polymorphisms was determined by direct sequencing of the gene fragments containing the C50R, N51I, C59R, S108N, and I164L mutations. Information on the use of antimalarial drugs and methods applied to prevent malaria were obtained by a questionnaire. The prevalence of asymptomatic *P. falciparum* infection was 30% (60/200). The frequency of the *pfdhfr* triple-mutant alleles (N51I, C59R, and S108N) was 63% (38/60); none of the isolates carried the I164L mutation. Among the investigated pregnant women, 40% used un-prescribed antimalarials such as dihydroartemisinin (18%), chloroquine (14%) or pyrimethamine (9%), while only 20.5% used sulfadoxine-pyrimethamine for prevention and 39.5% did not use any drug. The prevalence of *P. falciparum* parasitemia (37%) was higher among pregnant women who had not taken any antimalarial drugs. A significant difference in the prevalence of the *pfdhfr* triple-mutant alleles was observed among women who took SP (90%) compared to those who did not take any drug (82%) and women who took dihydroartemisinin (67%) *p* = 0.007. Poor adherence to the World Health Organisation (WHO) strategies for malaria prevention among pregnant women was observed in addition to high prevalence of *pfdhfr* mutations. These findings underline the need to improve control of malaria among pregnant women in the study area.

Introduction

Plasmodium falciparum malaria during pregnancy is a major public health concern with approximately 30 million pregnant women at risk in sub-Saharan Africa [1,2]. Malaria infection under such circumstances pose significant risks for both mother and fetus, and has been associated with intrauterine growth restriction, preterm and preventable low birth weight deliveries, as well as maternal anemia [3–5]. To reduce risk, morbidity, and mortality associated with malaria during pregnancy, the World Health Organization (WHO) recommends the use of intermittent preventive treatment with sulfadoxine-pyrimethamine (IPTp-SP), use of insecticide-treated bednets (ITNs), and effective and prompt case management of malaria [6,7]. According to these

recommendations, preventive treatment with SP should be given at every antenatal care visit except in the first trimester [8]. In countries where IPTp-SP has been adopted, the use of two or three doses of SP has led to a substantially reduced risk of low birth weight deliveries [9,10], reduced anemia, and improved pregnancy outcome [11]. Additionally, the beneficial effect of IPTp-SP in areas with treatment failure below 39% is higher compared to areas with high treatment failure [12].

Resistance to SP has been documented in most places where it was initially used after chloroquine (CQ) treatment failure, suggesting that its continuous and widespread use in these areas will further increase the level of resistance, eventually rendering it ineffective [13]. A study among pregnant women in an area with widespread SP resistance of Tanzania, reported that IPTp-SP may be

harmful by increasing placental malaria and contributing to fetal anemia [14,15], including high prevalence rates of SP-associated resistance, with multiple mutant alleles among pregnant women in Congo [16], Malawi [17], and Uganda [18]. These observations emphasize the importance of molecular surveillance studies to ascertain the current state, investigate the spread and understand the mechanisms driving SP resistance, especially in Nigeria.

SP is a combination of antifolates with sulfadoxine targeting the parasite's dihydropteroate synthase (*dhps*) and pyrimethamine targeting dihydrofolate reductase (*dhfr*) genes. Resistance to SP is associated with the presence of single nucleotide polymorphisms (SNPs) in the *pfdhfr* and *pfdhps* genes [19,20], with resistance initiated with a core mutation at codon 108 (S108N) of *pfdhfr*, followed by mutations at codon 51 (N51I) and 59 (C59R), all three referred to as 'triple mutation' [21,22]. Another SNP at codon 164 has been associated with high levels of SP resistance in Southeast Asia and Latin America, but has been rarely reported in Africa [23]. Further, mutations in the *pfdhps* gene at codons 437 and 540 (A437G and K540E) are related to higher risks of SP treatment failure [24]. Therefore, these polymorphisms are useful molecular markers for tracking the emergence and spread of SP resistance.

Nigeria records a worrisome high prevalence of malaria in pregnancy ranging from 19.7 to 72 % [25–27] in different parts of the country, and about 11% maternal deaths [28], leading policy-makers to adopt IPTp-SP as national policy in 2005 [9,29,30]. Till date, no significant reduction in malaria infection rates among pregnant women in Nigeria has been recorded, compared to similar countries with good surveillance and high intervention coverage [28]. Though published reports have shown that SP has had a positive impact on pregnancy outcomes, there are concerns about the waning efficacy of SP due to increase in parasite resistance [31,32]. Loss of IPTp-SP efficacy was associated with the recent emergence of a highly resistant 'sextuple mutant' parasite in Tanzania [33]. Resistance of *P. falciparum* against SP is reportedly rising in most West African countries, but IPTp-SP is still considered efficacious [18,34], emphasizing the need for regular monitoring of SP resistance to ensure timely revision in policy, if needed. This study therefore reported the prevalence of asymptomatic *P. falciparum* parasite positivity and *dhfr* mutations in parasite isolates from pregnant women who reportdely use SP in Southwest Nigeria.

Materials and methods

Study area

The study was carried out at General Hospital, Ikirun a neighboring town to Osogbo, Osun State capital, Nigeria. The entire state covers an area of about 835 hectares, with a population of over 3 million people, with Ikirun having an estimated population of about 60,000 people. The climate is tropical with two seasons, the dry season (from October to February) and a rainy season (from March to September). Average daily temperature is 32°C with a minimum temperature of 19°C and a maximum temperature of 35.9°C [35]. Malaria occurs holoendemically with perennial high and stable transmission in the study area.

Study population and recruitment

This is a cross-sectional study carried out between June and December 2015, that recruited all pregnant women attending antenatal clinic at the study center, who willingly gave consent to participate. Participants were informed about the advantages of the study, potential discomfort associated with sampling and verbal informed consent was received, recorded as a 'yes' or 'no' in sample collection form. In all, 200 pregnant women who gave their consent were recruited into the study. Approval for the study was obtained from Ethical Review Committee of the Osun State Ministry of Health, Osogbo (OSHREC/PRS/569/33).

Sample collection and processing

Demographic data including age, gravidity, parity, gestational age, and clinical information such as packed cell volume (PCV), weight and blood pressure were assessed at recruitment. Finger-prick blood was collected with a sterile lancet onto two separate slides and on a Whatman filter paper. Thin and thick Giemsa stained blood smears were examined under a light microscope for parasite identification and quantification, as previously described [36]. Blood was also collected into heparinized capillary tubes, sealed with plasticin, and spun in a micro-hematocritc centrifuge for hematocrit determination.

A semi-structured interviewer-administered questionnaire was used to collect information on the socio-demographic characteristics of respondents, types of antimalarial drug used in the last one month and types of malaria prevention currently being used before and during the pregnancy.

Parasite genotyping

Genomic DNA was isolated from dried blood stored on Whatman filter paper using the QIAamp DNA mini blood kit (Qiagen, Hilden, Germany), following manufacturer's guidelines. Polymerase chain reaction (PCR) and direct Sanger sequencing were subsequently used for the genotyping of the *pfdhfr* mutations C50R, N51I, C59R, S108N, and I164L, utilizing primers and protocols as previously described [37]. Conserved regions of the *msp-2* gene were used to classify the genetic diversity of parasite isolates into FC27 and 3D7-specific family alleles [38].

The multiplicity of infection (MOI), defined as the mean number of different *P. falciparum* strains co-infecting one individual, was determined by dividing the number of detected *msp-2* fragments by the number of positive samples.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism v. 5.0 for windows (Graphpad Software Inc., San Diego, U.S.A.). Two-sided Fisher's exact test was performed to analyze possible associations of *pfdhfr* polymorphisms with the use of IPTp-SP. The level of significance was set to a *p* value < 0.05.

Results

A total of 200 pregnant women (mean age: 26.7 ± 5.34 years; range 15–41 years; 78 (39%) primigravida and 122 (61%) multigravida); mean packed cell volume 35.94 ± 6.50%, attending the antenatal clinic and who gave their consent were recruited into the study. The baseline characteristics of participants in this study including additional data are provided in Table 1. Sixty (30%) and 58 (29%) of these women were positive for *P. falciparum* parasitemia both by PCR and microscopy, respectively (Table 1). The highest positivity rate (36.5%) was observed in the age range 26–30 years, with the difference in the malaria parasite positivity across all age groups not significant. Furthermore, there was no difference between the multiplicity of infection (MOI) determined by *msp-2* genotyping and the occurrence of the triple *pfdhfr* mutation across the stratified age groups (Table 2).

The prevalence of *P. falciparum* parasitemia, MOI and triple *pfdhfr* mutation with respect to gravidity and period of pregnancy (trimester) are shown in Table 3. Number positive for *P. falciparum* was higher among primigravidae (34.6%) than multigravidae, but the difference was insignificant. Additionally, the MOI and triple *pfdhfr* mutation were neither associated with gravidity, nor with age of pregnancy (first, second, or third trimesters) (Table 3).

Associations between intake of different antimalarial drugs, parasitemia, and triple *pfdhfr* mutation are as shown in Table 4. The highest *P. falciparum* parasite positivity was observed among women who took no prophylaxis (36.7%), though not significant when compared to others who had one kind of prophylaxis or the other. However, a significant difference in the frequency of the triple *pfdhfr* mutation was observed among the group that had taken antimalarial drugs (*p* = 0.007) with those who took SP recording the highest prevalence (90%). Pregnant women who took SP had a higher frequency of mutant alleles (90.0%) (Table 4).

With respect to malaria prevention methods, the highest proportion of pregnant women with the highest prevalence of *P. falciparum* parasitemia was observed among those who used insecticide sprays (44.4%) (not significant; Table 5), compared to other forms of prevention methods. Similarly, the highest and statistically significant prevalence of the triple *pfdhfr* mutation occurred among these women who used insecticide spray (*p* = 0.03) (Table 5).

Discussion

Malaria associated with drug resistance significantly contributes to high mortality rates among pregnant women and children less than 5 years in many endemic regions[39]. The WHO currently recommends that pregnant women should receive IPTp-SP at each antenatal visit, starting as early as possible in the second trimester. Several studies have reported the beneficial effects of IPTp-SP in preventing maternal malaria, improving the outcome of pregnancy in Africa and significantly lowering the risk of placental malaria in areas of high transmission [29,40]. However, other reports suggest that IPTp-SP is not effective in areas where SP is already failing and that the continuous use of SP as prophylaxis will increase the further spread of resistant parasites [14]. In this study, assessment of the efficacy of SP by determining polymorphisms in the *pfdhfr* gene reveal high prevalence of the triple *pfdhfr* mutations (81.8%) in our study population. This observation was comparable to our previous study (showing triple *pfdhfr* mutation of 86.5%) among children with uncomplicated *falciparum* malaria in this area [41]. This current observation confirms published reports that SP resistance is now a major concern in Nigeria [32] and that a revamping of prophylactic strategies for pregnant women might be imperative.

Among the 41 (20.5%) pregnant women that reportedly took SP, 16% had malaria parasitemia of which 90% of the parasites had the triple *Pfdhfr* mutation, revealing no protection whatsoever from malaria infection in this study. The fact that we did not directly administer or monitor SP intake by these women hampers the conclusion this study demands. However, this opens up the need to further investigate the impact of IPTp-SP in the prevention of malaria and its associated complications among pregnant women in the study area. Significantly, the I64L mutation, which has been associated with higher levels of resistance to SP was not found in this or

Table 1. General characteristics of the pregnant women recruited into the study.

Characteristics	Pregnant (*N* = 200)
Mean Age (yrs) ±SD	26.54 ± 5.34
Mean Gestational Age (weeks) ± SD	17.37 ± 5.80
Mean packed cell volume ± SD	35.94 ± 6.50
Mean weight ± SD	58.8 ± 8.20
Prevalence of *P. falciparum*	30 % (60/200)
Number positive by Microscopy	29 % (58/200)
Number positive by PCR	30 % (60/200)
Primigravida vs. Multigravida	78 vs. 122

Table 2. Prevalence of *Pfdhfr* triple mutation and MOI by age among pregnant women in the study area.

Age group	Number examined (%)	Number positive (%)	3D7 (%)*	FC27 (%)*	MOI*	Triple *Pfdhfr* mutation (%)*	
						Mutant	Wild type
15–20	30 (15.0)	8 (13.4)	6	8	2.1	4 (50.0)	4 (50.0)
21–25	60 (30.0)	18 (30.0)	14	16	1.8	10 (55.6)	8 (44.4)
26–30	73 (36.5)	22 (36.6)	13	20	1.6	17 (77.3)	5 (22.7)
31–35	26 (13.0)	10 (16.6)	6	8	1.8	5 (50.0)	5 (50.0)
>36	11 (5.5)	2 (3.4)	1	1	1.0	2 (100.0)	0(0)
Total	200	60 (30.0)					

*The difference between groups was not statistically significant.

Table 3. *pfdhfr* triple mutation and MOI according to gravidity and age of pregnancy.

Gravidity	Number examined (%)	Number positive (%)*	3D7 (%)*	FC27 (%)*	MOI	Triple *pfdhfr* mutation (%)*	
						Mutant	Wild type
1	78	27 (34.6)	17 (63.)	25 (92.6)	1.8	15 (55.6)	12 (44.4)
2	60	17 (28.3)	12 (70.3)	15 (88.2)	1.8	12 (70.6)	5 (29.4)
3	62	16 (25.8)	10 (62.5)	13 (81.3)	1.7	10 (62.5)	6 (37.5)
Total	200	60 (30.)	40 (66.7)	53 (88.3)		37 (61.7)	22 (36.7)
Trimester							
1	63	16 (26.7)	10 (62.5)	13 (81.3)	1.63	6 (37.5)	10 (62.5)
2	120	40 (33.3)	30 (75.0)	38 (95)	1.87	29 (72.5)	11 (27.5)
3	17	4 (23.5)	0	2 (50)	1.0	2 (50)	2 (50)
Total	200	60 (30.0)	40 (66.7)	53 (83.3)			

*The difference between groups was not statistically significant.

Table 4. Association of *pfdhfr* triple mutations and different drug intakes among pregnant women.

Drug intake	Number examined (%)	Number positive (%)*	Triple *pfdhfr*#	
			Mutant	Wildtype
Dihydroartemisinin	36 (18.0)	13 (21.0)	6 (46.2)	7 (53.8)
Chloroquine	27 (13.5)	9 (15.0)	1 (11.1)	8 (88.9)
Pyrimethamine	17 (8.5)	6 (10.0)	4 (66.7)	2 (33.4)
Sulfadoxine- Pyrimethamine	41 (20.5)	10 (16.7)	9 (90.0)	1 (10.0)
None	79 (39.5)	26 (36.7)	18 (81.8)	4 (18.2)

*The difference between groups was not statistically significant;
#The difference between groups was statistically significant ($p = 0.007$).

Table 5. Prevalence of malaria with respect to prevention methods.

Prevention methods	Number examined (%)	Number positive (%)	
Coil	47 (23.5)	15 (32.0)	$\chi^2 = 5.765$;
Door Net	30 (15.0)	5 (16.6)	$p = 0.03$
Insecticide spray	27 (13.5)	12 (44.4)	
TBNet	15 (7.5)	4 (26.7)	
UNTBnet	43 (21.5)	14 (32.6)	
None	38 (19.9)	10 (26.3)	
Total	200	60 (30.0)	

TBNet – Treated bed net
UNTBnet – Untreated bed net.

previous reports [37,42], an encouraging observation on SP usefulness for the time being, that is similar to findings from other parts of West Africa. The high prevalence of the triple *pfdhfr* mutation consistently reported in this area indicates that SP efficacy might be deteriorating, implying that prolonged use of SP in this region may cause its inefficacy. The high prevalence of 90% of the triple *pfdhfr* mutation observed among the pregnant women that reportedly took SP supports the idea that continuous use of the drug may promote selection of resistant alleles as a consequence of drug pressure. This phenomenon is the driving force behind the evolution of drug resistance [43].

An important observation is the poor adherence of pregnant women to the WHO recommended SP for malaria prevention. Other drugs like dihydroartemisinin, chloroquine, and pyrimethamine that are not recommended for treatment or prevention of malaria in pregnancy are still commonly used by pregnant women in this study area. Pyrimethamine was the most commonly prescribed drug for malaria prevention during pregnancy in Nigeria [44] before the advent of ITPp-Sp. It was also previously recommended for weekly use of non-immune individuals traveling to malaria endemic regions [24,25]. Pyrimethamine, as monotherapy, is no longer recommended due to widespread resistance to the drug [47]. Mutations in the *pfdhfr* gene may reduce the effectiveness of pyrimethamine [48]. These mutations decrease the binding affinity between pyrimethamine and dihydrofolate reductase via loss of hydrogen bonds and steric interactions [49]. Its continual use, as evidenced by study subjects in our study, reveals it remains a major contributory factor to the high rate of resistance and triple mutations among pregnant women specifically and the region in general. This observation is highlighted by the recent report of significant oral artemisinin monotherapy in Nigeria, contrary to policy, as a significant contributor to drug resistance and treatment failures [50]. We recommend stringent monitoring policies as well as improvement in import guidelines regarding what antimalarials can be imported and from which countries.

Few of the enrolled women (7.5%) reportedly used insecticide-treated bednets (ITNs) as anti-vector measure. A study conducted by Nigeria Demographic and

Health Survey (NDHS) reported that only 3% of pregnant women slept under ITNs in Southwest Nigeria [51]. Despite the proven efficacy and availability of ITNs in Nigeria, many pregnant women are still not comfortable with its use. A recent study among postpartum women in Ibadan, Southwest Nigeria reported that majority of the women did not use ITNs because of lack of access to free distribution [52]. Our results show that the current situation of low ITNs use among pregnant women remains unchanged. This situation underscores the need for urgent intervention from all stakeholders including Federal, State, and Local Governments, health care workers and the media to encourage the use of ITNs towards reducing the scourge of malaria infection in pregnant mothers and the unintended consequence of pregnancy complications and high rates of resistance.

A high prevalence (30%) of *P. falciparum* parasitemia was detected among pregnant women attending routine antenatal clinics in this study. This prevalence was comparable to the 44% earlier reported among pregnant women in Ilorin, Kwara State Nigeria [37] and 31.8% among pregnant women with asymptomatic *P. falciparum* infection in Lagos, Nigeria [53]. However, a previous study from this study area has reported a higher prevalence of 75% among pregnant women [27]. This high prevalence is not surprising as it has been previously observed that in areas of stable transmission in sub-Saharan Africa like our study area, one in every four pregnant women has evidence of peripheral or placental infection with malaria parasites [54].

A major drawback of this study is our inability to genotype mutations of the *dhps* gene, which is partly due to failure to generate good DNA sequence data and limited funding. Future studies of both the *dhfr* and *dhps* genes in our study area is therefore essential to investigate the distribution of these genes and their impact on SP efficacy in the control of malaria in pregnancy. In addition, larger sample sizes and a longitudinal approach rather than a cross-sectional study will assist to validate the association between the observed high rate of *dhfr* gene mutations, SP resistance, and pregnancy outcome. Beyond this, there is an urgent need to revise malaria prevention policy for pregnant women in sub-Saharan Africa. Published reports, from all over Africa, abounds confirming the reduced efficacy of SP for malaria prophylaxis among pregnant women, and the consequent effect on birth weight and other pregnancy-related outcomes [16,18,31,34,37] .In addition, new *Pfdhfr* and *Pfdhps* mutations [55,56], previously unknown within the continent, are being reported which should heighten our concern on the protection available to pregnant women and their unborn children, and the need for all stakeholders to address this looming problem.

In conclusion, this study reports a high prevalence of *pfdhfr* triple mutations in *P. falciparum* parasites among pregnant women in Osun State, Nigeria. The prevalence of this triple mutations was also significantly higher among pregnant women who took SP for IPTp. Furthermore, a poor adherence to the WHO recommendations for malaria prophylaxis among pregnant women was observed. There is therefore the need for improved awareness with regard to the application of WHO strategies to reduce maternal and child mortality resulting from *P. falciparum* infection in this area.

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