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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

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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/year; 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 × 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 others with some modifications described by Pearce and others. An estimated polymerase chain reaction (PCR) (was used to amplify fragments of the *Pfdhfr* and *Pfdhps* genes as described by Alifrangis and others. 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...
of DNA HotStart polymerase (Ampliqon III; VWR-Bie Bernten, 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 Pf dhfr/Pf dhps haplotypes. The nested Pf dhfr and Pf dhps PCR reaction mixture was the same as the outer PCR mixture using primer sets M3b/M9 and R2/R2 for the dhfr and dhps PCR, respectively. Amplifications were performed in 96-well PCR microplates. The M9 and R/primers for the Pf dhfr and Pf dhps, 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 Pf dhfr (position 50/51, 59, and 108), Pf dhps (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 of Velingara and Saraya where IPTi/c has been implemented (IPTi/c zone).

Statistical analysis. Statistical analyses of data were performed using Epi Info (version 06; CDC, Atlanta, GA). The χ² test was used to compare differences in proportions in Pf dhfr/Pf dhps 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 studies conducted in the same study area using 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).

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 (χ² = 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 (χ² = 32.57, P ≤ 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 in 2009 and 88 in 2010) were randomly selected in each zone for further PCR analysis.

Prevalence of SNPs in the Pf dhfr and Pf dhps 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 Pf dhfr 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 (χ² = 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). Though a trend for an increase in the prevalence of all mutant SNPs was observed, only S108N was significant (χ² = 8.30, P = 0.003).

For the Pf dhps gene, in the control zone, a trend for a decrease in the prevalence of Pf dhps A437G including mixed infections was observed from 44.6% (25 of 56) in 2009 to 28.6% (16 of 56) in 2010 (χ² = 3.12; P = 0.08), 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 (χ² = 5.92, P = 0.02). Similarly, in the IPTi/c zone the prevalence of Pf dhps C59R and S108N including mixed infections, were 60.8% (48 of 79), 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 Pf dhfr (data not shown).

The Pf dhps A581G was identified
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 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 and 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 zones, 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 plus ARTESUNATE treatment arm than the placebo group.10 reversed, in Mozambique, Mayor and others (2008) documented by Faye and others29 in the same study area, a general tendency of an increase in the prevalence of 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. In 2010, a combined, high prevalence of quadruple mutants (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 others30 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 control group and thus, IPTI control 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 group; possibility of availability of SP in the private market and as well, the use of other sulfas-drugs such as cotrimoxazole. Similarly, in Mali, a study showed that the prevalence of SP resistance markers nearly doubled in the IPTI-SP group compared with the placebo group.31 For IPTC, 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.11 The same tendency was also observed in Mali; the post-intervention prevalence of Pfdhfr/Pfdhps quadruple mutants was significantly higher in the SP-ARTESUNATE 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/SP 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 IPTS strategy in Senegal according to WHO recommendations.

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