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High prevalence of sulphadoxine–pyrimethamine resistance-associated mutations in *Plasmodium falciparum* field isolates from pregnant women in Brazzaville, Republic of Congo

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ABSTRACT

Intermittent preventive treatment during pregnancy with sulfadoxine–pyrimethamine (IPTp-SP) has not been evaluated in the Republic of Congo since its implementation in 2006 and there is no published data on molecular markers of SP resistance among *Plasmodium falciparum* isolates from pregnant women. This first study in this country aimed to describe the prevalence of dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) point mutations and haplotypes in *P. falciparum* isolates collected from pregnant women with asymptomatic infection. From March 2012 to December 2013, pregnant women attending Madibou health centre (in Southern Brazzaville) for antenatal visits were enrolled in this study after obtaining their written informed consent. Blood samples were collected and *P. falciparum* infections were characterized using PCR. A total of 363 pregnant women were enrolled. *P. falciparum* infection was detected in 67 (18.4%) samples as their PCR amplification of *dhfr* and *dhps* genes yielded bands and all the PCR products were successfully digested. Out of these 67 isolates, 59 (88%), 57 (85%) and 53 (79.1%) carried 51I, 59R and 108N *dhfr* mutant alleles, respectively. The prevalence of *dhps* 436A, 437G and 540E mutations were 67.1% (45/67), 98.5% (66/67) and 55.2% (37/67), respectively. More than one-half of the isolates carried quintuple mutations, with highly resistant haplotype *dhfr*51I/59R/108N + *dhps*437G/540E detected in 33% (22/67) whereas 25% (17/67) were found to carry sextuple mutations. We observed significantly higher frequencies of triple *dhps* mutations 436A/437G/540E and quintuple mutations *dhfr*51I/59R/108N + *dhps*437G/540E in isolates from women who received IPTp-SP than those who did not. Overall, this study shows high prevalence rates of SP-associated resistance mutations in *P. falciparum* isolates collected from pregnant women. The presence of the *dhps* mutant allele 540E and the high prevalence of isolates carrying quintuple *dhfr*/*dhps* mutations are here reported for the first time in the Republic of Congo. The increasing prevalence of multiple mutant alleles observed in this study is alarming and may present a challenge for the future interventions including IPTp-SP in the country.

1. Introduction

Malaria is a major parasitic disease in sub-Saharan Africa and South-East Asia where it remains a major public health problem. According to the last World Health Organization (WHO) report, 198 million clinical cases of malaria occurred globally in 2013 and the disease led to 584,000 deaths (WHO, 2014). Approximately 90% and 78% of clinical cases and malaria deaths, respectively, occurred in children and pregnant women in sub-Saharan Africa (WHO, 2014, 2012a).

Over the past decade, malaria elimination and/or eradication have been placed on the agenda of the Global Malaria Action Plan (WHO, 2007). Successful achievements in malaria control are reported from several countries like Eritrea, Tanzania (in Zanzibar islands), Senegal, Sao Tome and Principe, and Zambia based essentially on concerted efforts using artemisinin-based combination therapies (ACTs), insecticide treated nets (ITN),

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insecticide indoor residual spraying, and intermittent preventive treatment (IPT) in children and pregnant women (WHO, 2012b). To reduce disease burden in pregnant women exposed to malaria infection, more specifically to prevent deleterious effects of pregnancy-associated malaria, sulphadoxine–pyrimethamine (SP) has been recommended by the WHO for IPT of malaria during pregnancy (IPTp) in this vulnerable population (WHO, 2004). Studies conducted in Mozambique (Menéndez et al., 2010), Burkina Faso (Grietens et al., 2010), Senegal (Olliaro et al., 2008) and Ghana (Wilson et al., 2011) have shown that IPTp reduces the prevalence of maternal malaria infection, especially reduction in peripheral and placental blood, anaemia and episodes of malaria. This preventive measure has also been reported to have an impact on the outcomes of pregnancy (e.g. newborn's weight at birth and neonatal mortality) (Menéndez et al., 2010; Tonga et al., 2013) even though data from Tanzania (Harrington et al., 2013, 2011) modulates these positive findings. Indeed, IPTp-SP in Tanzanian pregnant women has been shown to be associated with increased risk of severe malaria in their offspring and the cost-benefit analyses of IPTp-SP should also consider the long term effects on offspring in addition to pregnancy outcomes (Harrington et al., 2013).

Sulphadoxine–pyrimethamine efficacy is dependent on a number of mutations which may accumulate in *Plasmodium falciparum* dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes which code for the proteins DHFR and DHPS, respectively (Moussiliou et al., 2013). Mutations in codons 51 (N51I), 59 (C59R), 108 (S108A) and 164 (I164L) of the *dhfr* gene and in codons 436 (S436A), 437 (437G), 540 (G540E), 581 (A581G) and 613 (A613S) of the *dhps* gene are associated with resistance to pyrimethamine and sulphadoxine *in vitro*, respectively (Gregson and Plowe, 2005; Plowe et al., 1998). Parasite resistance to SP *in vivo* is largely associated with a triple mutation in *dhfr* gene (51I/59R/108A) coupled with a double mutation in *dhps* gene (437G/540E) (Omar et al., 2001; Kublin et al., 2002; Kyabayinze et al., 2003). Recently, a study conducted in Benin reported a high prevalence of *dhfr*/*dhps* quadruple mutants (triple *dhfr* + single *dhps*) in isolates from pregnant women and a frequency of recrudescence reaching 76% after the second dose of IPTp-SP (Moussiliou et al., 2013). Investigations conducted in endemic countries such as Gabon (Mombo-Ngoma et al., 2011), Ethiopia (Mula et al., 2001), Kenya (Iriemenam et al., 2012), and Malawi (Lin et al., 2013) showed that the prevalence of variants in these genes is different according to the level of local SP resistance.

In the Republic of Congo, a country with a perennial transmission, malaria remains the leading cause of consultations, accounting for 55% of hospital admissions and 35% of children death in health facilities of Brazzaville and Pointe-Noire, the two largest cities (Ministère de la santé et de la population, 2010). Since 2006, the national guidelines have been supporting the distribution of insecticide-treated nets to each pregnant woman at the first antenatal visit and administration of two or three doses of SP for malaria prevention during pregnancy. The effectiveness of IPTp-SP has not been evaluated so far and no published data on molecular markers of SP resistance among *P. falciparum* isolates from pregnant women is available. Previous studies conducted before the change of national guidelines for the treatment of uncomplicated malaria (in 2006) in Makélékélé district in the Southern part of Brazzaville reported approximately 10–47% *in vivo* resistance to SP in children (Ndounga et al., 2007a; Nsimba et al., 2004) and high prevalence rates of *dhfr* and *dhps* mutant genotypes (Ndounga et al., 2007b; Nsimba et al., 2005).

In the present work, we propose to investigate in the same area, the prevalence of *dhfr* and *dhps* point mutations in *P. falciparum* isolates collected from pregnant women who either received IPTp-SP or not.

2. Methods

2.1. Study site

This study was conducted at Madibou Integrated health centre located in the semi-urban area of Makélékélé, currently named Madibou district, in Southern Brazzaville, Republic of Congo. This area gathers about 6000 inhabitants and is located along the Congo river where malaria transmission is high and occurs all year round with an entomological inoculation rate of 22.5 infective bites/person/year estimated several years ago (Trape and Zoulani, 1987). *P. falciparum* is the predominant plasmodial species and *Anopheles gambiae* s.s the main mosquito vector.

2.2. Study population, ethical consideration and blood sample collection

Pregnant women attending Madibou health centre for their antenatal visits were enrolled in this cross-sectional study. From March 2012 to December 2013, these pregnant women were adequately informed about the purpose of the study and consecutively included after obtaining their written informed consent and that of their parents for those who were less than 18 years old. This study was approved by the Institutional Ethics Committee of the Fondation Congolaise pour la Recherche Médicale (Avis N° 001/CEI/FCRM/2012). For all eligible women, demographic data were obtained through interviews using a structured questionnaire and from medical records. About 4 ml of whole blood were collected in an EDTA tube for haemoglobin concentration measurement using the haematology analyzer (ABX Micros ES60) and DNA extraction.

2.3. Genomic DNA extraction

Genomic DNA was extracted from 200 µl of whole blood sample using the QIAmp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. DNA was recovered in 150 µl of elution buffer and stored at −20 °C until use.

2.4. Genotyping of dhfr and dhps point mutations

PCR-restriction fragment length polymorphism (PCR-RFLP) was performed to screen different variants in *dhfr* (codons 51, 59 and 108) and *dhps* (436, 437 and 540) genes as previously described (Duraisingh et al., 1998). For enzymatic digestion reactions, 5 µl of PCR products were incubated with specific restriction enzymes (*Tsp509I*, *XmnI*, *BsrI*, *Mn1I*, *AvaII*, and *FokI* for codons 51, 59, 108, 436, 437 and, 540 respectively) (New England Biolabs, Beverly, MA, USA) following the manufacturer's instructions. In all PCRs and enzymatic digestion reactions, a set of positive controls was included. All PCR products and digested products were electrophoresed on 2% agarose gels, and visualized under UV transillumination after SYBR Green staining.

2.5. Data analysis

Statistical analysis was done using XLSTAT software, version 2011.2.08. Haplotypes were assigned based upon the number of codons considered. For instance, the "quadruple" or the "quintuple" mutant haplotype consisted of at least "4" or "5" mutant alleles. The χ^2 test and the fisher exact *T* test were used to compare quantitative variables between groups. Differences were considered statistically significant at a two-sided *P* values of <0.05.

3. Results

3.1. Characteristics of study participants

A total of 363 pregnant women participated to this study and their characteristic as shown in Table 1. Overall, the mean age of these women was 25.7 years old and the mean gestational age was 26.3 weeks. The majority of the women, 205 (56.5%), attended Madibou health centre for their first antenatal visit, while, 43 (11.8%) and 115 (31.7%) had 2 and ⩾3 visits, respectively. More than one-half of the women (53.5%) were multigravidae. Almost two-third of the study population (62.8%) had not received any IPTp-SP dose and only 21% had received 2 or 3 doses. Anaemia, defined as haemoglobin level <11 g/dl, was observed in 184 (51%) women, while, *P. falciparum* infection was detected in 67 (18.4%) samples as their PCR amplification of *dhfr* and *dhps* genes yielded bands and all the PCR products were successfully digested. Out of these 67 *P. falciparum* positive samples, 61 were from the women who had not received any IPTp-SP dose.

3.2. Prevalence of P. falciparum dhfr and dhps mutant alleles and haplotypes

Of 67 *P. falciparum* isolates, 59 (88%), 57 (85%) and 53 (79.1%) carried 51I, 59R and 108N *dhfr* point mutations, respectively (Table 2). Almost 60% (40/67) of the isolates harboured the triple *dhfr* mutation (51I/59R/108N). The prevalence of *dhps* 436A, 437G and 540E mutations were 67.1% (45/67), 98.5% (66/67) and 55.2% (37/67), respectively. Triple *dhps* mutation 436A/437G/540E was detected in 43.3% of the isolates. When considering both, *dhfr* and *dhps* genes, more than one-half of the isolates carried quintuple mutations with highly resistant haplotype *dhfr*51I/59R/108N + *dhps*437G/540E detected in 33% (22/67) whereas 25% (17/67) were found to have the sextuple mutation *dhfr*51I/59R/108N + *dhps*436A/437G/540E (Table 2).

We compared the frequency of multiple mutations between isolates from pregnant women who had not received any IPTp-SP and that of those who had at least one dose (Table 3). We found that the frequency of the triple *dhps* mutation 436A/437G/540E was significantly higher in isolates from pregnant women who had received at least 1 IPTp-SP dose (*P*-value = 0.038). The same observation was made with the quintuple mutation *dhfr*51I/59R/108N + *dhps*437G/540E, however, the difference was only marginally significant (*P*-value = 0.064).

Table 1
Characteristics of pregnant women enrolled in the study.

Characteristics	Values (*N* = 363)
Mean age ± SD (years)	24.7 ± 6.4
Mean gestational age ± SD (weeks)	26.3 ± 5.1
Antenatal visits	
1	205 (56.5%)
2	43 (11.8%)
⩾3	115 (31.7%)
Gravidity	
Primigravidae	95 (21.1%)
Secundigravidae	70 (19.3%)
Multigravidae	190 (53.5%)
Unknown	8 (2.2%)
IPTp-SP	
No	228 (62.8%)
1 dose	59 (16.3%)
2 or 3 doses	76 (20.9%)
Haemoglobin level	
<11 g/dl	184 (51%)
⩾11 g/dl	173 (48.5%)
Unknown	6 (1.6%)
P. falciparum infection	67 (18.4%)

Table 2
Prevalence of *dhfr* and *dhps* mutant alleles and haplotypes.

Mutated codons						*n* (%) of isolates (*N* = 67)
dhfr			*dhps*			
51I	59R	108N	436A	437G	540E	
+	+	+	+	+	+	17 (25.8)
+	+	+	+	+	–	9 (13.4)
–	–	–	+	–	–	1 (1.5)
–	+	+	+	+	+	2 (3)
+	–	+	+	+	+	3 (4.5)
+	+	–	+	+	+	5 (7.4)
+	+	+	–	+	+	5 (7.4)
–	+	+	+	+	–	2 (3)
–	+	–	+	+	–	1 (1.5)
+	+	–	+	+	–	2 (3)
–	+	–	+	+	+	2 (3)
+	–	+	+	+	–	1 (1.5)
+	–	+	–	+	–	3 (1.5)
+	–	+	–	+	+	2 (3)
+	+	+	–	+	–	9 (13.4)
+	+	–	–	+	+	1 (1.5)
+	+	–	–	+	–	2 (3)

4. Discussion

The present study is the first to report the prevalence of *dhfr* and *dhps* point mutations in *P. falciparum* isolates from pregnant women in the Republic of Congo. The infection was characterized in pregnant women with asymptomatic malaria infection attending Madibou integrated health centre for their antenatal visits. The prevalence of asymptomatic infection was 18% which is relatively low compared to 90% recently reported in Gabon (Tshibola Mbuyi et al., 2014) but in Gabon asymptomatic carriage was not influenced by IPT-p dose.

The findings show high prevalence rates of mutant alleles in *dhfr* codons 51, 59 and 108 as well as in *dhps* codons 436, 437 and 540 with the mutant allele 437G being nearly fixed (98.5%). Accordingly, previous studies analysing molecular markers of SP resistance at the time that the SP was used as second line treatment of uncomplicated malaria (1999–2005) showed high prevalence rates of mutations at codons 51, 59 and 108 of the *dhfr* gene and at codon 437 of *dhps* in *P. falciparum* isolates collected from children and non-pregnant adults (Ndounga et al., 2007b; Nsimba et al., 2005). SP treatment failure rate when used for uncomplicated malaria was reported to be about 30% in children aged from 6 months to 5 years in 2003–2005 (Ndounga et al., 2007b). However, in contrast to these previous studies whereby the frequency of the *dhps* mutant allele 436A was low and 540E allele was absent, we found higher frequencies of these two mutations in this study with the presence of the *dhps* mutant allele 540E here reported for the first time in the Republic of Congo. Our findings point to a continue accumulation of additional resistant variants in *dhfr* and *dhps* genes such as the *dhps* 581G and consequently to aggravation of SP resistance. This was recently demonstrated in studies conducted in Malawi and Tanzania where the presence of the *dhps* mutant allele 581G was associated with reduced efficacy of IPTp-SP (Gutman et al., 2015; Gesase et al., 2009). In the present study, the mutation 581G was not investigated.

The combination analyses of these mutations also revealed high prevalence of triple, and quadruple or more mutations. Notably, more than one third of the isolates (32%) harboured the quintuple mutant haplotype which is known as a better predictor of SP

Table 3
Frequencies of multiple mutant haplotypes in *P. falciparum* isolates from pregnant women who received IPTp-SP and those who did not.

Gene	Mutations	No IPTp *n*/*N* (%)	IPTp taken* *n*/*N* (%)	*P*-value
dhfr	51I + 59R + 108N	36/61 (59%)	4/6 (66.6%)	0.715
dhps	436A + 437G + 540E	24/61 (39.3%)	5/6 (83.3%)	0.038
dhfr + *dhps*	51I + 59R + 108N + 436A + 540E	14/61 (23%)	3/6 (50%)	0.146
	51I + 59R + 108N + 437G + 540E	18/61 (29.5%)	4/6 (66.6%)	0.064
	51I + 59R + 108N + 436A + 437G + 540E	14/61 (23%)	3/6 (50%)	0.146

* ⩾1 IPTp-SP dose, *n*: number of isolates carrying the considered haplotype, *N*: total number of isolates in the group.

treatment failure consisting of the triple *dhfr* haplotype 51I/59R/108N and the double *dhps* haplotype 437G/540E. Our findings are similar to those previously reported by Bouyou-Akotet et al. (2010) at the time of introduction of IPTp-SP in Gabon where the prevalence rates of triple, quadruple and quintuple mutations were 80%, 53% and 22%, respectively. Recent studies from other African countries also reported increased prevalence of multiple mutations in *dhfr* and *dhps* genes in *P. falciparum* isolates collected from pregnant women (Lin et al., 2013; Iriemenam et al., 2012; Bertin et al., 2011; Mockenhaupt et al., 2008). Despite the absence of previous data on the prevalence of SP resistance markers in isolates from pregnant women in the Republic of Congo for making a clear comparison, the increased prevalence of multiple mutant alleles observed in this study is alarming and may present a challenge for the future usefulness of IPTp-SP intervention in the country. This calls for regular monitoring of this strategy, through multi-site assessment of SP resistance markers in *P. falciparum* isolates from pregnant women as well as periodic evaluation of IPTp-SP efficacy with measurement of prophylactic endpoints.

The results of studies assessing the effect of IPTp-SP on selection of parasites harbouring multiple SP resistance alleles are controversial. In studies conducted in Malawi and Tanzania it was observed that IPTp-SP use increases the fraction of *dhfr*/*dhps* resistance alleles (Lin et al., 2013; Harrington et al., 2009). In contrast to these findings, investigations in Ghana by Mockenhaupt et al. (2008) demonstrated that IPTp-SP is not causally involved in selection of resistance alleles. In this study, we compared the frequencies of multiple *dhfr*/*dhps* mutations in isolates from women recruited before taking IPTp and those who had previously received IPTp. We observed higher frequencies of parasites carrying multiple mutations in IPTp-SP group, particularly that of triple *dhps* mutations 436A/437G/540E with a significant difference while the difference of quintuple mutations *dhfr*51I/59R/108N + *dhps*437G/540E was marginally significant. This suggests that IPTp-SP appears to be causally involved in selection of resistant parasites. However, the limited number of *P. falciparum* isolates obtained from pregnant women who received SP and the cross-sectional approach used in this study may render our observation speculative. Therefore, investigations on this population should be further conducted using a larger sample size and a follow-up of pregnant women until delivery and recording of parameters such as maternal anaemia and neonates' weight should provide important data for the evaluation of this preventive intervention in the Republic of Congo. Additionally, it is important to note that SP is still available in the local pharmacies and auto-medication remains an important issue in the country. Therefore, authorities should also consider restricting SP to IPTp only, by allowing its availability in antenatal health centres only.

In conclusion, this study shows high prevalence of SP-associated resistance mutations in *P. falciparum* isolates collected from pregnant women. Importantly, the presence of the *dhps* mutant allele 540E and the high prevalence of isolates carrying quintuple mutations have not been yet reported so far in the Republic of Congo. The increasing prevalence of multiple mutant alleles observed in this study is alarming and may present a challenge for the future malaria control intervention including IPTp-SP in the country. In addition, evaluation of alternative preventive treatment options should be prioritized.

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