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Carriage of Mutant Dihydrofolate Reductase and Dihydropteroate Synthase Genes among *Plasmodium falciparum* Isolates Recovered from Pregnant Women with Asymptomatic Infection in Lagos, Nigeria

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Key Words

Sulphadoxine-pyrimethamine resistance · Asymptomatic malaria · Dihydrofolate reductase gene · Dihydropteroate synthase gene · Single-nucleotide mutations · Pregnant women · Nigeria

Abstract

Objective: To assess N51I, C59R and S108N polymorphisms of dihydrofolate reductase (dhfr) and A437G and K540E of dihydropteroate synthase (dhps) genes of *P. falciparum* isolates recovered from pregnant women with asymptomatic malaria in a coastal setting in Nigeria. ***Subjects and Methods:*** A total of 107 consenting and consecutively enrolled pregnant women (mean age ± standard deviation, 26.6 ± 4.5 years) attending antenatal care at the Iru/Victoria Island Primary Health Centre, Lagos, were screened for peripheral malaria by microscopy, by a histidine-rich protein-2-based rapid diagnostic test (RDT) and by polymerase chain reaction (PCR) using finger-pricked and dot blood samples. DNA was extracted from the blood and used for dhfr and dhps gene polymorphism analyses by PCR and restriction fragment length polymorphism. The sociodemographic and parasite data obtained were analysed. ***Results:*** Of the 107 patients, 34 (31.8%), 46 (43%) and 40 (37.4%) were found to be *P. falciparum* infected using microscopy, RDT and corrected RDT-PCR, respectively ($p < 0.05$). The prevalence of *P. falciparum* isolates with mutant and mixed genotypes of dhfr at codons 51, 59 and 108 was 70, 75 and 80%, respectively, and the triple mutation in the homozygous form was 35%. The prevalence of the homozygous quintuple dhfr plus dhps mutant was 5%, while that of the *P. falciparum* isolates with mutant or mixed genotypes of dhps at codons 437 and 540 was 37.5 and 22.5%, respectively. ***Conclusion:*** This study revealed the emergence of the K540E mutation among the parasite population in Lagos. However, it supports the implementation of the intermittent preventive treatment of malaria during pregnancy with sulphadoxine-pyrimethamine with continuous effectiveness monitoring in the study area.

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Introduction

In sub-Saharan Africa, an estimated 35 million pregnant women remain at risk of *Plasmodium falciparum* infection and associated clinical effects such as maternal anaemia, placental parasitaemia and low-birth-weight babies every year [1, 2]. These effects also affect pregnant women with asymptomatic *P. falciparum* infection. To prevent malaria during pregnancy in sub-Saharan Africa, the WHO recommends the administration of at least 2 doses of sulphadoxine-pyrimethamine (SP) for the intermittent preventive treatment of malaria during pregnancy (IPTp) to every pregnant woman during the attendance of at least 4 antenatal care (ANC) services. By this policy, the 2 doses of SP are to be administered after quickening and at least 1 month apart [3]. Currently, SP remains the only drug of choice for IPTp because of its safety, tolerability, ease of use and proven efficacy in reducing the risk of low-birth-weight babies by 29% and clearing asymptomatic parasitaemia in pregnant women at booking [4, 5]. The prophylactic effect of SP also makes it desirable in preventing re-infection in pregnant women, ensuring a parasite-free delivery [4]. SP has also been found effective for IPTp in areas with high treatment failure of uncomplicated malaria in children <5 years [4]. A recent systematic review and meta-analysis by Kayentao et al. [6] has further reported an improved efficacy of 3 doses compared to 2 doses of SP for averting the clinical effects of malaria during pregnancy. Nigeria is one of the three countries responsible for 40% of the global malaria mortality in 2012 [7]. The disease accounts for 11% of the total maternal mortality in the country every year [8]. The government of Nigeria adopted the IPTp-SP policy in 2005, when previous drug efficacy studies reported an adequate clinical and parasitological response rate of 56.7% for SP as a treatment for uncomplicated malaria in children <5 years in the country [8]. Since its adoption and despite the scaling up of malaria control interventions with SP, compliance to IPTp-SP policy remains low in Nigeria. It was 5 and 13% in 2008 and 2010, respectively [9, 10]. A recent study by Fehintola et al. [11] reported SP to promote *P. falciparum* gametocytogenesis among pregnant women in Ibadan, South-West Nigeria. The increasing spread of SP resistance among circulating *P. falciparum* strains in Africa has further made it imperative to locally monitor the effectiveness of SP for IPTp. High SP resistance and treatment failure has been attributed to triple mutations at codons 51 (N51I), 59 (C59R) and 108 (S108N) of the dihydrofolate reductase *(dhfr)* gene and to quintuple mutations mediated by the triple *dhfr* mutant plus double dihydropteroate synthase *(dhps)* mutant gene at codons 437 (A437G) and 540 (K540E) of the parasite in West, East and South Africa [12, 13]. Unlike in other West African countries, studies monitoring the effectiveness of SP in the context of IPTp using the molecular surveillance approach have been grossly limited in Nigeria. Only two studies in 2010 and 2011 have documented the prevalence of SP resistance mutations in the country [14, 15]. However, both studies investigated mutations in the *pfdhfr* gene only in children of <5 years and in pregnant women who had received IPTp. Since these markers determine pyrimethamine resistance [12, 13], it is not clear whether sulphadoxine resistance is also spreading. However, these studies suggest a possible regional variation in the level of SP use and necessitate the need to continue to survey for SP resistance markers in other malaria-endemic settings in Nigeria. The present cross-sectional study was conducted among pregnant women at their booking in a primary health centre in Lagos, South-West Nigeria, where SP resistance has not been investigated. The study was aimed at assessing the N51I, C59R and S108N polymorphisms of *dhfr* and of the A437G and K540E polymorphisms of *dhps* of *P. falciparum* isolates recovered from pregnant women with asymptomatic infection. The relationship between age, gravidity and triple and quintuple mutations was also investigated.

Subjects and Methods

Study Area

The study was carried out at the Iru/Victoria Island Primary Health Centre on pregnant women at their booking for ANC between April and July 2011. This health facility is located on Victoria Island, coastal lowland in Lagos state, South-West Nigeria, which is equatorial. The coastal lowland is holoendemic for malaria with a transmission driven by female mosquitoes belonging to the *Anopheles gambiae* complex during the rainy season and by *A. arabiensis* during the dry season. Other mosquito species, including *A. melas and A. moucheti*, are found in low abundance throughout the year [16]. The long-lasting insecticide net (LLIN) survey of 2012 revealed a LLIN coverage rate of 88.1% as a result of the distribution of 4.1 million LLINs in the state [16]. With regard to the IPTp, there is also evidence that an estimated 118,999 doses of SP have been distributed to the primary health centres of the state via the Eko Free malaria programme [17].

Each apparently healthy pregnant woman at booking was approached to participate in the study. Inclusion criteria were residence within the 5 wards/communities (Takwa-Bay, Kuramo, Apese, Victoria Island I and Victoria Island II) under the administrative control of the Iru/Victoria Island local council development area and willingness to be screened for malaria as per the national ANC policy and respond to the study questionnaire. Exclusion criteria were subjects with pregnancy complications or fe-

Table 1. Primer sequences and amplification conditions for msp-2 (block 3) polymorphism genotyping

Gene	Primer	Sequence	Amplification profile, °C			
			D	A	E	C
msp-2	MSP2-F	5′-ATGAAGGTAATTAAAACATTGTCTATTATA-3′	94 (1)	55 (1)	72 (2)	30
	MSP2-R	5′-CTTTGTTACCATCGGTACATTCTT-3′				
	FC27-F	5′-GCAAATGAAGGTTCTAATACTAATAG-3′				
	FC27-R	5′-GCTTTGGGTCCTTCTTCAGTTGATTC-3′				
	3D7-F	5′-AGAAGTATGGCAGAAAGTAAGCCTCCTACT-3′				
	3D7-R	5′-GATTGTAATTCGGGGGATTCAGTTTGTTCG-3′				

Values in parentheses represent minutes.

ver (axillary temperature ≥37.5°C) at presentation or within 72 h prior to presentation or with treatment of malaria in the previous 2 months and those whose clinical records indicated HIV seropositivity and prior use of cotrimoxazole or SP at booking. Written informed consent of each pregnant woman was obtained before enrolment. Although a pregnant woman can book for ANC at any time of the week at the primary health care centre, routine ANC services occur every Wednesday. ANC services are implemented by trained nurses and midwives of the health facility under the headship of a medical officer of health.

Data Collection Methods

The study participants were consecutively enrolled during their ANC visits within the study period. At enrolment, a pretested structured questionnaire written in English and translated back into the Yoruba language was administered in any of the two languages as appropriate to each pregnant woman by a trained health worker of the primary health care centre to capture sociodemographic data. Data on the stage of pregnancy and parity/gravity status were also extracted from the antenatal card into the study form. This study was approved by the Ethic Committee of the Lagos State Health Management Board.

Blood Sampling

Each pregnant woman was finger pricked using a sterile lancet to obtain capillary blood. The blood was collected into a heparinized capillary tube for haematology. Blood samples were used to prepare thick and thin blood films for microscopic tests. They were also used for a rapid diagnostic test (RDT) for malaria and were spotted on 3MM Whatman filter paper for parasite genotyping. The collected dot blood samples on the filter paper were kept inside sealed plastic envelopes and maintained dry by storage in a desiccator prior to use for analyses.

Parasite Detection

Parasite detection was performed by the microscopic examination of thick and thin blood smears prepared in duplicates on grease-free prelabelled slides. Speciation was done by examining the thin film, and the parasites were counted relative to 200–500 leukocytes by examining the thick smear under oil immersion (×1,000 magnification). The slides were read by two trained microscopists per sample, and those samples with discrepant results >20% were read by a third expert microscopist. A slide was considered negative if no parasites were seen after viewing 100 high-power fields. The level of parasitaemia (parasites/μl of blood) was estimated based on the assumption of 8,000 leukocytes/μl of blood. An average of 2 parasite counts was used for parasitaemia estimation.

RDT based on *P. falciparum*-specific histidine-rich protein-2 (Bioline, USA) was also performed according to the manufacturer's directives to detect parasites as recommended by the national malaria programme [9]. Briefly, 5 μl of finger-pricked blood was placed directly on the test cassette, and 5 drops of clearing buffer were added, followed by incubation for 15 min. The presence of parasites was indicated by the appearance of 2 bands for control and test. The appearance of only the control band indicated a valid result that was negative for *P. falciparum* parasites.

The molecular assay for parasite detection employed DNA extracted by the Chelex method [18] from capillary blood samples also spotted in duplicates on 3MM Whatman filter paper (~25 μl/spot). The filter papers selected were those of the blood samples that were *P. falciparum* positive by microscopy and RDT. Here, parasite detection was by nested polymerase chain reaction (PCR) using primers that target the conserved region of the block 3 domain of the merozoite surface protein-2 (*msp-2*) gene in the primary reaction and of the FC27 and 3D7 alleles in the nested reaction using the PCR composition and amplification conditions previously described by Aubuoy et al. [19] (table 1). Both the initial (outer) and the nested PCR amplification reactions were performed in a 20-μl volume PCR tube containing 10× PCR buffer, 200 μM each of the dNTPs, 2.0 mM of $MgCl_2$, 20 pmol of each primer, 1.25 U of Taq DNA polymerase (Promega, USA) and 4 μl of genomic DNA (outer PCR) or 1 μl of outer PCR product. All PCR reactions were carried out in a thermal cycler (Techne™ Thermal cycler TC-312, Fisher Scientific, UK) with similar amplification conditions for both the outer and the nested PCR. An initial denaturation step at 94°C for 5 min was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, a final extension at 72°C for 10 min and cooling to 4°C. Template-free water and standard 3D7 and FC27 genomic DNA samples were used as controls. Parasite positivity was confirmed by the presence of msp-2 DNA fragments of 100–500 bp irrespective of the allele family on the agarose gel after electrophoresis. The absence of DNA indicated parasite negativity in the dot blood sample. The frequency of infection based on msp-

Table 2. Baseline characteristics of the studied pregnant women

Characteristics	Total (n = 107)	Malaria positivity[1]		p value
		present (n = 40, 37.4%)	absent (n = 67, 62.6%)	
Mean age ± SD, years	26.6±4.5	26.8±5.3	26.3±2.9	0.63
Age group, n (%)				
≤20 years	12 (11.2)	7 (17.5)	5 (7.5)	0.11
≥21 years	95 (88.8)	33 (82.5)	62 (92.5)	
Education, n (%)				
Primary	18 (16.8)	6 (15)	12 (17.9)	0.7
Secondary	83 (77.6)	31 (77.5)	52 (77.6)	0.99
Tertiary	6 (5.6)	3 (7.5)	3 (4.5)	0.51
Employment status, n (%)				
Working	59 (55.1)	25 (62.5)	34 (50.7)	0.34
Not working	48 (44.9)	15 (37.5)	33 (49.3)	
Gravidity, n (%)				
Primigravida	24 (22.4)	12 (30)	12 (17.9)	0.15
Secundigravida	49 (45.8)	21 (52.5)	28 (41.8)	0.52
Multigravida	34 (31.8)	7 (17.5)	27 (40.3)	0.005
Gestation age at booking, n (%)				
First trimester	25 (23.4)	9 (22.5)	16 (23.9)	0.87
Second trimester	65 (60.7)	26 (65)	39 (58.2)	0.49
Third trimester	17 (15.9)	5 (12.5)	12 (17.9)	0.46
LLIN use				
Yes	46 (43)	11 (27.5)	35 (52.2)	0.01
No	61 (57)	29 (72.5)	32 (47.8)	
Anaemia, n (%)				
Yes	70 (65.4)	27 (67.5)	43 (64.1)	0.26
No	37 (34.6)	13 (32.5)	24 (35.9)	
Mean Hb ± SD, g/dl	10.6±0.7	10.3±0.8	10.8±0.7	0.009

[1] *P. falciparum*-positive cases based on 34 microscopy- and RDT-positive cases plus 6 of the 12 RDT-positive PCR-corrected cases that were negative by microscopy.

2 was calculated counting the number of DNA samples genotyped that were positive for only FC27 allele and 3D7 allele infections and for both allele infections (i.e. mixed infection). The multiplicity of infection was calculated as the mean number of parasite genotypes per infected pregnant woman.

Haematology

The blood haemoglobin (Hb) concentration in 10 μl of capillary blood sample was measured in g/dl using HemoCue® Hb 201+ System, a point-of-care test based on the formation of azidemethemoglobin (Hemocue America, USA). Anaemia was defined as a Hb concentration <11 g/dl [20].

dhfr and dhps Gene Polymorphism Assessment

Polymorphisms in the *dhfr* and *dhps* genes were genotyped by the PCR and restriction fragment length polymorphism method previously described by Lopes et al. [21], Figueiredo et al. [22] and Mendes et al. [23]. For the *dhfr* gene, DNA fragments of 206, 320 and 504 bp were amplified by PCR for codons 51, 59 and 108, respectively, using codon-specific primers (table 1), while for the *dhps* gene, a common DNA fragment of 438 bp was amplified for codons 437 and 540, respectively. For the detection of single nucleotide polymorphisms at codons 51, 59 and 108, their respective PCR products were digested with *Tas1, Pdm1* and *Alu1*, while for codons 437 and 540, digestion was performed with HpyF10VI and Bseg1, respectively. A DNA sample of the *P. falciparum* clone *Dd2* was used as a control. For the analysis of these polymorphisms, the digested products were electrophoresed on 2% agarose gel prestained with ethidium bromide and visualized under UV light to size the DNA bands relative to a 100-bp DNA ladder and control DNA fragments. To further confirm the results of the dhfr and dhps polymorphisms obtained by PCR and restriction fragment length polymorphism, 10 PCR product pairs of *pfdhfr* and *pfdhps* were randomly selected, purified with a QIAquick PCR purification kit (Qiagen, Germany) and then sequenced directly using amplification primers on the 3130 Genetic analyser (Applied Biosystems). Sequence alignment and analysis were subsequently performed online using the BLAST programme (www.ncbi.nlm.nih.gov).

Data Analysis

The analyses were performed using SPSS 17.0. The data are presented as means ± standard deviation. The prevalence was cal-

Table 3. *P. falciparum* infection profile of the ASM subjects

Samples screened	107
Microscopy- or RDT-positive and PCR-confirmed cases	34 (31.8)
Microscopy-negative, RDT-positive cases	12 (11.2)
RDT-positive cases	46 (43)
PCR-confirmed, RDT-positive cases	40 (37.4)
GMPD, parasites/μl	970 (420–2,300)
msp-2 genotyping (n = 34)	
FC27 allele only	19 (55.9)
3D7 allele only	4 (11.8)
FC27 + 3D7	11 (32.4)
MOI	1.41

Values are numbers with percentages or range in parentheses. GMPD = Geometric mean parasite density; MOI = multiplicity of infection.

culated as a percentage of the wild, mutant or mixed genotype per analysed codon in the *pfdhfr* and *pfdhps* genes out of the total samples (n = 40) genotyped. All mixed genotypes for each of the studied SP resistance mutations were grouped together with their respective mutant genotypes for analysis to determine the prevalence of parasites with pfdhfr and pfdhps mutations at the genotype and haplotype levels. The χ^2 and Student's t test were used to evaluate the differences in percentages and mean values between asymptomatic malaria (ASM)-positive (n = 40) and ASM-negative (n = 67) pregnant women enrolled. Univariate logistic regression based on Fisher's exact test with the outcome measured as odds ratio and 95% confidence interval was used to assess the association between triple mutation prevalence and the derived sociodemographic variables (age group, education, gestational age at booking, anaemia, employment status and LLIN use).

Table 4. Frequency of SP resistance-associated mutations in the *dhfr* and *dhps* genes of the asymptomatic *P. falciparum* isolates

Gene	Codon	Genotype	Frequency (n = 40)
pfdhfr	51	Wild (N)	12 (30)
		Mixed (N + I)	10 (25)
		Mutant (I)	18 (45)
		Mutant or mixed genotype	28 (70)
	59	Wild (C)	10 (25)
		Mixed (C + R)	16 (40)
		Mutant (R)	14 (35)
		Mutant or mixed genotype	30 (75)
	108	Wild (S)	8 (20)
		Mixed (S + N)	10 (25)
		Mutant (N)	22 (55)
		Mutant or mixed genotype	32 (80)
pfdhfr single mutants	59	Mixed genotype	2 (5)
	108	Mutant or mixed genotype	3 (7.5)
pfdhps	437	Wild (A)	25 (62.5)
		Mixed (A + G)	12 (30)
		Mutant (G)	3 (7.5)
		Mutant or mixed genotype	15 (37.5)
	540	Wild (K)	31 (77.5)
		Mixed (K + E)	7 (17.5)
		Mutant (E)	2 (5)
		Mutant or mixed genotype	9 (22.5)
pfdhps double mutants		Mutant or mixed genotype	6 (15)

Values are numbers with percentages in parentheses. n = Number of *P. falciparum* isolate DNA samples genotyped for *dhfr* and *dhps* mutations.

Results

The mean age of the women was 26.6 ± 4.5 years. Of the 107 women, 34 (31.8%), 46 (43%) and 40 (37.4%), respectively, were found to be *P. falciparum* infected by microscopy, RDT and corrected RDT-PCR ($p < 0.05$; tables 2, 3). Of the 12 microscopically negative cases, 6 were RDT-positive cases and confirmed by PCR. The occurrence of asymptomatic *P. falciparum* parasitaemia was significantly associated with a lower mean blood Hb level (10.3 ± 0.8 vs. 10.8 ± 0.7 g/dl; $p < 0.05$) and a lower rate of LLIN use (27.5 vs. 52.2%). The LLIN use rate, prevalence of anaemia (Hb <11 g/dl) and adolescent pregnancy were 43, 65.4 and 11.2%, respectively (table 2). Of the 107 infected pregnant women, 17 (15.9%) were booked in the third trimester of pregnancy, while 5.6% of the participants had a tertiary education (table 2).

The infection profiles of asymptomatic *P. falciparum* malaria in the infected pregnant women are presented in table 3. Among the 34 microscopy-positive cases, the level of parasitaemia ranged from 420 to 2,300 parasites/μl, producing a geometric mean parasite density of 970 parasites/μl. msp2 genotyping further showed that 32.4, 55.9 and 11.8% of the asymptomatic infections were mixed infections, caused by FC27 and 3D7 allele strains. The overall multiplicity of infection was calculated to be 1.41.

The genotypes of the *dhfr* and *dhps* genes based on the analysis of 40 DNA samples of the *P. falciparum* isolates are summarized in table 4. The prevalence of *P. falciparum* isolates with mutant or mixed genotypes at codons 51, 59 and 108 of the *dhfr* gene was 70, 75 and 80%, respectively, while the prevalence of the *P. falciparum* isolates with mutant or mixed genotypes at codons 437 and 540 of the *dhps* gene was 37.5 and 22.5%, respectively. The

Table 5. Association between sociodemographic parameters and the occurrence of triple and quintuple mutations in the *dhfr* and *dhps* genes of the asymptomatic *P. falciparum* isolates

Parameter	Triple dhfr mutations		OR	95% CI	p value
	+	–			
Age group					
≤20 years	1	6	1		
>20 years	19	14	8.1	0.8–75.5	0.09
Gravidity					
Primigravida	2	10	1		
Secundigravida	13	8	8.1	1.4–46.9	0.027
Multigravida	5	2	12.5	1.3–116.8	0.045
Education					
Primary	2	4	1		
Secondary	17	14	2.4	0.05–18.1	1.0
Tertiary	1	2	1	0.2–29.6	0.58
Employment					
Working	13	12	1		
Not working	7	8	1.2	0.3–4.5	0.94
Gestational age at booking					
First trimester	2	7	1		
Second trimester	16	10	5.6	1–32.5	0.059
Third trimester	2	3	2.3	0.2–25.2	0.58
Anaemia					
Yes	16	11	3.2		
No	4	9	1	0.8–13.4	0.18
LLIN use					
Yes	6	5	1		
No	14	15	1.29	0.3–5.2	0.93

+ = Present; – = absent; OR = odds ratio; 95% CI = 95% confidence interval. $p < 0.05$ was considered to be significant.

prevalence of triple and quintuple mutations in these genes was 35 and 5%, respectively, for the mutant genotype forms, and 50 and 15%, respectively, for both the mutant and the mixed genotype forms. Univariate logistic regression analysis revealed a significant ($p < 0.05$) 8.1–12.5-fold increase in the risk of triple *dhfr* mutation in secundigravida and multigravida pregnant women. However, the observed 5.6–8.1-fold increased risk of this mutation among the pregnant women aged >20 years and among those who booked during the second trimester of pregnancy was not significant ($p > 0.05$; table 5).

Discussion

The 43% higher detection rate of ASM by RDT than the 31.8% rate by microscopy showed an improvement in malaria detection using histidine-rich protein-2 RDT and further confirmed the ability of this immunochromatographic test to detect submicroscopic parasitaemia common with ASM, which is more possible during pregnancy due to the placental sequestration of the parasite [2, 24]. However, the observed 6 false negative RDT results that were further confirmed by msp2 genotyping to be truly false highlights one of the drawbacks of using RDT for case management because of the persistence after the clearance of parasitaemia. In fact, studies have shown that this antigen can persist in the peripheral circulation for 28–42 days [25]. It is important to note that these truly negative RDT results occurred among the multigravida subjects (results not shown). A plausible explanation of this observation could be due to the influence of antiparasitic immunity, which improves with subsequent pregnancies [2, 11, 24]. Despite this drawback, our finding supported the scaling up of histidine-rich protein-2 RDT for the diagnosis of ASM in the context of malaria elimination in the study area, as this could further enhance the early detection of parasite reservoirs among the pregnant women population of the study area. This approach has also been demonstrated to be more effective, cost saving and cost effective compared to microscopy in the Democratic Republic of Congo [26]. With regard to the presence of SP resistance in the study area, the present study revealed prevalence rates of 70, 75 and 80% for single mutations at codons 51, 59 and 108 in the homozygous or heterozygous forms that resulted in a 50% prevalence rate of triple dhfr mutant parasites circulating in the study area. These molecular indices are higher than the 64.1, 61.5, 38.5 and 17.5% reported by Ojurongbe et al. [15] for *P. falciparum* among ASM pregnant women in Lafia, North-Central Nigeria. But they are lower than the 84–91% for 51I, 88 versus 87% for 59R and 95–97% for 108N reported by the same group among the *P. falciparum* population recovered from children with uncomplicated malaria in Osogbo, South-West Nigeria [14, 15]. This difference could be attributed to a variation in the level of use of SP and sulpha-related drugs such as pyrimethamine and cotrimoxazole in the country [9, 11]. For the first time in Lagos, Nigeria, the present study has found an emergence of mutant *P. falciparum* isolates carrying sulphadoxine resistance-associated A437G and K540E mutations in the *dhps* genes at rates of 37.5 and 22.5%, respectively, resulting in 15% of the parasite population carrying quintuple mutations in the *dhfr* plus *dhps* genes. Several studies have shown that the K540E mutation is not very common in West Africa compared to the East African and South African regions of the continent [18, 22, 27, 28]. The double dhps

mutant rate found in this study not only validates low SP resistance in the West African subregion compared to East Africa [12, 13], but also indicates varying levels of sulphadoxine pressure among countries in West Africa. The use of cotrimoxazole for prophylaxis or treatment is common among HIV patients and children with pneumonia, of which Nigeria carries a huge burden in Africa [29]. This has been shown to drive the emergence, spread and intensification of the A437G and K540E mutations in *pfdhps* [30]. With regard to the level of triple dhfr mutation found in this study, the disuse of pyrimethamine for prophylaxis especially among the sickle cell population, of which Nigeria also bears a huge burden in Africa, is also recommended both in the study area and in Lagos as a whole. This study also supports the replacement of SP with the artemisinin combination therapy for treating uncomplicated malaria in children and adults proposed in 2005 despite the lack of molecular evidence at the time of the policy change [8, 9]. However, in spite of reduced SP pressure, findings from this study have further confirmed the circulation of *P. falciparum* with SP resistance in Lagos with the implication of the SP pressure in the study area being higher than that of Lafia among the pregnant women population [15]. Generally, the higher prevalence rate of the 108N mutation is not surprising, since this mutation has been established by previous studies as the first to be selected in *dhfr* following exposure to pyrimethamine ex vivo to cause low-level resistance to pyrimethamine [12, 13]. An additional selection of mutations at codons 51 and 59 would only increase the level of this resistance [12, 13]. Also, in this study, the mutation rates of single mutations in the *dhfr* and *dhps* genes of the parasites were higher than the rates of the triple and quintuple mutant parasites, because 25–40% heterozygotes for these mutations were analysed as mutants. Based on this finding, the present study suggests a low to moderate level of SP resistance in the study area, thereby supporting the continued use of SP for IPTp. This is in view of the fact that the mutation rates of the studied SP resistance markers have not reached fixation levels in the study area compared to many endemic areas in East and South Africa [12, 30]. Meanwhile, these findings validate the previous studies in the West-African subregion in which non-fixation of SP resistance markers was reported and the continued use of IPTp-SP was recommended [13, 27, 28]. Our findings reveal multigravida and older age as risk factors for the asymptomatic carriage of *P. falciparum* and thus identify older and multigravida pregnant women as a potential target population for malaria elimination in the country. This population is also likely to harbour parasites with low-to-moderate levels of SP resistance and may warrant being treated according to the national treatment guidelines to further avert the dissemination of SP resistance in the study area in particular and in Lagos as a whole.

Based on these findings, the following necessities are pointed out: (a) the need for the clinical trial of SP as presently done for the artemisinin combination therapy at sentinel sites and other study sites like the present one to further monitor the effectiveness of the IPTp-SP intervention, and (b) the strengthening of strategies that will prevent an irrational use of SP especially in the informal health system and in the private sector where monotherapies such as chloroquine and SP are still prescribed for malaria treatment in the country [10]. The limitations of this study include the small size of the studied population and the cross-sectional approach used. The latter prevented the investigation of the association between the observed level of SP resistance and pregnancy outcome. Moreover, the II64L mutation in *pfdhfr*, associated with placental parasitaemia and low-birth-weight babies, was not investigated in this study. Despite evidence that SP resistance is associated with maternal morbidity, the lack of association between the triple dhfr mutation and maternal anaemia observed in this study may also be attributed to the small sample size of the study.

Conclusion

This study revealed the emergence of the K540E mutation among parasite populations in coastal Lagos and established the usefulness of PCR-corrected histidine-rich protein-2 RDT for an improved detection of ASM in pregnancy. While supporting its use due to non-fixation of the SP resistance markers, continuous monitoring of IPTp-SP effectiveness in the study area is recommended.

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