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Characterization of asymptomatic *Plasmodium falciparum* infection and its risk factors in pregnant women from the Republic of Congo

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ABSTRACT

Malaria in pregnancy remains a serious public health problem in the Republic of Congo despite the implementation of intermittent preventive treatment with sulfadoxine-pyrimethamine (IPTp-SP) in 2006. The aim of this cross-sectional study was to characterize *Plasmodium falciparum* infections and determine possible risk factors in pregnant Congolese women attending an antenatal clinic in a periurban area of southern Brazzaville. This study was conducted from March 2012 to December 2013 in a site where several years ago, high malaria resistance to SP was reported. Pregnant women were enrolled during antenatal visits and the number of received IPTp-SP doses was recorded as well as individual sociodemographic data. Peripheral blood was collected and *P. falciparum* infection was checked by microscopy and by PCR targeting *P. falciparum* merozoite surface protein gene (msp2). Haemoglobin concentration was measured and *P. falciparum* positive samples were typed for msp2 allelic diversity. A total of 363 pregnant women were recruited. The prevalence of asymptomatic *P. falciparum* infection was 7% and 19% by microscopy and by PCR, respectively. More than one half (51.5%) of the pregnant women were anaemic. Multivariate analysis indicated that *P. falciparum* infection was associated with anaemia. It was also observed that women who have received IPTp-SP have significantly lower prevalence of infection. The administration of IPTp-SP did not influence the multiplicity of infection (MOI). This first study investigating asymptomatic malaria infection on pregnant women of the Republic of Congo shows that *P. falciparum* infections were clearly associated with maternal anaemia, and use of IPTp-SP reduced the risk of carrying asymptomatic infections.

1. Introduction

Malaria during pregnancy is a major public health problem in endemic countries of sub-Saharan Africa. Indeed, malaria-associated maternal anaemia and deaths as well as premature delivery and low birth weight are reported consequences (Dicko et al., 2003; WHO, 2013). In high malaria transmission settings, carriage of *Plasmodium falciparum* (*P. falciparum*) parasites without presenting symptoms (asymptomatic infection) reflects acquisition of semi-immunity (Matangila et al., 2014). This so called "asymptomatic infection" during pregnancy is also associated with placental sequestration of parasites and significant reduction of maternal haemoglobin level (Douamba et al., 2012; Matangila et al., 2014). Additionally, age and the number of pregnancies of the mother are both factors influencing the course of *P. falciparum* infection during pregnancy (Campos et al., 2012; Jäckle et al., 2013; Kurth et al., 2010; Steketee et al., 1996).

In order to prevent malaria infections during pregnancy, the World Health Organization (WHO) recommends a package of interventions including the use of long-lasting insecticide nests (LLINs) and administration of intermittent preventive treatment with sulfadoxine-pyrimethamine (IPTp-SP) at each routine antenatal visit starting from the second trimester of pregnancy (WHO, 2004, 2012). However, some reports pointed out the negative impact of IPTp-SP in areas of existing high-grade *P. falciparum* resistance to SP

Abbreviation: WHO, World Health Organization; LLINs, long-lasting insecticide nests; IPTp-SP, intermittent preventive treatment during pregnancy with sulfadoxine-pyrimethamine; *P. falciparm*, *Plasmodium falciparum*; ACTs, artemisinin-based combination therapies; *msp2*, merozoite surface protein 2 gene; MOI, multiplicity of infection; AOR, adjusted odd ratio.

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(Harrington et al., 2011; Lin et al., 2013). Therefore, it appears crucial to monitor the effectiveness of these prevention interventions. Screening for the presence of asymptomatic malaria infection during pregnancy, not only by using microscopy which is the standard tool but also by polymerase chain reaction (PCR) techniques which allows detection of parasites far below the threshold of microscopy, may contribute to better understand malaria infection and also to determine the importance of this population as a reservoir for transmission.

The Republic of Congo (RoC), located in Central Africa, is an endemic malaria country with a perennial transmission (WHO, 2013). The use of IPTp-SP (2 or 3 doses) has been recommended in the country since 2006. For the treatment of uncomplicated malaria, arthemeter–lumefantrine and artesunate–amodiaquine are recommended as first and second line, respectively. So far, only one study was performed to evaluate the prevalence of uncomplicated clinical malaria cases among pregnant women (Ntoumi et al., 2013) which was found to be about 9% and 15% by microscopy and PCR respectively. No investigation was conducted so far in asymptomatic pregnant women.

In order to investigate the impact of implementation of malaria prevention strategies among pregnant women and to prepare sites for future malaria interventions in RoC, the present cross-sectional epidemiological study was conducted at antenatal care unit of a public health centre located in Southern area of Brazzaville. Our previous studies in this site showed about 30% *in vivo* resistance to SP in children and high frequencies of point mutations in *P. falciparum* dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps) genes; specifically *P. falciparum* isolates were harbouring 99% and 85% mutation on point 108 for dhfr, and on point 437 for dhps, respectively (Ndounga et al., 2007a,b). The objectives of the present study were: (i) to assess the prevalence of asymptomatic *P. falciparum* infection and possible associated risk factors in pregnant women, and (ii) using *msp2* as a marker, to investigate parasite diversity and multiplicity of infections (MOI) in field isolates collected from infected women.

2. Methods

2.1. Study site

This study was conducted at Madibou health division in southern area of Brazzaville which is the capital of the RoC. This area is semi-urban with about 6000 inhabitants and located along the Congo River where malaria transmission is high and occurs all year round with an entomogical 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. Malaria surveillance studies recently conducted in this area reported a prevalence of asymptomatic *P. falciparum* infection of 16% among children aged less than 10 years old (Koukouikila-Koussounda et al., 2012).

2.2. Study population, ethical consideration, demographic data collection and blood sampling

Pregnant women attending integrated health centre of Madibou for their antenatal visit participated in this cross-sectional study. Inclusion criteria were: no history of clinical malaria for at least two weeks before the day of enrolment, no history of fever for at least 48 h before enrolment and an axillary temperature $\leq$37.5 °C during the examination. Asymptomatic malaria infection was defined as presence of *P. falciparum* parasites detected by microscopy or by PCR. Positive detection by PCR only was considered to be asymptomatic sub-microscopic malaria infection.

From March 2012 to December 2013, pregnant women were informed about the study purpose and those who fulfilled the inclusion criteria were consecutively enrolled after obtaining their written informed consent and that of their parents for those who were less than 18 years old. 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 thick and thin blood smears, haemoglobin concentration measurement using the haematology analyzer (ABX Micros ES60) and parasite DNA extraction. This study was approved by the Institutional Ethics Committee of the Fondation Congolaise pour la Recherche Médicale (N° 001/CEI/FCRM/2012).

2.3. Microscopic examination

Thick and thin blood films were prepared and stained with 10% Giemsa for 15 min. After cleaning, these slides were read by two independent competent laboratory technicians to determine malaria species and the parasite density. Asexual parasites were counted against 200 leucocytes and expressed as the number of asexual parasites/μl of blood, assuming the leukocyte count of 8000/μl of blood.

2.4. 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.

2.5. Plasmodium falciparum msp2 genotyping

In order to (1) detect asymptomatic submicroscopic infections and (2) characterize *P. falciparum* isolates (by) determining the genetic diversity and multiplicity of infection), nested PCR assays for *P. falciparum msp2* central region, which comprises repeats of varying lengths were performed on all samples. The specific primers and PCR conditions were as previously described by Ntoumi et al. (2000). The FC27 and 3D7-type allele families were identified and analyzed.

3. Data analysis

P. falciparum infections were classified as microscopically positive and/or PCR positive (which includes microscopically negative blood smears). Sub-microscopic infections were identified as infections detected only by PCR. The prevalence of *msp2* FC27 and 3D7-type alleles was determined as the number of all alleles for each type out of the total number of alleles. The MOI, defined as the mean number of *msp2* alleles per infected pregnant women positive by PCR (Ntoumi et al., 2000). Statistical analysis was done using XLSTAT software v. 2011.2.08. The association between the prevalence of *P. falciparum* infection (screened by microscopy or by PCR) and independent variables, including age, gravidity, use of IPTp-SP and haemoglobin level, was first assessed by simple logistic regression with each independent variable. The consistency of these associations was then tested by multivariate logistic regression models to control for possible influencing factors and to identify only true independent associations. The Kruskal–Wallis one-way analysis of variance by ranks and chi-squared tests were used to compare the prevalence of multiclonal *P. falciparum* infection and MOI in different groups. Differences were considered statistically significant at P values <0.05.

Table 1
Characteristics of Congolese pregnant women recruited in the study from March 2012 to December 2013.

Characteristics	Values (N = 363)
Age (years)	
Mean (SD)	24.7 (6.4)
Range	12–44
< 18	37 (10.2%)
≥ 18	326 (89.8%)
Gestational age (weeks)	
Mean (SD)	26.3 (5.1)
Antenatal visits	
1	205 (56.5%)
2	43 (11.8%)
≥ 3	115 (31.7%)
Education level	
Illiterate	3 (0.9%)
Primary school	72 (19.9%)
Secondary school	207 (57.3)
High school	64 (17.7%)
University	15 (4.2%)
Do not know	2 (0.5%)
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	184 (51.5%)
≥11	173 (48.5%)
Not determined	6 (1.6%)
P. falciparum infection	
Microscopy	25 (7%)
GMPD (SD) in (P/μl)	1188.35 (301.42)
PCR	68 (19%)

4. Results

4.1. Characteristics of the study population

A total of 363 pregnant women participated in the study with a mean age of 24.7 years (Table 1). The mean gestational age (SD) was 26.3 (5.1) weeks and the majority of the participants were adults (89.8%), attending the health centre for their first antennal visit (56.5%), had received basic education (19.9% primary school and 57.3% secondary school), were multigravidae (were pregnant ≥3 times) (53.5%) and had not yet received any IPTp-SP (62.8%). Among the pregnant women, 184 (51.5%) were anaemic (haemoglobin level <11 g/dl) (Table 1).

4.2. Prevalence of P. falciparumn asymptomatic infection

We observed that 25 (7%) of the studied pregnant women were found to harbour asymptomatic *P. falciparum* infection detectable by microscopy, while 68 (19%) had *P. falciparum* infection detected by PCR (Table 1). All microscopically positive samples yielded a positive PCR result and *P. falciparum* was the solely plasmodial species identified microscopically. 43 (12%) pregnant women found negative by microscopy were revealed positive by PCR therefore having a submicroscopic infection.

4.3. Prevalence of P. falciparum infection according to age, gravidity, use of IPTp-SP and haemoglobin level

The prevalence of asymptomatic *P. falciparum* infection in pregnant women (determined by microscopy and by PCR) was classified according to their haematological and socio-demographic status (Table 2).

Based on the results of microscopy, the multivariate analysis revealed that the risk of experiencing asymptomatic infection was significantly reduced in multigravidae than in primigravidae (adjusted odd ratio, AOR (for age and IPTp-SP) 0.23, 95% CI [0.08–0.60]). However, no association was found when considering submicroscopic infection.

Pregnant women who received 2 or 3 IPTp-SP doses had lower risk (odds ratio adjusted for age and gravidity) of harbouring microscopically detectable asymptomatic *P. falciparum* infection compared to those who didn't received any IPTp-SP or those who received only one dose (adjusted odd ratio (AOR), 0.11, 95% CI [0.01–0.87]). However, when considering submicroscopic infection, any IPTp was significantly at lower risk of experiencing infections than no IPTp-SP (AOR 0.09, 95% CI[0.02–0.42)]; AOR 0.18, [0.06–0.48]).

An association was also demonstrated between asymptomatic infections and anaemia (haemoglobin level <11 g/dl). Regardless of the diagnosis technique, non-infected women had decreased risk (adjusted for age, gravidity and IPTp-SP) of experiencing anaemia than those who were infected (Table 2).

Parasite densities did not differ significantly according to clinical and sociodemographic status (data not shown). No significant association was also observed between IPTp-SP and anaemia (data not shown).

4.4. Determination of Plasmodium falciparum msp2 allele diversity

Of the 68 positive samples for *P. falciparum* detected by PCR using *msp2* marker (Block 3), FC27 and 3D7 alleles were successfully defined for 62 (91.18%) isolates. Overall, for the 62 isolates successfully characterised, 84 distinct *msp2* fragments or alleles were detected, out of which, 32 (38%) of the FC27 allelic family and 52 (62%) of the 3D7 family. Fragments of FC27 allelic family belonged to 11 different allele types with the sizes ranging from 300 to 600 bp while that of 3D7 allelic family belonged to 18 different allele types with the length variation of 200 to 700 bp.

4.5. Multiplicity of P. falciparum infection according to age, gravidity, IPTp-SP doses and haemoglobin level

As presented in Table 3, we observed that 25/62 (40.3%) of the isolates carried more than one *msp2* genotype and the overall MOI was 1.6. The proportion of isolates carrying multiclonal infections and the MOI were comparables across all the considered status (Table 3).

5. Discussion

The present study conducted in the RoC is the first to investigate the prevalence of asymptomatic *P. falciparum* infection and possible associated risk factors in pregnant Congolese women.

Our findings showed that the prevalence of asymptomatic *P. falciparum* infection was 7% and 19% using microscopy and PCR, respectively. These results, demonstrating that almost two third of infections cases were missed by microscopy, are in line with

Table 2
Prevalence of asymptomatic *P. falciparum* infections in Congolese pregnant women in relation to age, gravidity, IPTp-SP intake and haemoglobin level.

	Microscopy				PCR			
Profiles	Positive	OR(95%CI)	AOR(95%CI)	*P*	Positive	OR(95%CI)	AOR(95%CI)	*P*
Age								
<18 (*N* = 37)	4 (10.8%)	1.00	1.00		12 (32.4%)	1.00	1.00	
≥18 (*N* = 326)	21 (6.4%)	0.56(0.17–2.41)	0.70(0.21–2.46)	0.4	56 (17.2%)	0.43(0.19–1.00)	0.73(0.28–1.90)	0.5
Gravidity								
Primigravidae (*N* = 95)	14 (14.7%)	1.00	1.00		25 (26.3%)	1.00	1.00	
Multigravidae (*N* = 260)	10 (3.8%)	0.23(0.08–0.58)	0.23(0.08-0.60)	0.003*	43 (16.5%)	0.55(0.30–1.02)	0.60(0.29–1.26)	0.2
IPTp-SP								
No (*N* = 228)	22 (9.7%)	1.00			61 (26.8%)	1.00	1.00	
1 dose (*N* = 59)	2 (3.4%)	0.32(0.03–1.40)	0.29(0.06–1.36)	0.1	2 (3.4%)	0.09(0.01–0.38)	0.09(0.02–0.42)	0.002*
2 or 3 doses (*N*=76)	1 (1.3%)	0.12(0.00–0.80)	0.11 (0.01–0.87)	0.03*	5 (6.7%)	0.19(0.05–0.50)	0.18(0.06–0.48)	<0.001*
Haemoglobin level								
<11 g/dl (*N* = 184)	22 (12.0%)	1.00	1.00		44 (23.9%)	1.00	1.00	
≥11 g/dl (*N* = 173)	3 (1.7%)	0.12 (0.02–0.44)	0.12(0.03–0.43)	0.001*	22 (12.7%)	0.46(0.25–0.83)	0.39(0.22–0.72)	0.002*

OR: odds ratio, AOR: adjusted odds ratio, CI: confidence interval.
* Significant at $P < 0.05$.

Table 3
P. falciparum multiclonal infections (based on *msp2* marker) in isolates from Congolese pregnant women attending antenatal health care in Madibou, Brazzaville.

Characteristics	Multiclonal infection	*P*	MOImean(range)	*P*
Age (years)				
<18	6/12 (50.0%)	0.4	1.58 (1–3)	0.3
≥18	19/50 (38.0%)		1.55 (1–4)	
Gravidity				
Primigravidae	9/22 (40.9%)	0.9	1.5 (1–3)	0.5
Multigravidae	16/40 (40.0%)		1.64 (1–4)	
IPTp-SP				
No	21/55 (38.2%)	0.9*	1.56 (1–4)	0.9*
1 dose	1/2 (50.0%)		1.5 (1–2)	
2 or 3 doses	2/5 (40.0%)		1.6 (1–3)	
Haemoglobin level				
< 11	18/41 (43.9%)	0.2	1.66 (1–4)	0.1
≥ 11	6/21 (28.6%)		1.36 (1–3)	
Total	25/62 (40.3%)		–	–

MOI: multiplicity of infections.
* *P* value calculated using Kruskal–Wallis test.

previous studies showing that microscopically detectable *P. falciparum* peripheral blood is a poor indicator of infection status in pregnant women (Desowitz and Alpers, 1992; Leke et al., 1999; Mockenhaupt et al., 2000; Ntoumi et al., 2013). One explanation could be that, during pregnancy, placental sequestration of parasitized red blood cells leads to low grade or even the absence of peripheral parasitaemia (Brabin, 1983). We found a similar prevalence of asymptomatic infection in under 10 years old children living in the same area (Ntoumi et al., 2000). However, considering Central Africa region, the prevalence of asymptomatic *P. falciparum* infection in Congolese pregnant women is lower than that reported from the Democratic Republic of Congo and Gabon (Matangila et al., 2014; Mayengue et al., 2004; Tshibola Mbuyi et al., 2014). This can be explained by the difference in transmission intensity between these endemic areas and also by the difference of the period for blood collection (dry or rainy season) and the effectiveness of IPTp-SP.

This study showed an association between gravidity and microscopic *P. falciparum* infection based on both univariate and multivariate analyses. More specifically, we found a significantly lower prevalence of infection in multigravidae than in primigravidae. Our finding agrees with the observation made in highly endemic areas stating that women in their first pregnancy are more likely than multigravidae to experience malaria parasitaemia, because immunity to malaria associated pregnancy is not yet developed (Diagne et al., 1997; Kurth et al., 2010; Steketee et al., 1996; Zhou et al., 2002). This specific immunity during pregnancy is related to specific strains of *P. falciparum* which are able to adhere to placental tissue and immune mechanisms against these parasites are gradually acquired with successive pregnancies (Beeson et al., 2000; Mockenhaupt et al., 2000).

To reduce the burden of malaria among pregnant women and prevent deleterious effects of pregnancy-associated malaria, WHO monthly recommends administration of IPTp-SP (starting as early as possible in the second trimester) to this vulnerable population (WHO, 2004). The administration 2 or 3 doses of IPTp-SP has been used in the Republic of Congo since 2006. In this study, we observed that 2 or 3 doses of IPTp-SP were associated with a lower prevalence of asymptomatic infection regardless of screening method (microscopy or PCR). This is similar to findings from Burkina Faso by Douamba et al. (2012), and from Ghana by Wilson et al. (2011).

Malaria is known to be a common cause of anaemia in pregnant women (WHO, 1992) which is reflected by a low haemoglobin level (<11 g/dl) and this have been demonstrated to result in poor perinatal outcomes (WHO, 1992). A proportion of 51.5% of Congolese pregnant women recruited in this study were anemic. This observation is in line with the WHO estimates for the prevalence of anaemia between 35 and 75% in African pregnant women (WHO, 1992). With regard to the genetic diversity of *msp2* gene in *P. falciparum* isolates collected from pregnant women, no difference was found according

to IPTp-SP doses. Importantly, in the present study, occurrence of asymptomatic *P. falciparum* infection was significantly associated with anaemia even when adjusted for age, gravidity and IPTp-SP doses.

6. Conclusion

Our study suggests that *P. falciparum* infection (detected by microscopy or PCR) is common in pregnant women attending Madibou health centre for antenatal visits and contributes to occurrence of maternal anaemia. The results also revealed that administration of 2 or 3 IPTp-SP doses was associated with a reduced prevalence of infections but did not reduce the risk of anemia.

Conflict of interests

The authors declare that they have no conflict of interest.

Author's contributions

NF was responsible for the protocol development, study design, data interpretation and writing of the manuscript. BD participated in study design, recruitment of pregnant women, collection of data and molecular analysis. FA participated in molecular analysis. KM was involved in data collection and microscopic examination of blood films. VJC was responsible for data entry and statistical analysis. KKF supervised the conduct of the study, participated in statistical analysis and drafted the manuscript. All authors read and approved the final manuscript.

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