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# Prevalence of *Plasmodium falciparum* Resistance Markers to Sulfadoxine-Pyrimethamine among Pregnant Women Receiving Intermittent Preventive Treatment for Malaria in Uganda

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**The aim of this study was to assess the prevalence of mutations in *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) genes among pregnant women using sulfadoxine-pyrimethamine (SP) as an intermittent preventive treatment (IPTp). A molecular epidemiological study of *P. falciparum* parasite resistance markers to SP was conducted from August 2010 to February 2012 in Mukono district in central Uganda. DNA was extracted from 413 *P. falciparum*-positive samples. Real-time PCR, followed by melting curve analysis, was used to characterize point mutations in the *Pfdhfr* and *Pfdhps* genes that are associated with SP resistance. The prevalence of the single-nucleotide mutations in *Pfdhfr* at codons 51I, 59R, and 108N and in *Pfdhps* at codons 437G and 540E was high (>98%), reaching 100% fixation after one dose of SP, while the prevalence of 581G was 3.3% at baseline, reaching 12.5% after one dose of SP. At baseline, the prevalence of *Pfdhfr* and *Pfdhps* quintuple mutations was 89%, whereas the sextuple mutations (including 581G) were not prevalent (3.9%), reaching 16.7% after one dose of SP. However, the numbers of infections at follow-up visits were small, and hence there was insufficient statistical power to test whether there was a true rise in the prevalence of this allele. The overall high frequency of *Pfdhfr* and *Pfdhps* quintuple mutations throughout pregnancy excluded further analyses of possible associations between certain haplotypes and the risk of lower birth weight and anemia. However, women infected with *P. falciparum* had 1.3-g/dl-lower hemoglobin levels ($P = 0.001$) and delivered babies with a 400-g-lower birth weight ($P = 0.001$) compared to nonparasitemic women. Despite this, 44 women who were *P. falciparum* positive at baseline became negative after one or two doses of SP (i.e., 50.5%), implying that SP-IPTp still has some efficacy. *P. falciparum* resistance markers to SP are high in this population, whereas *P. falciparum* infection was associated with poor birth outcomes.**

Malaria in pregnancy is a major public health problem in areas where malaria is endemic (1, 2). The current policy in Uganda for treating malaria in pregnancy in the first trimester is to give quinine (10 mg/kg). During the second and third trimesters, the following artemesinin-based combination therapy is recommended: Coartem (artemether [20 mg] and lumefantrine [120 mg]) at four tablets twice a day for 3 days. For malaria prevention, the policy recommendation is at least two doses of sulfadoxine-pyrimethamine (SP) as an intermittent preventive treatment of malaria in pregnancy (IPTp) (3). Recently, the World Health Organization (WHO) issued new guidelines on IPTp for countries in areas of moderate to high transmission: IPTp with SP is now recommended for all pregnant women at each scheduled antenatal care (ANC) visit, and each SP dose should be given at least monthly (4). This recommendation is based on evidence that three or more doses of SP as IPTp are more beneficial in reducing the risk of low-birth-weight babies compared to two doses (5). In Mali, the addition of a third dose of SP halved the risk of placental malaria, low infant birth weight, and preterm births compared to two doses of SP in an area with highly seasonal malaria and low SP resistance (6).

The rapid spread of *P. falciparum* parasites that are resistant to SP in areas of malaria endemicity poses a major threat to the prevention of malaria in pregnancy (7, 8). SP resistance is mediated through mutations at the genes encoding *P. falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) (9–12). Specific mutations at three codons of the *Pfdhfr* gene, namely, N51I, C59R, and S108N, are commonly referred to as the triple mutation; mutations in the *Pfdhps* gene at codons A437G and G540E are known as the double mutation. Mutations at codon 613S have been documented in Africa (13). In Tanzania, the prevalence of a triple *Pfdhfr* mutation was recently found to be >84% in all regions, whereas prevalence of the *Pfdhps* double mutation ranged from as low as 43.8 to 95% (14). Recently, studies in some countries of East Africa have observed the emergence of the *Pfdhps* 581G mutation with quintuple mutants, resulting in sextuple mutants (15).

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Although SP-IPTp seems to be effective even in areas with a high prevalence of quintuple *P. falciparum* mutants (16), recent studies have shown that the presence of the 581G mutation may compromise continued IPTp using SP. For instance, in Tanzania, there is now evidence of the negative impact of resistance to SP on birth outcomes (17, 18). A high prevalence of circulating *P. falciparum* sextuple mutants was associated with increased parasitemia and more intense placenta infection, suggesting that the use of SP exacerbated malaria infection (17). It has further been shown that sextuple mutants were associated with reduced birth weight (19). In Uganda, a recent study in an area of intense malaria transmission has shown that the prevalence of placental malaria by histopathology was high among women who reported taking at least two doses of SP during pregnancy, indicating that SP was not associated with protection against individual birth and maternal outcomes pointing to high resistance to SP (20).

In West African countries, namely, Benin, Mali, and Senegal, resistance to SP has increased to moderate levels. In a recent study in Benin, the prevalence of *Pfdhfr* and *Pfdhps* quadruple mutations was high, and SP could not clear parasites in half of the women infected with malaria with recrudescence in 76% of women after the second dose. Although in these countries *Pfdhfr* and *Pfdhps* triple and quadruple mutations were frequent, there was no evidence of a correlation between these haplotypes and the efficacy of SP-IPTp (21–23).

In Uganda, SP resistance markers were identified early in 2004 when SP was still used as a monotherapy to treat malaria. At that time, high levels of *Pfdhfr* mutations at codons S108N and *Pfdhps* at codons A437G and K540E were not associated with clinical outcomes or parasitological failure (24, 25). Another study in Uganda assessing the effect of co-trimoxazole prophylaxis on the incidence of malaria documented a high prevalence of the quintuple mutations (26).

Since SP is the recommended drug for IPTp in Uganda (3), continuous monitoring of SP efficacy for IPTp and for molecular markers of resistance is important (7). To our knowledge, this is the first study to assess resistance markers among pregnant women using SP as an IPTp in Uganda. The main objective of the study was to assess the prevalence of *Pfdhfr* and *Pfdhps* mutations among pregnant women using SP-IPTp.

## MATERIALS AND METHODS

**Study setting.** The study was conducted in Mukono district in central Uganda. The total population of the district is 850,900, with an annual growth rate of 2.3%. Mukono district consists predominantly of subsistence farmers of the Baganda ethnic group. The majority of the population (88%) live in rural areas. The prevalence of *P. falciparum* parasitemia in children younger than 5 years was 42.4% in 2011 (27). Access to ANC (at least one visit) was 94.1%, and 25.9% of these pregnant women received the two recommended doses of SP-IPTp (28).

Mukono district has one hospital, three health centers IV, and 15 health centers III. A health center IV serves a population of approximately 100,000 people and has a theater, inpatient facilities, a maternity dispensary unit, and an outpatient unit. A health center III has an outpatient unit and a maternity dispensary unit and serves a population of 50,000 people. These health facilities attract a large number of pregnant women seeking routine ANC care for various illnesses. The study was carried out at two representative health facilities: Kawolo hospital and Mukono health center IV.

**Study participants.** Pregnant women attending routine ANC and those presenting with fever in outpatient clinics were enrolled in the study.

**Study design.** A molecular epidemiological study of *P. falciparum* parasite resistance markers to SP was undertaken. Pregnant women were recruited from August 2010 to February 2012. Pregnant women visiting Kawolo hospital and Mukono health center IV for ANC were approached and offered information about the study. Women were recruited from August 2010 to February 2012. Those who consented to participate were given SP as directly observed therapy during the second and third trimesters, as recommended by the policy in Uganda. Women who refused to give consent to the study and those who had a history of allergy to sulfonamide-containing drugs were excluded from the study. A pelvis examination was performed by trained midwives to confirm pregnancy and assess the gestation period. Any history of taking SP was noted prior to the visit. To assess hemoglobin (Hb) levels and parasitemia, blood was drawn from pregnant women at three time points: (i) at recruitment before receiving the first dose of SP (at 20 to 24 weeks of gestation), (ii) before receiving the second dose of SP (at 28 to 30 weeks of gestation), and (iii) after delivery. At delivery, the weights of the babies were measured using baby-weighing scales (UNICEF-Super Samson Salter), measuring to the nearest 50 g.

For women presenting with fever in outpatient clinics, recruitment started from January to November 2011. Inclusion criteria were (i) the presence of fever or a history of fever during the last 14 days, (ii) a temperature of at least 37.5°C (this was measured using a digital thermometer), and (iii) menstrual history and a pelvic examination by trained midwives and clinical officers to confirm pregnancy. Any history of taking SP was noted prior to the visit.

The aim of including this sample was to estimate the prevalence of gene mutations circulating in a population of pregnant women with acute malaria. Women who were *P. falciparum* positive and receiving ANC or at outpatient clinics were treated according to standard guidelines, i.e., in the first trimester the patients were given quinine (10 mg/kg), and during the second and third trimesters the patients received the recommended artemesinin-based combination therapy, i.e., Coartem (artemether [20 mg] and lumefantrine [120 mg]) administered as four tablets twice a day for 3 days.

The sample size for the study was based on the proportion of pregnant women who completed two doses of SP-IPTp and were positive for *P. falciparum*, estimated to be 5.3%. In order to estimate the proportion positive for *P. falciparum* with a ±2% absolute precision, at a power of 80% and a 5% level of significance (two sided), a minimum of 800 pregnant women was needed, allowing for a 10% loss to follow-up. In total, 1,315 pregnant women who received two doses of SP and delivered at the two studies health units and 998 pregnant women with fever were included in the sample.

**Collection of blood samples.** Blood samples were collected in 10-ml EDTA Vacutainers and immediately tested for malaria, hemoglobin levels, and HIV, and the remaining samples were temporarily stored at 4°C for genotyping. To assess parasitemia, thick blood smears were stained with Giemsa, and malarial parasites were counted against 200 leukocytes and expressed as the number of parasites/μl of blood, assuming a standard leukocyte count of 8,000/μl of blood. A blood smear was regarded as negative after the examination of at least 100 high-power fields with no parasites seen. This cutoff point was selected based on the high level of malarial endemicity in the study area. All buffers, solutions, and stains were stored appropriately, and the cleanliness and sterility of all equipment was maintained.

Blood specimens were transported to the reference laboratory at the Uganda Virus Research Institute (UVRI) in cool packs and immediately frozen at −20°C. Samples were sorted according to origin, visits, and malaria status. For quality assurance, all malaria-positive slides examined in the field laboratory were reread at the UVRI. The discrepancy between the two readings was <5%. In preparation for genotyping, the total genomic DNA was extracted from 200 μl of malaria-positive blood samples by using a QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The genomic DNA was eluted in 200 μl of elution buffer.

**Molecular genotyping.** Extracted DNA was tested for the presence of *P. falciparum* DNA by using a real-time PCR assay (29). Only positive samples were genotyped for SP resistance. A molecular assay was used for genotyping SP-resistant strains of *P. falciparum* that identifies single-nucleotide polymorphisms (SNP) within the *Pfdhfr* and *Pfdhps* genes that are associated with resistance to SP (30). The assay targets three SNPs within the *Pfdhfr* gene at codons 51I, 59R, and 108N that confer resistance to pyrimethamine and four SNPs within the *Pfdhps* gene at codons 437G, 540E, 581G, and 613G that confer resistance to sulfadoxine (8, 30). This method is based on high-resolution melting curve analysis using a LightCycler (Roche), as previously described (31). Asymmetric PCR was performed in the presence of an unlabeled probe specific to the SNP of interest, followed by melting curve analysis to identify the wild-type or mutant form. This methodology has been described to effectively genotype using a closed-tube system and has many advantages, including minimizing cross-contamination and high-throughput capacity (30, 32, 33). Parasite DNA was successfully extracted from 413 *P. falciparum*-positive samples.

**Statistical analyses.** Data were entered into Microsoft Access 2007 (Microsoft, Inc., Redmond, WA), and statistical analyses were performed using Stata v11.0 (Stata Corp., College Station, TX) statistical software. Anemia was defined as an Hb level of ≤11.0 g/dl. Low infant birth weight was defined as a birth weight of ≤2,500 g. A two-sample proportion test was used to compare differences in Hb concentration at the time of delivery and the infant birth weight between mothers with or without malaria parasitemia after receiving one to two doses of SP-IPTp at ANC visits. Univariate analyses were carried out to determine the proportions of background characteristics, the prevalence of parasitemia, anemia, and low infant birth weight. The risk of anemia and low infant birth weight among women with malarial parasites compared to women without parasitemia was measured as odds ratios (OR) using logistic regression analysis while controlling for the confounding effects of parity, gravidity, education, age, occupation, and gestational age. To determine the predictors of pregnancy outcomes among the study population, covariates were fitted in the multivariable logistic regression analysis. All significant variables with a *P* value of <0.1 from the bivariate analyses were included in multivariable analysis. A backward elimination method was used to develop the final multivariable model. A *P* value of <0.05 was considered significant.

**Analyses of *Pfdhfr* and *Pfdhps* mutations.** The analyses calculated the proportions and estimated 95% binomial confidence intervals (CI) of malarial infections with mutant alleles present in samples from pregnant women seeking ANC at the time of enrollment and at the SP-IPTp follow-up visits, as well for pregnant women attending outpatient clinics and presenting with fever or history of fever in the last 2 weeks. Each isolate was coded based on the presence or absence of a resistance-associated codon. Two infections with mixed wild-type and mutant alleles were treated as mutant, and data from all analyzed sites in the *Pfdhfr* and *Pfdhps* genes were combined to construct haplotypes. For frequency analyses, one haplotype with the majority type mutation at all analyzed alleles was scored from each sample. The prevalences of *Pfdhfr* and *Pfdhps* mutations at delivery in the cohort of women on SP-IPTp and for pregnant women with fever at outpatient clinics were calculated for each mutant allele. Associations between infection with *Pfdhfr*/*Pfdhps* mutations, and other variables, including sociodemographic details (age, education level, and marital status), the use of an insecticide-treated net (ITN), parity, gestational age, a history of using antimalarial drugs, and hemoglobin levels, were explored using a logistic regression model.

**Ethics.** Ethical approval for the study was granted by review boards at the UVRI, the Uganda National Council for Science and Technology (HS.747), and the Health Research Ethics Board of the University of Alberta. Written consent was obtained from all participating pregnant women. The documentation of consent was through recording any refusals to participate in the study, the reasons for that refusal, and/or refusal to give blood samples.

## RESULTS

**Baseline characteristics of study participants.** At baseline, 1,387 pregnant women consented to participate and were given the first dose of SP. At the second ANC visit, 1,343 received the second dose of SP, with a loss to follow-up of 44. A total of 1,315 pregnant women received two doses of SP and delivered at the two study health units, with a loss to follow-up of 28 women (Fig. 1). Analyses were performed on 1,315 women, and 998 pregnant women with fever were recruited from outpatient clinics.

The mean age of pregnant women on SP-IPTp was 24.1 years (95% confidence interval [CI] = 23.6 to 23.9). The median gestational age at ANC visit to receive the first dose of SP was 24 weeks (interquartile range [IQR], 20 to 28 weeks). At recruitment, women were asked about their history of taking SP before the visit, and the median time when SP was taken before the ANC visit was determined to be 30 days (IQR, 7 to 60 days). A total of 109 (8.3%) women had taken SP before the first visit (Table 1). A total of 232 (17.6%) pregnant women seeking ANC had fever within the last 2 weeks, and 1,056 (80.3%) of the pregnant women slept under an ITN. The mean hemoglobin level was 11.9 g/dl (range, 11.8 to 12.0 g/dl). The mean age in pregnant women with fever receiving outpatient care was 23.9 years (standard deviation, 4.9; range, 14 to 42); median gestational age was 25.2 weeks (IQR, 22 to 30), and 12.8% were positive for *P. falciparum*. The percentage of ITN coverage was also high (91.1%), and the mean Hb level was 11.9 g/dl (range, 11.8 to 12.0 [data not shown]).

**Prevalence of *P. falciparum* infections.** At baseline, the prevalence of *P. falciparum* infections among pregnant women on SP-IPTp ($n$ = 1,315) was 55 (4.2%); after one dose of SP it was 26 (2.0%), and it was 22 (1.7%) after two doses of SP. At baseline, the proportion of *P. falciparum* infections was higher among pregnant women with fever: 128/998 (12.8%) compared to 55/1315 (4.2%) among afebrile women receiving ANC (Tables 1 and 3).

The proportion of new infections was calculated as the number of pregnant women who were *P. falciparum* negative at baseline but became positive sometime between receiving the first and the second doses of SP (15/1,235 [1.2%]) and/or those who were *P. falciparum* negative at baseline and after receiving the first dose of SP but became positive sometime between the second dose of SP and delivery (10/1,235 [0.8%]). A few pregnant women (7/80 [8.8%]) were considered to have persistent infections as defined by being *P. falciparum* positive at baseline and persistently positive at all ANC visits up to delivery. It is worth noting that the number of women who were *P. falciparum* positive at baseline but become negative after one or two doses of SP was 44 (50.5%) (Table 2).

**Frequency of *Pfdhfr* and *Pfdhps* single gene mutations.** Among afebrile pregnant women at ANC visits for SP-IPTp, the frequencies of mutations at codons 51I, 59R, and 108N *Pfdhfr* were high at baseline, reaching 100% fixation for codon 108N, 89.2% for codon 59R, and 98.2% for codon 51I. Similarly, there was a high frequency of mutation in *Pfdhps* codon 437G (99.1%), whereas the prevalence of *Pfdhps* codon 540E (98.2% at baseline) reached fixation and was 100% after one dose of SP. The prevalence of codon 581G was 3.3% at baseline, reaching 12.5% after one dose of SP, whereas only wild-type alleles were observed at codon 613A. The distribution of SNPs among febrile pregnant women attending outpatient clinics showed the same pattern (Table 3).

**Prevalence of *Pfdhfr* and *Pfdhps* mutant haplotypes.** SNPs in *Pfdhfr* were constructed into 51-59-108 haplotypes. The following

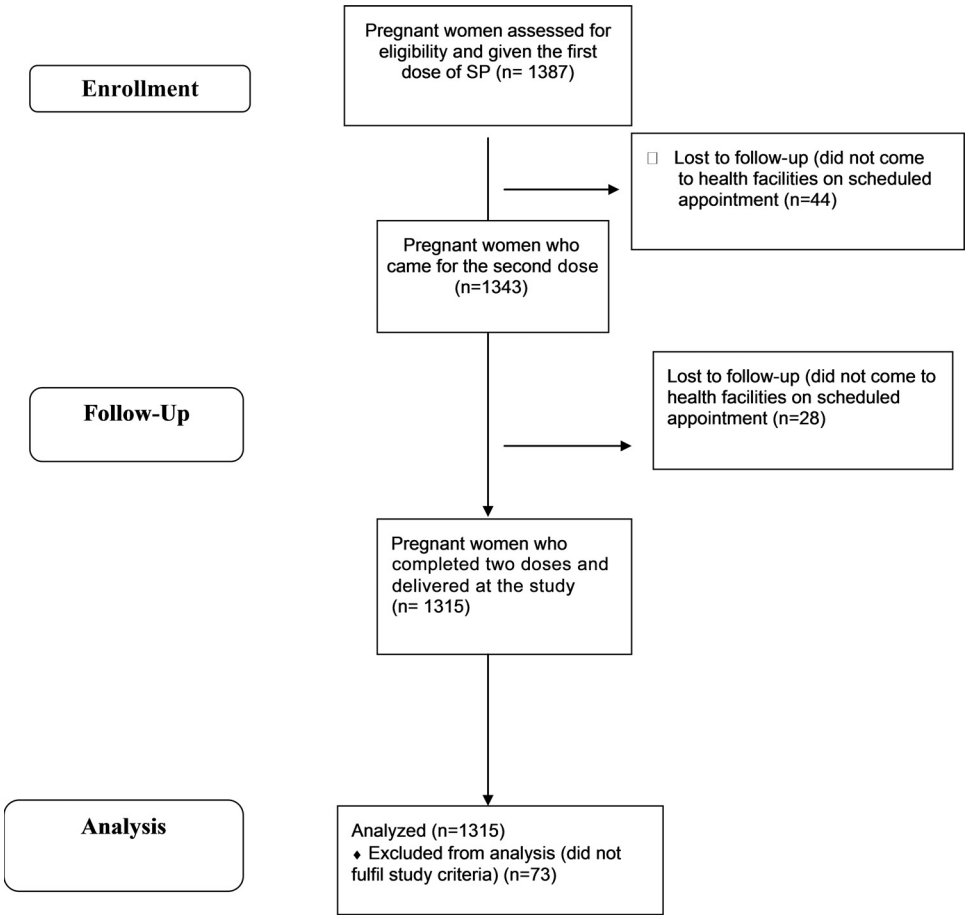


**FIG 1** Recruitment of patients.

haplotype frequencies were observed: at baseline, the double mutant 51I+108N frequency was 98.2%, and the double mutant 59R+108N was detected in 89.2% of samples, whereas the frequency of the triple mutant 51I+59R+108N was 90.7%. The frequency of the *Pfdhps* double mutant 437G+540E was 98.2%, and that of the *Pfdhfr* and *Pfdhps* combined quadruple mutation (51I+59R+108N)+437G was 90.7%. The frequency of the quintuple mutation, (51I+59R+108N)+(437G+540E), was 88.9%, and that of the sextuple mutation, (51I+59R+108N)+(437G+540E+581G), was 3.9% (Table 3). A similar pattern was observed among pregnant women with fever attending outpatient clinics (Table 4).

**TABLE 1** Characteristics of pregnant women receiving routine ANC enrolled in the study ($n$ = 1,315)[a]

| Parameters for pregnant women | Finding |
|---|---|
| Median age in yr (IQR) | 23 (20–27) |
| No. (%) of patients with fever in the last 2 weeks | 232 (17.6) |
| No. (%) of patients with fever in the last 2 weeks and treated with SP | 38 (16.4) |
| No. (%) of *P. falciparum*-negative patients given SP ($n$ = 1,260) | 37 (2.9) |
| Median no. of days (IQR) when SP was taken before the visit | 30 (7–60) |
| No. (%) of women who had taken SP before the first visit | 109 (8.3) |
| Median gestation period in wk (IQR) | 24 (20–28) |
| No. (%) of patients that slept under an ITN | 1,056 (80.3) |
| No. (%) of pregnant women positive for *P. falciparum* (microscopy) | 55 (4.2) |
| Mean Hb level in g/dl (range) | 11.9 (11.8–12.0) |

[a] ITN, insecticide-treated net; SP, sulfadoxine-pyrimethamine; IQR, interquartile range.

**Association of *P. falciparum* parasitemia, anemia, and birth**

**TABLE 2** SP-IPTp and *P. falciparum* parasitemia

| *P. falciparum* infection status at[a]: | | | |
|---|---|---|---|
| First visit | Second visit | Delivery | Frequency (%)[b] |
| Negative | Negative | Negative | 1,235 (93.9) |
| Positive | Negative | Negative | 40 (50.0) |
| Negative | Positive | Negative | 14 (1.1) |
| Negative | Negative | Positive | 10 (0.8) |
| Positive | Positive | Negative | 4 (5.0) |
| Positive | Negative | Positive | 4 (5.0) |
| Negative | Positive | Positive | 1 (0.1) |
| Positive | Positive | Positive | 7 (8.8) |

[a] First visit: baseline, first dose of SP. Second visit: second dose of SP.
[b] Frequency is expressed as the total number (% total) of patients with the indicated pattern of infection findings.

**TABLE 3** Prevalence of *Pfdhfr* and *Pfdhps* single and haplotype mutations among women on SP-IPTp[a]

| *Pfdhfr* and *Pfdhps* haplotype(s) | Prevalence of *Pfdhfr* and *Pfdhps* mutations at[b]: | | |
|---|---|---|---|
| | First ANC visit (baseline sample) | Second ANC visit (after one SP dose) | Delivery (after two SP doses) |
| *dhfr* 51I | 56/57 (98.2) | 13/13 (100) | 9/9 (100) |
| *dhfr* 59R | 74/83 (89.2) | 18/18 (100) | 9/10 (90) |
| *dhfr* 108N | 105/105 (100) | 23/23 (100) | 14/14 (100) |
| *dhps* 437G | 116/117 (99.1) | 26/26 (100) | 14/15 (93.3) |
| *dhps* 540E | 107/109 (98.2) | 24/24 (100) | 14/15 (93.3) |
| *dhps* 581G | 2/61 (3.3) | 2/16 (12.5) | 0/9 (0) |
| *dhps* 613S | 0/74 (0) | 0/18 (0) | 0/10 (0) |
| *dhfr* double mutant 51I+108N | 56/57 (98.2) | 13/13 (100) | 9/9 (100) |
| *dhfr* double mutant 59R+108N | 74/83 (89.2) | 18/18 (100) | 9/10 (90) |
| *dhps* double mutant 437G+540E | 107/109 (98.2) | 24/24 (100) | 14/15 (93.3) |
| *dhfr* triple mutant 51I+59R+108N | 49/54 (90.7) | 13/13 (100) | 7/8 (87.5) |
| Quadruple mutant (51I+59R+108N)+437G | 49/54 (90.7) | 13/13 (100) | 6/8 (75) |
| Quintuple mutant (51I+59R+108N)+(437G+540E) | 48/54 (88.9) | 13/13 (100) | 6/8 (75) |
| Sextuple mutant (51I+59R+108N)+(437G+540E+581G) | 2/52 (3.9) | 2/12 (16.7) | 0/8 (0) |

[a] The proportions of *P. falciparum*-positive mutations were as follows: first ANC visit, 55(4.2%); second ANC visit, 26 (2.0%); and delivery, 22 (1.7%). SP, sulfadoxine-pyrimethamine; IPTp, intermittent preventive treatment.
[b] Prevalence data are expressed as follows: number of mutations/total number of samples tested (%).

**weight.** The association of *P. falciparum* parasitemia at delivery with infant birth weight was analyzed among 1,315 women who received two doses of SP-IPTp at ANC clinics. Pregnant women with *P. falciparum* parasitemia at delivery had babies with an average weight that was 400 g (95% CI = 120 to 505; $P = 0.001$) lower than those without parasitemia after adjusting for the differences in age, study sites, and whether the child was born alive or not (Table 4). The numbers of low-birth-weight babies (<2,500 g) were 4/24 (18.2%) among parasitemic women and 104/1,291 (8.0%) among nonparasitemic women (OR 2.6; 95% CI = 0.6 to 8.0; $P = 0.08$) (Table 5).

Similarly, pregnant women who were *P. falciparum* positive at delivery had an average Hb level of 1.3 g/dl, a lower Hb level than those without parasitemia ($P = 0.001$) (Table 5). Anemia was significantly associated with malaria parasitemia (adjusted OR [AOR] = 4.5, 95% CI = 1.7 to 12.0). Educated pregnant women were less likely to have anemia than women with little education (AOR = 0.6; 95% CI = 0.4 to 0.9), and women with a gravidity score of <4 were more likely to have anemia than women with a gravidity score of ≥4. Maternal age was associated with delivering a low-birth-weight baby; older women were less likely to deliver babies with low birth weights (OR = 0.9, 95% CI = 0.9 to 0.1.0) (Table 6).

**TABLE 4** Prevalence of *Pfdhfr* and *Pfdhps* single and haplotype mutations among febrile pregnant women presenting at outpatient clinics[a]

| *Pfdhfr* and *Pfdhps* haplotype(s) | Prevalence of *Pfdhfr* and *Pfdhps* mutations[b] |
|---|---|
| *dhfr* 51I | 111/111 (100) |
| *dhfr* 59R | 112/116 (97) |
| *dhfr* 108N | 120/120 (100) |
| *dhps* 437G | 120/120 (100) |
| *dhps* 540E | 119/120 (99) |
| *dhps* 581G | 8/103 (8) |
| *dhps* 613S | 0/111 (0) |
| *dhfr* double mutant 51I+108N | 111/111 (100) |
| *dhfr* double mutant 59R+108N | 112/116 (97) |
| *dhps* double mutant 437G+540E | 119/120 (99) |
| *dhfr* triple mutant 51I+59R+108N | 107/110 (97) |
| Quadruple mutant (51I+59R+108N)+437G | 107/110 (97) |
| Quintuple mutant (51I+59R+108N)+(437G+540E) | 106/110 (96) |
| Sextuple mutant (51I+59R+108N)+(437G+540E+581G) | 8/101 (8) |

[a] The proportion of *P. falciparum*-positive mutations was 128/998 (12.8%).
[b] Prevalence data are expressed as follows: number of mutations/total number of samples tested (%).

## DISCUSSION

A high prevalence of *Pfdhfr/Pfdhps* single and haplotype mutations were found at baseline (prior to the first dose of SP), including a high prevalence of the K540E mutation (98.2%). However, only a few parasites (3.3%) had the additional 581G *Pfdhps* mutations that increased after the second dose of SP, whereas *P. falciparum* infection was associated with poor birth outcomes. Previous studies have associated the A581G and 540E point mutations with high SP treatment failure (15). In addition, a high proportion of women (50.5%) were *P. falciparum* positive at baseline but became negative after one or two doses of SP. Thus, SP-IPTp could still have some beneficial effect in Uganda. However, it is a concern that among pregnant women presenting with fever at outpatient clinics the prevalence of the A581G mutation was 8.0%, indicating that soon SP-IPTp may become ineffective.

There have been recent discussions at the WHO as to whether countries should discontinue IPTp-SP when the population prevalence of *Pfdhps* mutation K540E is >95% and the prevalence of mutation A581G is >10% (32). However, there is currently no clear policy recommendation on this cutoff point. In eastern and southern Africa, where antifolate resistance in *P. falciparum* is highest, monitoring of SP resistance should focus on the *Pfdhps* A581G and K540E mutations (34).

Similar findings of high prevalences of resistant haplotypes were reported in Tanzania (17) and Kenya (35). It has also been

**TABLE 5** Association of *P. falciparum* parasitemia at delivery with birth outcomes[a]

| Parameter | Findings for pregnant women at delivery after receiving two doses of SP ($n = 1,315$) | | Statistical significance ($P$) |
|---|---|---|---|
| | *P. falciparum* negative | *P. falciparum* positive | |
| Mean Hb level in g/dl (range) | 12.5 (12.3–12.6) | 11.2 (10.7–12.3) | 0.001 |
| Mean birth wt in kg (range) | 3.2 (3.15–3.20) | 2.8 (2.50–3.08) | 0.001 |
| Proportion (%) of low birth wt | 104/1,291 (8.0) | 4/24 (18.2) | 0.08 |

[a] Adjusted for differences in age, study sites, and whether the child was born alive or not.

shown that the use of SP for IPTp has negative effects on pregnancy outcomes in areas with high parasite resistance to SP (18). In the Democratic Republic of Congo, SP was documented to have a negligible effect on low infant birth weight in an area with high levels of therapeutic failure (36). In our study, lower hemoglobin levels and lower-birth-weight infants were observed outcomes among mothers infected with *P. falciparum* at delivery. Considering the high prevalence of multiple resistance alleles in these parasites, these negative outcomes may be associated with SP resistance.

In an earlier study in the same study population, it was found that a significant proportion of pregnant women with fever and those negative for *P. falciparum* were treated with SP (37). This widespread use of SP for treatment of malaria could have resulted in high drug pressure and could account for the high SP resistance observed in the present study. Policy decisions urgently need to be enacted to restrict the use of SP for the treatment of malaria.

The low parasitemia prevalence of 4.2% at baseline could be attributed to the high level of ITN use (80.3%). Previously, parasitemia among pregnant women in the study area was 24.5%, and

**TABLE 6** Risk factors associated with anemia and low birth weight in pregnant women attending routine ANC visits[a]

| Variable(s) | $n$ (%) | OR (95% CI) | | | |
|---|---|---|---|---|---|
| | | Low birth wt (≤2.5 kg) | | Anemia (≤11.0 mm/dl) | |
| | | Unadjusted | Adjusted | Unadjusted | Adjusted |
| *P. falciparum* parasitemia | | | | | |
| Absent | 1,298 | 1.0 | | 1.0 | |
| Present | 17 (1.3) | 2.6 (0.9–8.0) | 2.3 (0.8–7.0) | 4.36 (1.79–10.59) | 4.5 (1.7–12.0) |
| Birth outcome | | | | | |
| Live birth | 1,293 | | | 1.0 | |
| Stillbirth | 16 (1.2) | | | 0.61 (0.14–2.71) | |
| Study site | | | | | |
| Kawolo hospital | 738 | 1.0 | | 1.0 | |
| Mukono health center | 577 (43.8) | 1.3 (0.8–1.9) | | 0.9 (0.8–1.3) | |
| Mother's education | | | | | |
| No education | 160 (12.9) | 1.0 | | 1.0 | |
| Completed primary/secondary/tertiary | 1,086 | 0.8 (0.4–1.3) | | 0.6 (0.4–0.9) | 0.6 (0.4–0.9) |
| Mother's occupation | | | | | |
| Peasant/agriculture | 125 (10.1) | 1.0 | | 1.0 | |
| Earns salary/business | 383 (31.0) | 0.94 (0.43–2.06) | | 1.21 (0.71–2.07) | |
| Student/no job/activity that earns money | 728 (58.9) | 1.26 (0.61–2.61) | | 1.13 (0.68–1.87) | |
| Parity score | | | | | |
| 3+ | 335 | 1.0 | | 1.0 | |
| 0–2 | 982 (74.6) | 0.9 (0.6–1.5) | | 1.3 (0.9–1.8) | |
| Gravidity score | | | | | |
| 3+ | 353 | 1.0 | | 1.0 | |
| 1–2 | 962 (73.2) | 1.18 (0.69–1.68) | | 1.36 (0.98–1.89) | 1.39(0.99–1.96) |
| Mother's age | | | | | |
| Median yr (range) | 23 (20–27)* | 0.9 (0.9–1.0) | 0.9 (0.9–1.0) | 0.98 (0.96–1.0) | |
| Gestational age | | | | | |
| Median yr (range) | 24 (20–28)* | 0.9 (0.9–1.0) | | 1.0 (0.9–1.1) | |

[a] *, Interquartile ranges. OR, odds ratio; CI, confidence interval.

net use was 8.8% (38). On the other hand, the low prevalence of parasitemia could justify a focus on prevention interventions other than SP-IPTp, which is costly with less benefit. Currently, Uganda spends $428,453 annually on the procurement, storage, and distribution of SP for IPTp (39). Intermittent screening and treatment with an effective antimalarial drug could be a useful tool for malaria management in pregnancy, as recently shown in Ghana (40).

In the present study, women infected with *P. falciparum* had 1.27-g/dl-lower hemoglobin levels and delivered babies with 310-g-lower birth weights compared to nonparasitemic women. Although we wanted to look at the associations between *Pfdhfr* and *Pfdhps* and birth weight, the high prevalence of quintuple mutations reaching fixation with the sextuples made such an assessment impossible. We are thus cautious in generalizing our results, since the effect of SP on maternal Hb levels and the birth weights of babies could have been confounded by factors such as nutrition, access to treatment of anemia with folic acid and iron, deworming, and other health promotion services that were not evaluated. It was also difficult to get precise estimates of gestational age since this depended on pregnant women knowing the last day of their menstruation period, which was affected by poor memory recall in some cases. In the present study, we were unable to show the impact of the haplotype infections on birth outcomes, as a previous study in Tanzania demonstrated (19), because of the low prevalence of 581G mutations.

One of the limitations of our study is that we were not able to genotype the *Pfdhfr* I164L mutation, which has been associated with high SP resistance (26, 41). Similarly, we did not get a full haplotype on all of the SNPs due to poor-quality DNA in a few samples. The prevalence of the I164L mutation, which confers high-level resistance, has been documented in the Kabale and Rukungiri districts in southwest Uganda (41). More recently, in Kenya, as well as in Rwanda (43), the *Pfdhfr* I164L mutation has been detected (42). We recommend evaluating the resistance allele in future studies given the overall lack of evidence for SP-IPTp in areas of extensive SP resistance and the need for up-to-date information on all relevant mutations.

In interpreting our results, we are cautious because our data represent women who delivered at health facilities. However, in the study area, the majority of women (62%) deliver at health facilities, and we are confident that these data could be generalized to those women and to other women in other areas of Uganda where malaria is endemic (27).

This is the first report on *P. falciparum* SP-resistant haplotypes for IPTp in Uganda and the most recent in the East African region. Malaria is highly endemic in the study area (Mukono district, located in central Uganda near Lake Victoria), with year-round transmission. Based on these results, Uganda needs to identify an alternative to SP for IPTp or to broaden the use of ITNs and effective case management, as recommended by the WHO in areas where SP resistance is high (34).

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A.K.M. conceived the study. A.K.M., J.B., S.Y., and P.M. participated in the design, implementation, and coordination of the study and in drafting the manuscript. S.S. performed the genotyping. S.M. participated in data analyses. M.A. and S.M. participated in reviewing the manuscript and interpretation of findings. All authors read and approved the manuscript.

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