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Evaluation of the usefulness of intermittent preventive treatment of malaria in pregnancy with sulfadoxine-pyrimethamine in a context with increased resistance of *Plasmodium falciparum* in Kingasani Hospital, Kinshasa in the Democratic Republic of Congo

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ABSTRACT

Background: Increasing resistance of *Plasmodium falciparum* to sulfadoxine-pyrimethamine (SP) threatens its usefulness for intermittent preventive treatment in pregnancy (IPTp-SP). The prophylactic effects of IPTp-SP on maternal malaria and adverse pregnancy outcomes were evaluated in Kingasani Hospital, Kinshasa in the Democratic Republic of Congo (DRC).

Methods: Laboring women ($n = 844$) and respective newborns were investigated. Blood samples collected from women were tested for malaria using rapid diagnostic test (RDT), blood smears examination, and real-time PCR. The hemoglobin level was measured by HemoCue© analyzer. A PCR-RFLP method was applied for detecting N51I, C59R, and S108N mutations on *dhfr* along with A437G and K540E mutations on *dhps* in *P. falciparum* positive samples. Logistic regression models assessed relationships between IPTp-SP uptake and pregnancy outcomes.

Results: P. falciparum malaria was detected at delivery in 10.8% of women and was statistically associated with fever during the pregnancy (OR = 2.9 [1.5; 6.3]; $p = 0.004$) and maternal anemia (OR = 3.9 [2.4; 6.3]; $p < 0.001$). One out of five parasites was a quintuple mutant encoding *dhfr* mutations 51I, 59R, and 108 N along with *dhps* mutations 437G and 540E. The molecular profile of parasites (i.e., 32.6% of parasites carrying *dhps* K540E) was suitable with continued use of SP for IPTp. IPTp-SP uptake was not associated with reduced maternal malaria, fever reported in pregnancy, or fetal deaths ($p > 0.05$). Conversely, three or more doses of SP were

Abbreviations: DRC, Democratic Republic of Congo; LBW, Low birth weight; PTB, Preterm birth; NMCP, National Malaria Control Program; WHO, World Health Organization; ITN, Insecticide-treated mosquito net; IPTp, Intermittent preventive treatment in pregnancy; SP, Sulfadoxine-pyrimethamine; ANC, Antenatal care; *dhfr*, Dihydrofolate reductase gene; *dhps*, Dihydropteroate synthase gene; HIV/AIDS, Human immunodeficiency virus / Acquired immunodeficiency syndrome; RDT, Rapid diagnosis test; PCR, Polymerase chain reaction; DBS, Dried blood spot; RFLP, Restriction fragment length polymorphism; UV, Ultraviolet; AIC, Akaike's Information Criterion; qPCR, quantitative polymerase chain reaction; HRP2, Histidine-rich protein 2.

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associated with reduced maternal anemia at delivery (OR = 0.4 [0.2; 0.9]; $p = 0.024$), shortened gestation (OR = 0.4 [0.2; 0.8]; $p = 0.009$), and low-birth weights (OR = 0.2 [0.1; 0.5]; $p < 0.001$).
Conclusion: IPTp-SP was not associated with reduced maternal malaria in our study, but evidence was found of a prophylactic effect against adverse pregnancy outcomes. To counteract further loss of clinical effects of IPTp-SP in the study population, alternative strategies able to improve its anti-malarial efficacy such as combination of SP with partner molecules should be implemented.

1. Introduction

In the Democratic Republic of Congo (DRC), around 1.5 million pregnant women are exposed to *Plasmodium falciparum* malaria yearly (Walker et al., 2014). This infection has severe consequences for both the mother and the unborn child, causing maternal anemia, intrauterine growth restriction, low birth weight (LBW), preterm birth (PTB), fetal death, and congenital malaria (Fried and Duffy, 2017; McGready and Nosten, 2012). To avert these complications, the National Malaria Control Program (NMCP), along with the World Health Organization (WHO), recommends three main interventions including the use of insecticide-treated mosquito net (ITN), the uptake of intermittent preventive treatment in pregnancy (IPTp) with antimalarials, and a prompt management of malaria episodes occurring in pregnancy (USAID, 2017; WHO, 2018).

Sulfadoxine-pyrimethamine (SP) is an antimalarial combination drug currently recommended for IPTp in areas of moderate to high malaria transmission and whose clinical effects basically include major reductions in maternal anemia, LBW, and neonatal mortality while clearing existing *P falciparum* infections in pregnant women (Eisele et al., 2012; Kalilani-Phiri et al., 2013; ter Kuile et al., 2007; WHO, 2013). Currently, a minimum of three doses of SP are required, comprising one dose at each scheduled antenatal care (ANC) visit and starting in the second trimester of pregnancy (WHO, 2013). IPTp based on the SP drug combination (IPTp-SP) has been adopted in the DRC since 2003, but its coverage levels remain low (MPSMRM, 2014; PMI, 2013; USAID, 2017). Moreover, resistance of *P. falciparum* parasites to SP has emerged in the country for more than twenty years (like in other areas with stable malaria transmission) and is becoming a major threat for the effectiveness of the IPTp-SP strategy with potentially important implications for malaria control efforts (Alker et al., 2008; Fried and Duffy, 2017; Harrington et al., 2011; Kern et al., 2011; Mandoko et al., 2018; van Eijk et al., 2019).

In *P. falciparum* species, resistance to SP is due to a series of genetic mutations in the parasite's dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes (Fried and Duffy, 2017; Harrington et al., 2011; Ruh et al., 2018). These mutations have arisen as molecular markers for epidemiological surveillance of SP resistance. The five most common mutations include *dhfr* substitutions N51I, C59R, and S108N and *dhps* substitutions A437G and K540E (Menard and Dondorp, 2017; van Eijk et al., 2019). Quintuple mutant *P. falciparum* parasites defined as those that harbour a combination of these five mutations have been widely reported in Sub-Saharan Africa, including in DRC (Baraka et al., 2017; Vinayak et al., 2010). At the ecological level, a high prevalence of these quintuple-mutant parasite undermine substantially the efficacy of IPTp-SP (MPSMRM, 2014; USAID, 2017). Continuous spread of this SP resistance is expected with continued SP use according to recent reports, likely leading to poorer SP-IPTp outcomes despite continued recommendations of its use by the WHO (Mandoko et al., 2018; van Eijk et al., 2019). Hence, there is a need not only for continuously monitoring this resistance but also for assessing the clinical relevance of the use of IPTp-SP to inform guideline policies in the country. Therefore, we investigated laboring women and their respective newborns for assessing the effects of maternal malaria infection and IPTp-SP intake on pregnancy and birth outcomes in Kingasani Hospital, DRC. We additionally evaluated the frequency of molecular markers of SP resistance among infected laboring women.

2. Methodology

2.1. Study design, site and population

A cross-sectional study was carried out in the general reference hospital of Kingasani located in Kinshasa, the capital city of the DRC. From September 2019 to January 2020, consecutive laboring women were recruited upon arrival at the maternity ward and their respective newborns were examined immediately after delivery. All laboring women who agreed to participate in the study were basically eligible. However, women with comorbidities or conditions with reported effect on pregnancy outcomes were excluded from the study (e.g., women with chronic conditions such as diabetes mellitus or HIV/AIDS and women with twin pregnancy). Initially, the prophylactic success of SP against adverse birth outcomes (i.e., LBW) was assumed to be 93% when taking $\geq$2 doses and 89% when receiving $\leq$1 dose as suggested by previous data in the DRC (Likwela et al., 2012). Consequently, by considering a difference of less than 5% in prophylactic success as being clinically insignificant, a minimum sample size of 88 women under $\leq$1 dose of SP and 352 under $\geq$2 doses (total sample size = 440, $r = 4$) was deemed to be sufficient for reaching a statistical power of 80% when using a one-sided test size of 5%. Finally, 178 laboring women who had taken $\leq$1 dose of SP (prophylactic success: 87.6%) were included along with 666 women who had received $\geq$2 doses (prophylactic success: 95.5%); this enabled to obtain a total sample size of 844 ($r = 3.7$) with a statistical power of 99.9%.

2.2. Data collection and study parameters

Socio-demographic information and the data on the medical itinerary during pregnancy were collected through face-to-face interviews using a piloted standard questionnaire (See in **Appendix: Study questionnaire**, for details). This questionnaire initially conceived in English was translated into the national languages spoken in the collection site (i.e., Lingala, French) and back-translated to English. Delivery and birth outcomes were obtained from medical records. The schedule of IPTp-SP intake was reconstructed from the information in the booklet of routine antenatal care (ANC) of each pregnant woman. Biological data were obtained after laboratory analyses. All the data thus collected were compiled through double-blind data entry by two independent operators using an Excel database.

2.3. Definitions

Given challenges for the diagnosis of malaria that are specific or not to pregnancy (Kozycki et al., 2017; Uneke, 2008), the malaria infection in laboring women was defined as a composite indicator generated by including infections detected by either a rapid diagnosis test (RDT) targeting the *P. falciparum* histidine-rich protein 2 (*HRP2*) antigen or by microscopic examination of thick smears. Maternal anemia was defined as a hemoglobin (Hb) level < 11 mg/dL obtained in laboring women before the delivery. The term low-birth weight (LBW) referred to an absolute weight of <2500 g regardless of the gestational age of women. Preterm birth (PTB) was considered when the delivery of a living child occurred before the gestational age of 37 weeks of amenorrhea. The term shortened gestation was used to refer to both fetal death and PTB. The level of SP resistance used in this study was stratified into low,

moderate, and high using the following prevalence thresholds for particular mutations of malaria parasites: *dhps* A437G <90% or *dhps* K540E <30%; *dhps* A437G ≥90% or *dhps* K540E ≥30% and < 90%; and *dhps* K540E ≥90% respectively (van Eijk et al., 2019).

2.4. Biological samples and laboratory analyses

2.4.1. Measurement of the Hb level and detection of malaria infection

We collected venous blood samples from the left arm of each laboring woman before delivery. This blood was used for hematological analyses and rapid testing of malaria in the clinical laboratory of the Kingasani Hospital (DRC). A thick smear was made for qualitative detection of *Plasmodium* parasites using microscopic examination in the Laboratory of Molecular Biology at the University of Kinshasa (DRC). The Hb level was measured by using the HemoCue© analyzer (Quest Diagnostics, Ängelholm, Sweden) whereas the RDT of malaria was carried out with the SD Bioline Malaria Ag Pf test (Standard Diagnostics, Suwon city, Republic of Korea). All these analyses were performed following the corresponding manufacturer's instructions. The microscopic examination was performed by two microscopists and blindly confirmed by an independent third party in case of inconsistent results.

2.4.2. Molecular analyses

We collected also dried blood spot (DBS) from each woman by using Whatmann 3 MM filter paper (GE Healthcare, UK) as previously described (Grüner et al., 2015). These DBS from RDT positive women were used for the detection of *P. falciparum* species and molecular markers of SP resistance respectively in the Laboratory of Molecular Biology at the University of Kinshasa (DRC) and in the Laboratory of Clinical Microbiology at the University of Liège (Belgium). First, the DNA was extracted by using the QIAamp DNA Mini Blood kit (Qiagen, Germany) and following the manufacturer's instructions. The extracted DNA was stored at −20 °C in appropriately labeled tubes. The *P. falciparum* species was detected in RDT positive samples by using a real-time polymerase chain reaction (PCR) following a protocol described previously (Cnops et al., 2011). For the analysis of molecular markers, fragments of *dhfr* (including codon-positions 51, 59, and 108) and *dhps* (including codon-positions 540 and 437) genes of *P. falciparum* were amplified using nested PCR protocols that had been developed previously (See Appendix, Table S1) (Duraisingh et al., 1998). PCR amplicons were then screened for the presence of targeted mutations using restriction fragment length polymorphism (RFLP) method as described previously (Duraisingh et al., 1998). Shortly, 8 µl of each corresponding amplicon were incubated at 37 °C during 1 h in 25 µl final reaction volume with each of five different restriction enzymes (New England Biolabs, France) – i.e., MlUC I, *Xmn* I, and *Bsr* I for detecting N51C, C59R, and S108N on *dhfr*, as well as *Ava*II and *Fok*I for detecting A437G, and K540E on *dhps*. Restriction enzymes were then inactivated by heating to 65 °C for 20 min. Amplicons of PCRs and RFLP products were successively subject to electrophoresis on 2% agarose gel stained with ethidium bromide and were visualized under UV light. PCR amplicons from 3D7, Peru, and Dd2 clones were used as a positive internal controls (Molina-Cruz et al., 2012). PCRs were run in a conventional Verity thermocycler (Bio-Rad Laboratories, CA, US) and an ABI 7500 Fast real-time thermocycler (Applied Biosystems, USA). Samples containing no detectable PCR products were re-examined twice before being declared negative.

2.5. Statistical analyses

Statistical analyses were conducted using R version 3.5.3 (The R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). Relative and absolute frequencies were used to summarize qualitative variables. Uni- and multivariable logistic regression models were fitted to assess the association between various factors and malaria infection as well as the effect of different doses of SP on pregnancy and birth outcomes. When a multivariable model was considered, the final model was selected based on Akaike's Information Criterion (AIC) values calculated for different potential models using both a backward and forward approach. For all the statistical tests applied, the value of $p < 0.05$ was considered as the statistical significance threshold.

2.6. Ethical considerations

This study has been approved by the Ethics Committee of the School of Public Health of the University of Kinshasa (registration number: ESP/CE/235/2019).

3. Results

3.1. Basic characteristics of laboring women and newborns included in this study

A total of 844 laboring women and their respective newborns were recruited and included in this study (Table 1, Table 2). Most of laboring women were 18–35 years old (80.4%), married (82.3%), and had a high-school education level (83.1%). Preterm deliveries were noted in 29.3% of women. About one out of three laboring women were primigravidae (32.9%). The vast majority of women had visited ANC services (98.7%),

Table 1
Socio-demographic and obstetrical characteristics of laboring women.

Characteristics	n	%
Marital status (n = 838)		
Married	690	82.3
Divorced	13	1.6
Single	135	16,1
Age group (years)		
<18	24	2.9
[18–35]	673	80.4
[35–40]	103	12.3
>40	37	4.4
Education level		
None	6	0.7
Primary school	30	3.6
High school	701	83.1
College or University	107	12.7
Gravidity		
Primigravidae	193	22.9
Multigravidae	651	77.1
ANC Visit during pregnancy		
No	11	1.3
Yes	833	98.7
SP intake during pregnancy		
No SP intake	37	4.3
1 or 2 doses of SP	407	48.2
At least 3 doses of SP	401	47.5
History of at least one fever episode during pregnancy		
No	506	60
Yes	338	40
Possession of an ITN during pregnancy		
No	24	2.8
Yes	820	97.2
Sleeping under an ITN the night before the medical visit (n = 820)		
No	397	48.4
Yes	423	51.6
History of a curative anti-malarial treatment in pregnancy		
No	354	41.9
Yes	490	58.1
Maternal anemia (Hb < 11 g/dL)		
No	718	85.1
Yes	126	14.9
Dystocia at delivery		
Yes	214	25.4
No	630	74.6

(*) ANC: Antenatal consultations; Hb: Hemoglobin; LLIN: Long-acting insecticide-impregnated mosquito net; SP: Sulfadoxine-Pyrimethamine.

Table 2
Clinical characteristics of newborns.

Characteristics	n	%
Fetal death		
No	841	99.7
Yes	3	0.3
Shortened gestation (fetal deaths, PTB)		
Yes	239	28.3
No	605	71.7
Birth weight category		
LBW	51	6.0
NBW	793	94.0

(*) PTB: Preterm birth; LBW: Low birth weight; NBW: Normal birth weight; AW: Weeks of amenorrhea

had taken at least once a SP combination drug (95.7%), and reportedly had an ITN for use during pregnancy (97.2%). However, only 47.5% of them had achieved the three recommended doses of SP and only 48.4% admitted to have slept under an ITN during the previous night. About 40% of laboring women reported that at least one episode of fever occurred in pregnancy and 41.9% had even taken a curative anti-malarial treatment. On admission to the maternity ward, 14.9% of these women had anemia (i.e., Hb level < 11 mg/dL). Dystocia occurred in 25.4% of women. Adverse birth outcomes included shortened gestation, LBW, and fetal death that were observed in 28.3, 6.0, and 0.3% of newborns.

3.2. Malaria and associated variable in laboring women

The overall prevalence of malaria detected by RDT or thick smears examination in laboring women was 10.8% with fair agreement between the two diagnosis approaches (kappa = 0.35; $p < 0.001$) (See Appendix, Table S2). A logistic regression model was fitted to assess variables associated with malaria infection (Table 3). The final logistic regression model indicated that women with a history of fever in pregnancy were approximately 3 times more associated with malaria infection at delivery (OR = 2.9 [1.5; 6.3]; $p = 0.004$) than those with no history of fever. Likewise, women with anemia on arrival at the maternity ward were approximately 4 times more associated with malaria infection (OR = 3.9 [2.4; 6.3]; $p < 0.001$) than those with normal hemoglobin level.

3.3. Effect of SP intake on different pregnancy and birth outcomes

A univariable logistic regression was fitted to evaluate the relationship between SP doses taken by laboring women during pregnancy and

Table 3
Logistic regression model (final model) identifying variables associated with malaria at delivery.

Characteristics*	Malaria at delivery		Univariable model		Final multivariable model	
	Positive	Negative	OR [IC95%]	*p*-value	OR [IC95%]	p-value
Maternal age						
Minor (< 18 years old)	5	23	Ref.	0.226		
Major (≥ 18 years old)	86	730	0.5 [0.2; 1.7]			
Shortened gestation (fetal deaths, PTB)						
No	58	547	Ref.	0.076		
Yes	33	206	1.5 [0.9; 2.4]			
Gravidity						
Multigravidae	66	585	Ref.	0.269		
Primigravidae	25	168	1.3 [0.7; 2.1]			
ANC Visit during pregnancy						
Yes	89	744	Ref.	0.432		
No	2	9	1.9 [0.3; 7.4]			
SP intake during pregnancy						
No	7	30	Ref.	0.109		
Yes	84	723	0.5 [0.2; 1.3]			
History of at least one fever episode during pregnancy						
No	42	464	Ref.	0.005	Ref.	0.004
Yes	49	289	1.9 [1.2; 2.9]		2.9 [1.5; 6.3]	
Possession of an ITN during pregnancy						
Yes	86	734	Ref.	0.116	Ref.	0.089
No	5	19	2.2 [0.7; 5.7]		2.5 [0.8; 6.7]	
Sleeping under an ITN the night before the medical visit (n = 820)						
No	47	374	Ref.	0.721		
Yes	44	379	0.9 [0.6; 1.4]			
Maternal anemia (Hb < 11 g/dL)						
No	59	659	Ref.	<0.001	Ref.	<0.001
Yes	32	94	3.8 [2.3; 6.1]		3.9 [2.4; 6.3]	
Dystocia at delivery						
No	63	567	Ref.	0.210		
Yes	28	186	1.4 [0.8; 2.2]			
Prematurity						
No	82	715	Ref.	0.062		
Yes	9	38	2.1 [0.9; 4.2]			
Fetal death						
No	3	750	Ref.	0.981		
Yes	0	91	0.0 [−; $>10^{+23}$]			
History of a curative anti-malarial treatment in pregnancy						
Yes	56	434	Ref.	0.476	Ref.	0.085
No	35	319	0.9 [0.5; 1.3]		1.9 [0.9; 4.2]	
Birth weight category						
NBW	82	710	Ref.	0.122		
LBW	9	43	0.6 [0.3; 1.3]			

(*) PTB, Preterm; ANC, Antenatal consultation; SP, Sulfadoxine-Pyrimethamine; ITN, Insecticide-treated net; Hb, Hemoglobine; NBW, Normal birt weight; LBW, Low birth weight.

various adverse pregnancy and birth outcomes (Table 4). Compared to no SP uptake, taking the drug at one, two, or ≥ 3 doses during pregnancy was not significantly associated with the occurrence of maternal malaria, fever during pregnancy, fetal deaths, and dystocia ($p > 0.05$). Likewise, taking only one dose of SP (compared to no SP intake) had no significant effect on any adverse clinical outcome. In contrast, taking two doses of SP (compared to no SP intake) was associated with a reduction of LBW by 80% (OR = 0.2 [0.1; 0.7]; p = 0.004). Furthermore, taking at least three doses of SP (compared to no SP intake) was associated with adverse clinical outcomes reduced by 60% in terms of maternal anemia (OR = 0.4 [0.2; 0.9]; $p = 0.024$) and shortened gestation (OR = 0.4 [0.2; 0.8]; $p = 0.009$), and by 80% in terms of LBW (OR = 0.2 [0.1; 0.5]; $p < 0.001$).

3.4. Frequency of *P. falciparum* SP-resistance in laboring women

The *P. falciparum dhps* and *dhfr* genes were successfully amplified and genotyped for the detection of *dhfr* mutations N51I, C59R, and S108N and *dhps* mutaions A437G and K540E in 43 out of 91 RDT positive laboring women. The distribution of *different* genotypes detected in this study population is summarized in Table 5. Out of all genotyped parasites, 95.3% carried one or more mutations at analyzed codon-positions that encode for SP resistance. The most frequent mutations were *dhfr* N51I and S108N (88.4%), followed by *dhps* A437G and *dhfr* C59R (79.1%). The least frequently detected mutation was *dhps* K540E (32.6%). The most frequent *dhfr-dhps* alleles showed all three *dhfr* mutations (51I, 59R, and 108 N) carried together with *dhps* 437G (IRN-GK, 44.2%) and with an additional *dhps* 540E mutation (IRN-GE, 20.9%).

Table 4
Univariable logistic regression models evaluating the effect of increasing doses of SP (compared to no SP intake) on different adverse clinical outcomes in laboring women and their newborns.

Clinical outcomes and different doses of SP*	Adverse clinical outcome		OR [95% CI]	p-value
	Present	Absent		
Maternal malaria				
No SP intake	7	30	Ref.	
One SP dose	13	128	0.4 [0.2; 1.2]	0.103
Two doses of SP	31	235	0.6 [0.2; 1.5]	0.216
At least three doses of SP	40	360	0.5 [0.2; 1.2]	0.100
Maternal anemia				
No SP intake	9	28	Ref.	
One SP dose	26	115	0.7 [0.3; 1.7]	0.424
Two doses of SP	46	220	0.7 [0.3; 1.5]	0.301
At least three doses of SP	45	355	0.4 [0.2; 0.9]	0.024
Shortened gestation (fetal deaths, PTB)				
No SP intake	28	9	Ref.	
One SP dose	98	43	0.7 [0.3; 1.6]	0.464
Two doses of SP	157	109	0.5 [0.2; 0.9]	0.056
At least three doses of SP	211	189	0.4 [0.2; 0.8]	0.009
History of fever during pregnancy				
No SP intake	15	22	Ref.	
One SP dose	69	72	1.4 [0.7; 2.9]	0.363
Two doses of SP	108	158	1.0 [0.5; 2.1]	0.994
At least three doses of SP	146	254	0.8 [0.4; 1.7]	0.626
Fetal deaths				
No SP intake	0	37	Ref.	
One SP dose	0	141	1.0 [0.0; $>10^{+172}$]	1.000
Two doses of SP	1	265	8,765,540.1 [0.0; −]	0.997
At least three doses of SP	2	398	11,672,704.2 [0.0; −]	0.997
LBW				
No SP intake	7	30	Ref.	
One SP dose	15	126	0.5 [0.2; 1.4]	0.179
Two doses of SP	14	252	0.2 [0.1; 0.7]	0.004
At least three doses of SP	16	384	0.2 [0.1; 0.5]	<0.001

(*) SP: Sulfadoxine-Pyrimethamine; PTB: Preterm birth; LBW: Low birth weight.

Table 5
Frequency of *dhfr and dhps* genotypes in laboring women (n = 43).

Genotype	n	%
dhfr mutations		
N51I	38	88.4
C59R	34	79.1
S108N	38	88.4
dhps mutations		
A437G	34	79.1
K540E	14	32.6
dhfr-dhps alleles (with mutant amino acids being underlined)		
NCS-AK (wild-type)	2	4.7
NCS-GE (*dhps* A437G and K540E)	3	7.0
ICN-AK (*dhfr* N51I and S108N)	1	2.3
ICN-GK (*dhfr* N51I and S108N; and *dhps* A437G)	2	4.7
ICN-GE (*dhfr* N51I and S108N; and *dhps* A437G and K540E)	1	2.3
IRN-AK (*dhfr* N51I, C59R, and S108N)	5	11.6
IRN-AE (*dhfr* N51I, C59R, and S108N; and *dhps* K540E)	1	2.3
IRN-GK (*dhfr* N51I, C59R, and S108N; and *dhps* A437G)	19	44.2
IRN-GE (*dhfr* N51I, C59R, and S108N; and *dhps* A437G and K540E)	9	20.9

Rare quadruple mutants detected carried *dhfr* N51I and S108N along with *dhps* A437G and K540E (ICN-GE, 2.3%) or *dhfr* N51I, C59R, and S108N together with *dhps* K540E (IRN-AE, 2.3%). No statistical relationship could be observed between carriage of parasites encoding thes SP-resistance mutations by laboring women and adverse pregnancy outcomes (See Appendix, Table S3 & Table S4).

4. Discussion

This study indicates that overall one in ten laboring women was infected with malaria on arrival at the maternity ward in Kingasani. The prevalence of malaria in pregnancy varies widely in studies conducted within endemic areas, including in the DRC (Likwela et al., 2012; Radeva-Petrova et al., 2014; ter Kuile et al., 2007; van Eijk et al., 2019). Various factors such as methodological approaches (e.g., diagnostic approaches), baseline characteristics of women (e.g., gravidity), local epidemiology of malaria (e.g. transmission intensity), and uptake levels of malaria control interventions (e.g. the use of ITN, antimalarial drug intake during pregnancy) can potentially explain the variations noted between the prevalence of maternal malaria in different studies (Desai et al., 2018; Fried and Duffy, 2017; Uneke, 2008). Besides possible technical errors, the fair agreement (8.2% of results being discordant) obtained between thick blood smear examination and RDT in this study would be explained by pregnancy-specific challenges for the diagnosis of malaria (Uneke, 2008). These include the possibility of infected erythrocytes sequestering within the placenta that are likely to still be detectable by RDTs while being submicroscopic in peripheral blood (Cohee et al., 2014; Fried and Duffy, 2017). Placental infections can even affect RDTs' performance in case of lower parasite densities detectable by highly sensitive methods such as qPCR (Mayor et al., 2012). Additionally, other factors such as antigenic variation, histidine rich protein 2 (*hrp2*) gene deletions, presence of blocking antibodies, and the persistence of malaria antigens after recent parasite clearance may also contribute to the falsely negative HRP2-based RDTs (Baker et al., 2005; Dalrymple et al., 2018; Gamboa et al., 2010; Kozycki et al., 2017; Wongsrichanalai et al., 2007). This outcome highlights the urgent need for more accurate malaria diagnostic tools for pregnant women.

In the DRC, key strategies for pregnancy-specific protection implemented by the NMCP include the use of ITN and uptake of IPTp-SP (MPSMRM, 2014; PMI, 2013; USAID, 2017). In the current study, the ITN coverage among women was very high (97.2%). This probably reflects the achievements of mass campaigns and routine distribution at ANC and child vaccination clinics that have been carried out in recent years for the distribution of ITN to households and pregnant women (MPSMRM, 2014; OMS, 2015; PNLP, 2017). The low number of women without ITN coverage can explain the lack of association between ITN

possession during pregnancy and malaria at delivery in this study. However, it is important to note that almost half of laboring women admitted to have not slept under an ITN the night before visiting the maternity ward. This outcome suggest that, despite the high ITN coverage, its usage during pregnancy may still be far less than the NMCP's target in 2020 (>80%) (PMI, 2013). Additional communication efforts are needed to promote the effective use of these ITN in pregnancy (Likwela, 2014; PMI, 2013). On the other hand, only 45.5% of laboring women had completed the three doses of IPTp-SP that are recommended by the national guidelines. This coverage of IPTp-SP is slightly higher than that reported previously in different sites in DRC, including in Kinshasa (MPSMRM, 2014; PMI, 2013; USAID, 2017). However these figures are still low if we consider the deployed efforts to increase IPTp-SP in the RDC for almost 20 years, highlighting the importance to identify the obstacles for its implementation through operational research, and consequently to address them programmatically.

Otherwise, laboring women reporting fever episodes that occurred in pregnancy and those with anemia had significantly frequent malaria infection at delivery in this study. This may indicate that malaria at delivery may result from malaria infections that occurred and persisted throughout the pregnancy which is consistent with previous studies (Cohee et al., 2014; De Beaudrap et al., 2013; Kalilani-Phiri et al., 2013; Kapisi et al., 2017; Tran et al., 2020). Hence, in the study population, malaria is probably an important cause of fever and anemia in pregnancy despite their potential multifactorial origins (Le Gouez et al., 2016; More, 2017). Maternal anemia which affected 14.9% of women in this study, is known as able to increase the risk of LBW, PTB, perinatal and neonatal mortality in low- and middle-resource regions (Rahman et al., 2016). Fever episodes reported by 40% of explored women may present additional risks to pregnancy health such as teratogen effects (Botto et al., 2014; Graham Jr and Edwards, 1998; Yin et al., 2011). Therefore, beyond its potential direct effects, malaria infection threatens the neonatal and maternal health in the study population through possible indirect effects of maternal fever and anemia. Hence, this infection, including its asymptomatic forms, requires rigorous management in pregnant women in the study area. Efforts for scaling up the use of IPTp-SP could be thus seen as a beneficial step for reducing the impact of malaria in pregnancy in the study population.

However, given the nationwide spread of SP-resistant malaria, concerns have been raised and persist over the clinical usefulness of SP for prevention of malaria in pregnancy which remains very less investigated in DRC (Likwela et al., 2012; Verity et al., 2020). We thus assessed the prophylactic effects of the drug combination. Outcome measures for this evaluation comprised maternal malaria at delivery and various adverse pregnancy and birth outcomes. Our results show that SP uptake during pregnancy had no significant effect on maternal malaria at delivery as well as on the occurrence of fever episodes in pregnancy and fetal deaths. A plausible explanation for these results may be a lack of sufficient statistical power to detect differences in outcomes, especially for fetal deaths that occurred in small number of cases ($n = 3$). Another explanation would be a loss of the drug's prophylactic efficacy because of the emergence of SP-resistant parasites (van Eijk et al., 2019). However, an eventual relationship between the drug prophylactic effects and SP-resistant parasites could not be evaluated reliably in this study probably due to small number of genotyped parasites that lacked the needed statistical power. In addition, translating such association into a confirmed causal link would require a comprehensive analysis taking into account several other factors that can contribute to the reduced parasite clearance by the IPTp-SP, such as the malaria transmission dynamics in the area, effects of pregnancy on the pharmacokinetics of SP doses, schedule of administration of SP doses related to timing of infections, status of acquired immunity in pregnancy, pregnancy-related susceptibility to malaria (De Kock et al., 2017; Deloron et al., 2010; Fried and Duffy, 2017; van Eijk et al., 2019). Regardless to its possible cause, persistent malaria during pregnancy potentiates the placental proliferation of parasites that can have a direct impact on neonatal morbidity and mortality, as well as an indirect effect on malaria epidemiology by serving as a reservoir of parasites including those resistant to anti-malarial drugs in the community (Harrington et al., 2011; Kern et al., 2011). Conversely to expected anti-malarial effects, a significant prophylactic effect of SP uptake was noted against adverse pregnancy and birth outcomes, namely maternal anemia at delivery, shortened gestation, and LBW, especially at increased doses of the drug. Several studies from various area with high parasite resistance to SP have been pooled in recent meta-analyses and indicate a global loss of anti-malarial effect of IPTp-SP but preserved benefits for pregnancy and birth outcomes (Desai et al., 2016; Desai et al., 2018; Radeva-Petrova et al., 2014; van Eijk et al., 2019). These results show that the drug probably confers additional effects that are independent from its anti-malarial properties and which would comprise its broad-spectrum antibacterial and immunomodulatory effects similar to those described for co-trimoxazole (trimethoprim-sulfamethoxazole), another antifolate drug combination (Capan et al., 2010; Chico et al., 2017; Church et al., 2015; Desai et al., 2018; Roh et al., 2020). Through these additional effects, despite concerning amounts of parasite resistance, the IPTp-SP could still prevent the non-malarial causes of adverse pregnancy outcomes such as curable sexually transmitted infections and reproductive tract infections (Chico et al., 2017). Consequently, combining SP for its non-malarial benefits with partner molecules able to enhance anti-malarial effects (e.g., dihydroartemisinin-piperaquine) would be a more attractive strategy than the use of only SP for IPTp and should be investigated in future studies (Roh et al., 2020).

Resistance to SP results from an incremental accumulation of mutations in the *P. falciparum dhfr* and *dhps* genes. In this study, almost all genotyped isolates carried at least one molecular marker of SP resistance (95.3%). The prevalence of *dhfr* N51I and S108N mutations noted here (88.3% of isolates) is consistent with previous observations made in DRC (Alker et al., 2008; Mobula et al., 2009; Ruh et al., 2018). A *dhps* substitution K540E was the least detected mutation of *P. falciparum* parasites (32.5%) from laboring women. This mutation has been considered as an epidemiological marker for parasites carrying the quintuple mutation (e.g., co-occurring *dhfr* N51I, C59R, and S108N; and *dhps* A437G, and K540E mutations) in Africa which is a surrogate of parasite resistance to SP and loss of therapeutic efficacy of IPTp-SP against malaria (WHO, 2012). It thus enabled classifying regions as of high, moderate, and low resistance to SP when the K540E is <30%, from ≥30% to <90%, and ≥ 90% respectively (van Eijk et al., 2019). However, evidence have shown that the IPTp-SP remains protective against the adverse consequences of malaria in pregnancy across a wide range if SP resistance levels including in high prevalence of *P. falciparum* parasites carrying the quintuple mutations (van Eijk et al., 2019; WHO, 2012). Consequently, the WHO recommends that IPTp-SP should still be administered to women even in area with high K540E prevalence (van Eijk et al., 2019; WHO, 2013). The study population was thus still suitable for continuous use of SP for IPTp. However, the *dhps* K540E mutation occurred at rates almost two times higher than that recently reported in parasites sampled from the general population in the study area (Mandoko et al., 2018). This would tend to shift the sampling area from "low" to "moderate" level of resistance to SP according to the criteria established by van Eijk AM et al. (van Eijk et al., 2019). It is likely that the frequent use of IPTp-SP in the cohort of pregnant women would have selected more resistant parasites (including those carrying *dhps* K540E) compared to the general population. Consequently, genotyping parasites isolated at delivery from these women may have sampled higher levels of SP-resistance compared to parasites currently circulating in the wider population. Enhanced mitigating efforts are thus required to counteract any possible evolution towards a situation with higher level of resistance to SP in the study population. Overall, accumulated *dhfr-dhps* alleles have generated eight different haplotypes among the mutant parasites that could be identified in this study. Of these haplotypes, quadruple mutants were the most common (48.7%), including mainly IRN-GK (*dhfr* N51I, C59R, and S108N along with *dhps* A437G) but also IRN-AE (*dhfr* N51I, C59R, and

S108N together with *dhps* K540E) and ICN-GE (*dhfr* N51I and S108N with *dhps* A437G). This profile is consistent with several reports in pregnant women from African countries, including the DRC, where parasites with an IRN-GK haplotype (*dhfr* N51I, C59R, and S108N along with *dhps* A437G) may exceed 50% (Naidoo and Roper, 2013; Ruh et al., 2018). Moreover, one out of five parasites in the study population (20.9%) was found to be a quintuple *dhfr-dhps* IRN-GE mutant which carries N51I, C59R, and S108N on *dhfr* gene additional to A437G and K540E on *dhps* gene. These quadruple and quintuple mutants should attract the maximum attention of health decision-makers in the country. First, they are known to reduce the efficacy of SP as an intermittent preventive treatment in pregnant woman as well as in children and infants, by undermining the ability of SP to clear existing *P. falciparum* infections and by shortening the post-treatment prophylactic period following IPTp (Alker et al., 2008; Desai et al., 2016; Gosling et al., 2009; Kalilani-Phiri et al., 2013; Nankabirwa et al., 2010; Ruh et al., 2018; Swarthout et al., 2006). Furthermore, they can offer a genetic background for the emergence of specific quintuple (e.g., ICN-GEG emerging from mutants with *dhfr* N51I, C59R, S108N, and *dhps* A437G, K540E) and sextuple mutants (e.g., IRN-GEG emerging from mutants carying *dhfr* N51I, C59R, and S108N along with *dhps* A437G and K540E) by generating an additional *dhps* A581G mutation involved in the transition to high-level resistance to SP and to a loss of the ability of IPTp-SP to prevent adverse birth outcomes such as LBW (Baraka et al., 2017; Minja et al., 2013; van Eijk et al., 2019). Recently, alarming data have already been reported by Mandoko K et al. who detected the emergence of this A581G mutation through septuple (e.g., IRNL-GEG made of *dhfr* N51I, C59R, S108N, and I164L with *dhps* A437G, K540E, and A581G) and even octuple mutants (e.g., IRNI-VAGKGS carrying *dhfr* N51I, C59R, and S108N, along with *dhps* I431V, S436A, A437G, A581G and A613S) in the study region (Mandoko et al., 2018). Hence, the study population is likely experiencing a transitory situation towards the loss of all prophylactic effects of IPTp-SP which deserves to be proactively managed. Indeed, the data reported in the current study cannot definitively rule out the possibility of these highly resistant haplotypes, as the applied RFLP approach was restricted only to the detection of five mutations (*dhfr* mutations N51I, C59R and S108N and *dhps* mutations A437G and K540E) and did not explore other relevant codon-positions such as *dhps* A581.

There are several limitations in this study. Firstly, the survey conducted may be subject to errors in the detection of malaria and SP resistance markers. In an endemic environment, malaria in pregnant women can be underestimated given the presence of infected erythrocytes sequestering within the placenta that ideally require histopathological examinations for diagnosis (Fried and Duffy, 2017; McGready and Nosten, 2012). RFLP allows the detection of only a very limited number of mutations (e.g., *dhfr* N51I, C59R, and S108N; and *dhps* A437G, and K540E). It would therefore be speculative to imagine the presence or absence of other mutations that were not analyzed (e.g., *dhps* I431V and A581G). However, though the prevalence of *dhps* A581G is expected to be lower in Kinshasa than in East Africa, we acknowledge that this specific mutation is worth being investigated in future given its clinical relevance for IPTp-SP failures and the growing concern raised by its emergence in other regions of Africa (Baraka et al., 2017; Mandoko et al., 2018; Minja et al., 2013; van Eijk et al., 2019). Secondly, the survey could not avoid the potential information and memory biases inherent in any self-reporting of information, even if the use of medical files and ANC booklets could have reduced these biases. Thirdly, a possible relationship between different *dhfr-dhps* alleles of *P. falciparum* parasites and adverse pregnancy outcomes could not be assessed reliably in this study. This was probably due to the small sample size of genotyped parasites ($n = 43$ including only two wild-type parasites) that did not enable reaching sufficient statistical power for detecting differences in clinical outcomes. Future studies that would genotype a large number of parasites would be able to address this limitation. Moreover, this study which has focused on a single collection site cannot be representative of the entire study population. Similar studies at other sites are needed to extend the observations made here. Finally, the cross-sectional design of this study may have not captured some information but was deemed to be an ethically acceptable design given the challenges of conducting clinical trials in pregnant women (Divala et al., 2015).

5. Conclusion

Despite the limitations, this study provides interesting information for the study population. First, the results indicate that malaria remains a serious and frequent health problem, associated with fever and anemia during pregnancy. Second, SP-resistant *P. falciparum* parasites seem more frequent in pregnant women than in the general population (Mandoko et al., 2018). However, the population profile is still suitable for continuous use of SP for IPTp (WHO, 2013). Although these resistant parasites may result in a decreased prophylactic effect of IPTp-SP against malaria at delivery, such association can only be confirmed with comprehensive analysis including other factors like host pharmacokinetics and timing of IPTp relative to timing of infection (De Kock et al., 2017; van Eijk et al., 2019). Finally, despite concerns on reduced parasite clearance at delivery, a protective effect of SP on adverse pregnancy and birth outcomes (e.g., maternal anemia, shortened gestation, and LBW) was still preserved. This suggested that the drug likely exerts beneficial effects that are independent from its antimalarial properties (Chico et al., 2017; Roh et al., 2020). Given all these outcomes, enhanced mitigating efforts are urgently needed to further control malaria and drug resistance during pregnancy. Though administration of IPTp-SP is still useful, its anti-malarial effects require to be reinforced through alternative strategies such as a combination with partner drugs. A monitoring of SP-resistant parasites in pregnant women should be pursued in the study area for continuously guiding malaria decision policies.

Contributors

NS, MPH, PDM, GLM, DMM, ARA, BD, DMY and NKK conceived the study. NKK wrote the protocol. NKK and DMY conducted the survey on the field and collected the data. DYM and VRTK did the laboratory analysis. NKK did the data analyses and wrote the first draft of the manuscript. DMY, VRTK, DMM, PZK, BD, EM, PDM, MPH, GLM, ARA, PLD, and NS revised the first draft of the manuscript. All authors provided conceptual input, and revised and approved of the final version of the manuscript.

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Declaration of Competing Interest

We declare no competing interests.

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Appendix A. Supplementary data

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