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Selection of Antimalarial Drug Resistance after Intermittent Preventive Treatment of Infants and Children (IPTi/c) in Senegal

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Abstract. Our study investigated the possible impact of SP-IPT given to infants and children on the prevalence of SP-resistant haplotypes in the *Plasmodium falciparum* genes *Pfdhfr* and *Pfdhps*, comparing sites with and without IPTi/c. *P. falciparum* positive samples (N = 352) collected from children < 5 years were analyzed to determine the prevalence of SP resistance-related haplotypes by nested PCR followed by sequence-specific oligonucleotide probe-enzyme-linked immunosorbent assay. The prevalence of the *Pfdhfr* triple mutant haplotype (CIRN) increased in both groups, but only significantly in the IPTi/c group from 41% to 65% in 2011 ($P = 0.005$). Conversely, the *Pfdhps* 437G mutation decreased in both groups from 44.6% to 28.6% ($P = 0.07$) and from 66.7% to 47.5% ($P = 0.02$) between 2010 and 2011 in the control and the IPTi/c groups, respectively. A weak trend for decreasing prevalence of quadruple mutants (triple *Pfdhfr* + *Pfdhps* 437G) was noted in both groups ($P = 0.15$ and $P = 0.34$). During the two cross-sectional surveys some significant changes were observed in the SP resistance-related genes.

INTRODUCTION

During the last decades, *Plasmodium falciparum* has become broadly resistant to widely used antimalarial drugs such as chloroquine (CQ) and sulfadoxine-pyrimethamine (SP).¹ Still, one of the essential strategies to control *P. falciparum* infections in malaria-endemic countries in sub-Saharan Africa is the use of SP as an intermittent preventive treatment (SP-IPT) for malaria in risk groups.² For instance, many African countries have through their National Malaria Control Program (NMCP) implemented the use of SP-IPT during pregnancy (IPTp).³ In recent years, several studies in Africa, including Senegal, have explored the use of SP for malaria prevention in infancy (IPTi) through the Expanded Program on Immunization (EPI). In a pooled data analysis of six clinical SP-IPTi trials, the strategy was shown to provide a 30% overall protection against clinical malaria episodes (95% confidence interval [CI] = 19.8–39.4%) and overall reduction in the prevalence of anemia (< 8 g/dL) by 21.3% (95% CI = 8.3–32.5%).^{4,5} Consequently, the World Health Organization (WHO) recommended the implementation of the IPTi strategy.⁴ However, a SP-IPTi study in Tanzania, not included in the pooled analysis, contrarily found no protection at all, most likely a result of the high level of SP resistance in the *P. falciparum* populations prevailing in the study area.⁶ Thus, the protective efficacy of IPTi is highly dependent on regional levels of SP resistance in the *P. falciparum* populations.

In areas of markedly seasonal malaria transmission, such as the Sahel and sub-Sahel regions of Africa, the main burden of malaria is in older children rather than infants, and the risk of clinical malaria is restricted largely to a few months each year.^{7,8} In such areas, administration of IPT to children (IPTc) 3 months to 5 years of age monthly during the seasonal peak in malaria transmission seems like an attractive strategy in preventing malaria. A meta-analysis of 12 clinical IPTc studies

in sub-Saharan Africa including Senegal using SP, but always combined with another drug (amodiaquine AQ), have demonstrated impressive protective efficacies against clinical malaria episodes ranging from 31% to 93% (overall protective efficacy of 82% (95% CI = 75–87%).⁹ Furthermore, it was shown that IPTc protected against all-cause mortality by 57% (95% CI = 24–76%).⁹ Thus, IPTc is highly effective and furthermore, well tolerated.^{10–12} Therefore, WHO recommended IPTc,¹³ now named Seasonal Malaria Chemoprevention (SMC) and the intervention will most likely be adopted large scale in areas where malaria transmission is seasonal. However, as long as SP is used, the protective efficiency of these strategies is highly dependent on the current level and spread of SP resistance in *P. falciparum* populations.

Plasmodium falciparum in vivo and in vitro resistance to pyrimethamine and sulfadoxine is associated with single nucleotide polymorphisms (SNPs) in the *P. falciparum* dihydrofolate reductase gene (*Pfdhfr*) and the dihydropteroate synthetase gene (*Pfdhps*), respectively. Resistance to pyrimethamine is related with SNPs in the *Pfdhfr* gene resulting in amino acid changes at mainly positions N51I, C59R, S108N/T, and I164L.^{14,15} In the *Pfdhps* gene, five SNPs, namely, S436A/F, A437G, K540E, A581G, and A613S/T is associated with *P. falciparum* resistance to sulfadoxine.^{16,17} The accumulation of SNPs in both *Pfdhfr* and *Pfdhps* genes leads to increased risk of clinical failures after SP treatment. The predictive value of the combination of these SNPs varies geographically, depending on, e.g., baseline prevalence, age, and levels of acquired immunity.^{18–22} In Africa, the *Pfdhfr* triple mutant, 51I-59R-108N, together with the *Pfdhps* double mutant, 437G-540E forms the quintuple mutant that predicts a high risk of treatment failure after SP treatment.^{23,21} Similarly, and specifically regarding IPTi, recent evidence suggests that high prevalence (> 50%) of the highly resistant *Pfdhps* 540E mutant—essentially the quintuple *Pfdhfr/Pfdhps* mutant, may undermine the required protective efficacy of SP-IPTi of 20%.²⁴

However, cumulative effects of IPTi/c interventions and what happens when follow-up is performed at long term remain largely unknown. The aim of this study was to investigate the possible impact of IPTi/c on prevalence of SNPs in

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Pfdhfr/Pfdhps after long-term follow-up in Senegal. The study was conducted in one area where both IPTi and IPTc strategies have been implemented since 2007 and 2009, respectively, and compared with a study site where none of these strategies have been applied.

METHODS

Study sites and sample collection. The samples for this study were collected in the southern part of Senegal in Velingara, Saraya, and Tambacounda health districts located 500, 400, and 700 km from the capital city of Dakar, respectively (Figure 1). In Velingara and Saraya districts, a pilot study of IPTi was conducted from 2006 to 2009. In the same area, IPTc was also implemented from 2009 until now. Tambacounda district was the control for both strategies. Malaria transmission in these areas is seasonal and in 2008 the entomological inoculation rate was 264-infected bites/person/year²⁵; before blood sample collection, written informed consent was obtained from the parent or guardian of each child. During the study, if children presented to health huts with symptoms consistent with uncomplicated malaria, including fever and a positive rapid diagnostic test (RDT), they were offered standard artemisinin combination therapy first-line treatment, and children with

severe malaria were referred to the nearest health center and received quinine treatment.

Blood samples were collected using finger prick blood. Thick and thin smears were stained with Giemsa. Parasite density was determined by counting the number of asexual parasites per 200 white blood cells, and calculated per μL using the following formula: number of parasites $\times 8,000/200$, assuming a white blood cell count of 8,000 cells/ μL . The absence of malaria parasites in 200 high power ocular fields of the thick film was considered as negative. Additionally, the finger prick blood was also blotted onto Whatman filter paper 3MM. Samples were stored at room temperature and protected with silica gel desiccant. Filter papers corresponding to thick *P. falciparum* positives were selected for later *P. falciparum* DNA isolation.

DNA extraction and *Pfdhfr/Pfdhps* SNPs analysis. DNA was extracted from positive *P. falciparum* blood spots by the Chelex-100 method described by Wooden and others²⁶ with some modifications described by Pearce and others²⁷. A nested polymerase chain reaction (PCR) (was used to amplify fragments of the *Pfdhfr* and *Pfdhps* genes as described by Alifrangis and others.²⁸ The 20- μL *Pfdhfr/Pfdhps* outer PCR mixture consisted of 0.3 mM of each dNTP, 0.25 μM of either primer set M1/M7 (*Pfdhfr*) or N1/N2 (*Pfdhps*), one unit

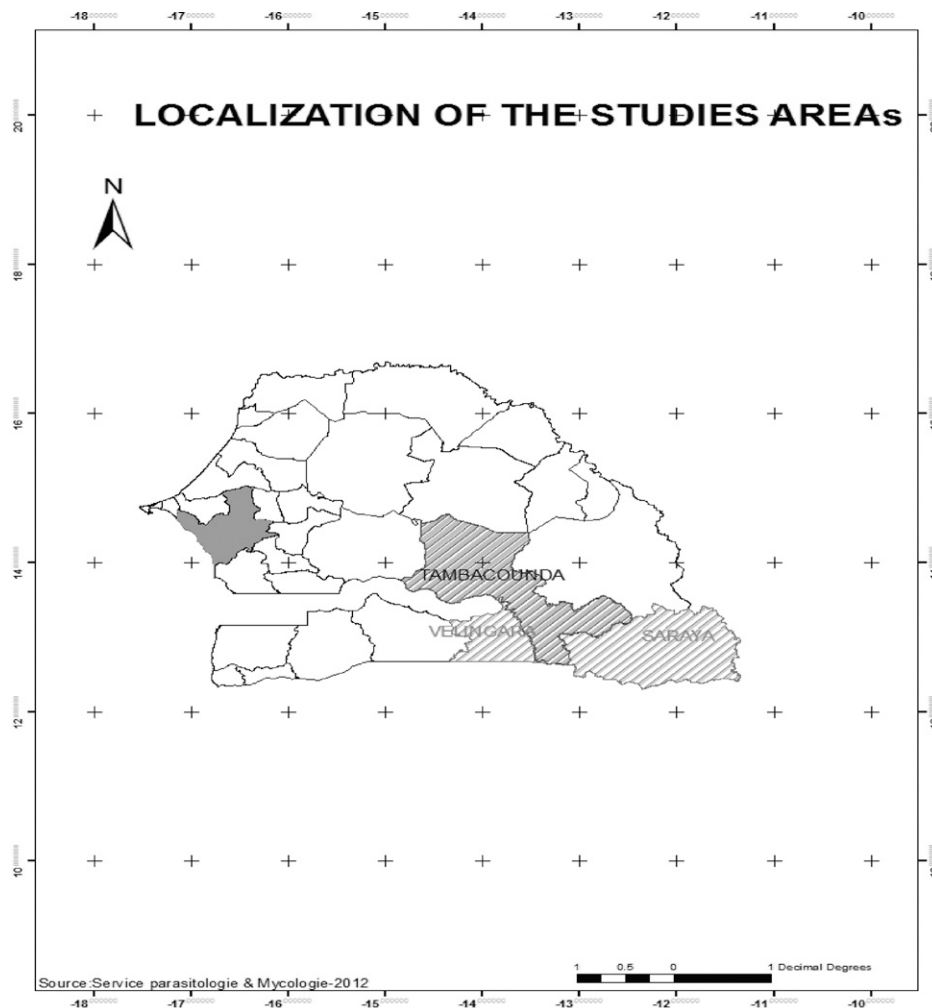


FIGURE 1. Study area Velingara and Saraya: IPTi/c districts Tambacounda: Control district (without IPTi/c).

of DNA HotStart polymerase (Ampliqon III; VWR-Bie Berntsen, Denmark), buffer containing 1.5 mM MgCl₂, as recommended by the manufacturer, and 1 µL of extracted DNA. Genomic DNA preparation of laboratory isolates 3D7, FRC3, K1, Dd2, and 7G8 were included as controls with known *Pfdhfr*/*Pfdhps* haplotypes. The nested *Pfdhfr* and *Pfdhps* PCR reaction mixture was the same as the outer PCR mixture using primer sets M3b/M9 and R2/R/ for the *dhfr* and *dhps* PCR, respectively. Amplifications were performed in 96-well PCR microplates. The M9 and R/primers for the *Pfdhfr* and *Pfdhps*, nested PCRs were biotinylated at the 5'-end by the supplier (MWG Biotech, Riskov, Denmark). The nested PCR products were confirmed by running the controls by electrophoresis on 1.5% agarose gel.

The SNPs at *Pfdhfr* (position 50/51, 59, and 108), *Pfdhps* (position 436/437, 540, 581, and 613) were determined by the sequence-specific oligonucleotide probe (SSOP)-enzyme-linked immunosorbent assay (ELISA)-based technique of PCR amplified fragments, as described in Reference 28. Briefly, biotin-conjugated nested PCR amplified DNA were fixed on streptavidin-coated ELISA plates and mixed with digoxigenin-labeled oligonucleotide probes with specificity for the SNPs of interest. The mixtures were washed with high stringency at set temperatures using Tetramethyl ammonium chloride (TMAC; Sigma Aldrich Chemie, Seelze, Germany) solution (3 M TMAC, 50 mM Tris, pH 8.0, 0.1% sodium dodecyl sulfate, 2 mM EDTA, pH 8.0), before incubated with peroxidase-conjugated anti-digoxigenin antibodies and visualized by o-phenylenediamine (OPD).²⁸ For each SNP analyses, parasite samples were categorized into single or mixed infections as follows: infections were considered to be single genotype when only one SNP was present at optical density (OD) values above the threshold of positivity. Samples were considered to be mixed but containing a majority SNP genotype when the OD value of the weakly reacting SSOP was less than half the OD value of the strongly reacting SSOP. Conversely, if the OD value of the weakly reacting SSOP was higher than half the OD value of the strongly reacting SSOP, the infection was categorized as mixed with no dominant genotype. For the construction of *Pfdhfr* or *Pfdhps* haplotypes, samples that contained mixed SNP genotypes without one being dominant at more than one codon were omitted from the analysis.

Statistical analysis. Statistical analyses of data were performed using Epi Info (version 06; CDC, Atlanta, GA). The χ^2 test was used to compare differences in proportions in *Pfdhfr*/*Pfdhps* comparing parasite populations. The significance level of statistical tests was set at 0.05, with a two-sided test. Data from this study were also compared with previous *Pfdhfr*/*Pfdhps* studies conducted in the same study area using the χ^2 test.

Ethical approval. This study received the approval of the National Ethical Committee (000169 MSP/DS/CNERS) and the administrative approval of the Ministry of Health and Prevention of Senegal (004883 MSP/DS/CNERS). Informed consent was signed by all parents or legal representatives of children before any blood sampling.

RESULTS

Overall, 1,903 samples were collected in the health district of Tambacounda without IPTi/c intervention; e.g., the control zone (804 in 2009 and 1,099 in 2010) and a total of 2,457 samples (1,215 in 2009 and 1,242 in 2010) in the health district of Velingara and Saraya where IPTi/c has been implemented (IPTi/c zone).

The prevalence of *P. falciparum* infections in the control zone (based on RDT determination) increased significantly from 10.1% (81 of 804) in 2009 to 13.9% (153 of 1,099) in 2010 ($\chi^2 = 5.00$, $P = 0.02$). Similarly, in the IPTi/c zone the prevalence increased significantly from 3.7% (46 of 1,215) in 2009 to 10.1% (125 of 1,242) in 2010 ($\chi^2 = 32.57$, $P \leq 0.001$). Overall, 234 samples were *P. falciparum* RDT positive in the control group and 176 in the IPTi/c zone. Out of these, 164 samples (88 samples in 2009 and 88 in 2010) were randomly selected in each zone for further PCR analysis.

Prevalence of SNPs in the *Pfdhfr* and *Pfdhps* genes in samples from IPTi/c and control zones in 2009 and 2010. In the control zone, the prevalence of mutant SNPs at N51I, C59R, and S108N in *Pfdhfr* including mixed infections, were 65.3% (47 of 72), 65.3% (47 of 72), and 72.6% (53 of 73), respectively, in 2009, whereas 75.0% (48 of 64), 76.6% (49 of 64), and 91.0% (69 of 76), respectively, in 2010 (Table 1). Though a trend for an increase in the prevalence of all mutant SNPs was observed, only S108N was significant ($\chi^2 = 8.30$, $P = 0.003$).

In the IPTi/c zone, the prevalence of N51I, C59R, and S108N including mixed infections, were 60.8% (48 of 79), 67.5% (54 of 80), and 73.0% (54 of 74), respectively, in 2009, whereas in 2010 it was 60.7% (51 of 84), 71.2% (52 of 73), and 79.7% (59 of 74), respectively, and no significant difference was observed between the years (Table 1). For both zones, only wild-type at codon 164 was detected. Between the control and the IPTi/c zone in 2009, there was no difference between the codons of *Pfdhfr* (data not shown).

For the *Pfdhps* gene, in the control zone, a trend for a decrease in the prevalence of *Pfdhps* A437G including mixed infections was observed from 44.6% (25 of 56) in 2009 to 28.6% (16 of 56) in 2010 ($\chi^2 = 3.12$; 0.07), whereas in the IPTi/c zone a significant decrease was noted from 66.7% (54 of 81) in 2009 to 47.5% (29 of 61) in 2010 ($\chi^2 = 5.92$, $P = 0.01$) (Table 1). The *Pfdhps* A581G was identified

TABLE 1
Prevalence of *Pfdhfr*/*Pfdhps* mutation before and after IPTi/c in Senegal

Mutations	Control zone		P values	IPTi/c zone		P values	
	2009	2010		2009	2010		
<i>Pfdhfr</i>	51I	65.3% (47/72)	75.0% (48/64)	0.21	60.8% (48/79)	60.7% (51/84)	0.99
	59R	65.3% (47/72)	76.6% (49/64)	0.14	67.5% (54/80)	71.2% (52/73)	0.61
	108N	72.6% (53/73)	90.7% (69/76)	10 ⁻³	73.0 (54/74)	79.7% (59/74)	0.33
<i>Pfdhps</i>	437G	44.6% (25/56)	66.7% (54/81)	0.01	28.6% (16/56)	47.5% (29/61)	0.03
	581G	0.0% (0/64)	2.7% (2/73)	–	2.4% (2/83)	0.0% (0/78)	–
	613S	4.8% (3/62)	7.1% (5/70)	0.85	4.9% (4/81)	0.0% (0/72)	–

in the IPTi/c zone at 2.4% (2 of 83) in 2009, however absent in the 2010 samples, whereas it was 2.7% (2 of 73) in 2010 in the control zone. In 2009, the prevalence of *Pfdhps* 613S was 4.8% (3 of 62) and 4.9% (4 of 81) in the control and IPTi/c zone, respectively, although in 2010 it rose to 7.1% (5 of 70) in the control zone and was absent in the IPTi/c zone (Table 1). For both zones, only wild-types (540 K) were detected.

Prevalence of constructed *Pfdhfr* and *Pfdhps* haplotypes in samples from IPTi/c and control zones in 2009 and 2010. In the control zone, the prevalence of parasites harboring the double mutant haplotypes (CICN, CNRN) including mixed haplotype infections (excluding haplotypes with more than one mixed SNP) was 9.3% (6 of 64) in 2009 and 5.1% (2 of 39) in 2010 with no significant difference between the years ($P = 0.68$) (Figure 2). In the IPTi/c zone, the prevalence of double mutant haplotype was 2.3% (1 of 43) in 2009, although increasing significantly to 20.0% (10 of 50) in 2010 ($\chi^2 = 6.92$, $P = 0.008$). Regarding the triple mutant *Pfdhfr* haplotype (CIRN), there was a small trend for an increase in prevalence in the control group from 52.1% (37 of 71) in 2009 to 64.3% (36 of 56) in 2010 ($\chi^2 = 1.90$, $P = 0.17$), whereas in the IPT group, it was significant, from 40.6% (28 of 69) in 2009 to 64.6% (42 of 65) in 2010 was observed ($\chi^2 = 7.75$, $P = 0.005$). A decrease of *Pfdhfr* wild-types and/or single mutants was noted in the IPTi/c zone.

There was a trend for a decrease in the prevalence of parasites harboring the *Pfdhps* 437G mutation (as SGKAA, AGKAA, and FGKAA) from 44.6% (25 of 56) in 2009 to 28.6% (16 of 56) in 2010 in the control group ($\chi^2 = 3.12$, $P = 0.08$), whereas this was significant in the IPT-group from 66.7% (54 of 81) in 2009 to 47.5% (29 of 61) in 2010 ($\chi^2 = 5.24$; $P = 0.02$).

When both *Pfdhfr* and *Pfdhps* haplotypes were jointly examined by constructing *Pfdhfr-Pfdhps* haplotypes, the prevalence of quadruple mutant parasites (CIRN/SGKAA or CIRN/AGKAA), a limited decrease in both groups from 36.8% (14 of 38) to 20.0% (5 of 25) ($\chi^2 = 2.03$, $P = 0.15$) and from 35.6% (21 of 59) to 27.1% (13 of 48) was observed in 2009 and 2010 for the control and the IPTi/c group, respectively ($\chi^2 = 0.88$, $P = 0.35$).

Temporal distribution of *Pfdhfr/Pfdhps* SNP/haplotypes prevalence in samples from IPTi/c and control zones between 2006 and 2010. Results of the *Pfdhfr/Pfdhps* SNPs/haplotypes

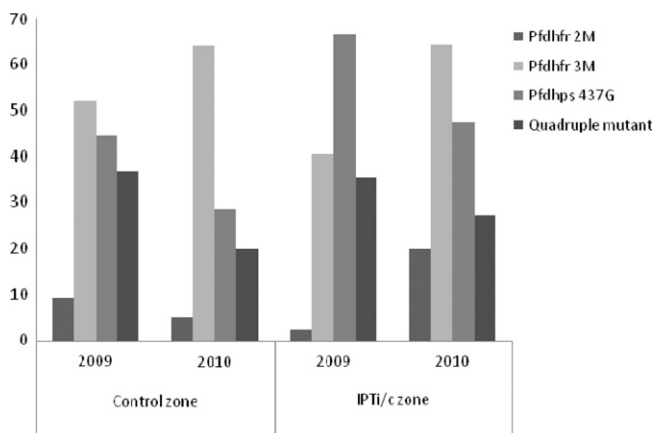


FIGURE 2. Prevalence of *Pfdhfr/Pfdhps* mutant haplotype in the study area.

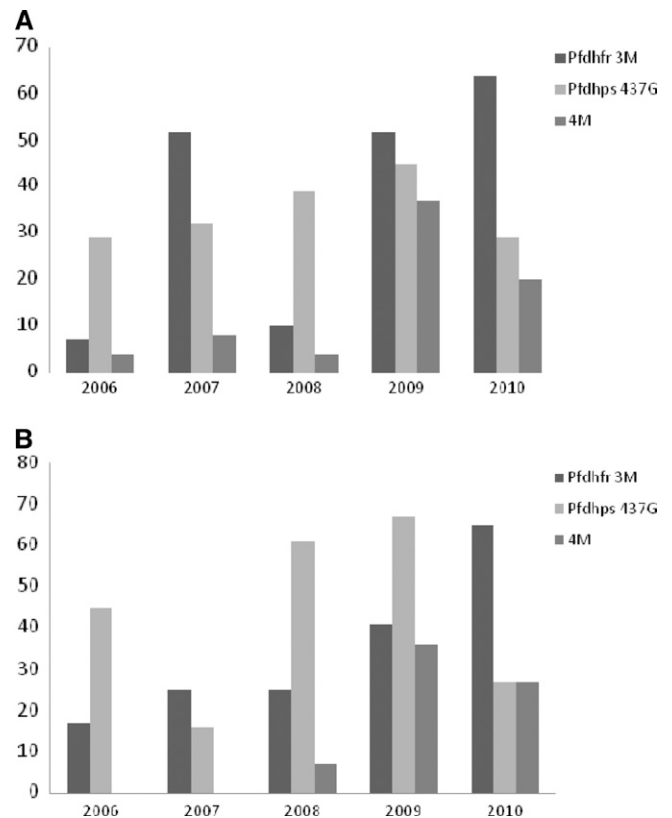


FIGURE 3. (A) Temporal distribution of *Pfdhfr/Pfdhps* single nucleotide polymorphisms (SNPs) mutant haplotype from 2006 to 2010 in control zone (without IPTi/c). (B) Temporal distribution of *Pfdhfr/Pfdhps* SNPs mutant haplotype from 2006 to 2010 in IPTi/c zone. *Pfdhfr* 3M: haplotype *dhfr* triple mutant (*Pfdhfr* 511, 59R, 108N) 4M: haplotype quadruple mutant (*Pfdhfr* 511, 59R, 108N + *Pfdhps* 437G) 2006: Baseline data IPTi: Pilot study from 2007 to 2008; after 2 years of IPTi a significant increase of *Pfdhps* 437G was noted IPTc: started from 2009 to 2010.

in samples from 2009 and 2010 was compared with previously published results obtained in the years 2006, 2007, and 2008 from the same study area, Faye and others²⁹ (Figure 3). Except for a sudden high prevalence of the *Pfdhfr* triple mutant haplotype (52%) observed in 2007 in the control zone (Figure 3A), a general trend of increase of *Pfdhfr* triple mutant (3M) parasites was noted from 2006 to 2010 in both groups: from a prevalence of 7% and 17% in 2006 to 64.3% and 64.6% in 2010 for the control and IPTi/c zones, respectively ($\chi^2 = 23.7$; $P \leq 0.001$) (Figure 3A and B). A significant increase of the quadruple mutation (*Pfdhfr* triple mutant plus *Pfdhps* 437G) was noted: from 3.7% and 4.4% in 2006 to 38.6% and 35.6% in 2009 in the control and IPT zone, respectively ($\chi^2 = 9.7$; $P \leq 0.001$; $\chi^2 = 33.2$; $P \leq 0.001$) (Figure 3B). However, in 2010, as described, there was a trend for a decrease in prevalence in both groups (Figure 3A and B).

DISCUSSION

In malaria-endemic settings, IPT using mainly SP have shown high protective efficacy against malaria, anemia, and death in IPTp,^{30–32} IPTi,⁴ and IPTc¹²; despite the beneficial impact of these strategies, mass implementation of IPT raises overall concern on whether the strategy may drive the spread of SP resistance further. Resistance to SP is well established

in East Africa, whereas not as pronounced in West Africa, mainly as a result of continuing low prevalence of the *Pfdhps* 540E mutation and thus, also low prevalence of the quintuple *Pfdhfr/Pfdhps* haplotype. In large areas of East Africa SP for IPTi and SMC should not be applied because above 50% prevalence of the *Pfdhps* 540E mutation and thus other options or combinations are currently tested such as artesunate plus amodiaquine (AS+AQ), artesunate plus sulfadoxine pyrimethamine (AS+SP), dihydroartemisinin plus piperazine (DHA+PPQ), sulfadoxine pyrimethamine plus piperazine (SP+PPQ). Regarding IPTc (now SMC), further research is also needed to evaluate the potential benefits of these drugs. For IPTc, to ensure a maximum protective effect in areas of highly seasonal malaria, it is recommended to combine two long half-life drugs and the combination of SP/AQ is currently the most optimal regimen.¹⁰ With concerns of continuing accumulation of SP resistance possibly establishing quintuple *Pfdhfr/Pfdhps* haplotypes (or similar) in West Africa as well, periodical monitoring of molecular markers of SP resistance is necessary to evaluate these strategies in the region. This study performed in Senegal evaluated the possible impact of IPTi/c on SP resistance markers after long-term follow-up in areas where both strategies were implemented.

The study observed a general trend of an increase in the prevalence of triple *Pfdhfr* haplotypes in both intervention and control areas over just 1 year. Furthermore, a high prevalence of the *Pfdhps* 437G mutants was observed in both groups in 2009, however the prevalence seemed to decrease in 2010. Finally, a combined, high prevalence of quadruple mutant (triple mutant *Pfdhfr* + 437G mutations) haplotype was noted in both areas in 2009 but as well seemed to decrease in 2010. Compared with the baseline study conducted by Faye and others²⁹ in the same study area, a general increase in all mutant haplotypes was noted. However, comparing the two groups in 2009 and 2010, it appears that, although changes are observed as a trend for an increase of triple *Pfdhfr* haplotypes and a trend for a decrease in *Pfdhps* 437G this is not only seen in the IPTi/c group and thus, IPTi/c is not driving the selection of SP resistance in the study area. Other factors may impose drug pressure in both the control and the IPTi/c zone; possibility of availability of SP in the private market and as well, the use of other sulfa-drugs such as cotrimoxazole. Similarly, in Mali, a study showed that the prevalence of SP resistance markers did not increase over a 1-year period of SP-IPTi intervention but remained unchanged at 34.6% and 8.1% regarding triple and quadruple mutations.³³ Conversely, in Mozambique, Mayor and others (2008) observed that the prevalence of *Pfdhfr/Pfdhps* quintuple mutants nearly doubled in the IPTi-SP group compared with the placebo group.³⁴ For IPTc in a study in Senegal, Cissé and others (2007) have shown that post-intervention prevalence of *Pfdhfr* triple mutants plus *Pfdhps* A437G mutants was significantly higher in the SP-artesunate treatment arm than the placebo arm.¹⁰ The same tendency was also observed in Mali; the post-intervention prevalence of *Pfdhfr/Pfdhps* quadruple mutants was significantly higher in the SP-amodiaquine group than the placebo group.¹² In areas with a long and high malaria transmission SMC will be given an all year-round treatment, there may then be a stronger selection of resistance. This could explain why this strategy is not recommended in areas of high malaria transmission (as Uganda).

Overall, during a short period of the two cross-sectional surveys some significant changes were observed in *Pfdhfr* and *Pfdhps*. However, because these changes were observed in both the intervention and in the control zones, the IPTi/c strategy does only seem to have limited impact on resistance development and other factors that impact as well. However, continuous monitoring will be needed as a result of the up scaling of the IPTc strategy in Senegal according to WHO recommendations.

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