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Selection of antimalarial drug resistance after intermittent preventive treatment of infants and children (IPTi/c) in Senegal

Sélection de la résistance aux médicaments contre la malaria après un traitement préventif intermittent de nourrissons et d'enfants (IPTi/c) au Sénégal

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ABSTRACT

Senegal has since 2003 used sulphadoxine-pyrimethamine (SP) for Intermittent Preventive Treatment (IPT) of malaria in risk groups. However, the large-scale IPT strategy may result in increasing drug resistance. 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* positives samples ($n = 352$) were collected from children under 5 years of age during two cross-sectional surveys in 2010 and 2011 in three health districts (two on IPTi/c and one without IPTi/c intervention) located in the southern part of Senegal. The prevalence of SP-resistance-related haplotypes in *Pfdhfr* and *Pfdhps* was determined by nested PCR followed by sequence-specific oligonucleotide probe (SSOP)–ELISA. The prevalence of the *Pfdhfr* double mutant haplotypes (CNRN and CICN) was stable between years at $< 10\%$ in the control group ($P = 0.69$), while it rose significantly in the IPTi/c group from 2% in 2010 to 20% in 2011 ($P = 0.008$). 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. Combined with *Pfdhfr*, there was a weak trend for decreasing prevalence of quadruple mutants (triple *Pfdhfr* + *Pfdhps* 437G) 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. However, since these changes were observed in the two groups, the IPTi/c strategy does only seem to have limited impact on resistance development and other factors as well. However, continuous monitoring will be needed, due to the up-scaling of the IPTi/c strategy in Senegal according to WHO recommendations.

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RÉSUMÉ

Au Sénégal, depuis 2003, le traitement préventif intermittent utilisant la sulphadoxine-pyriméthamine (TPI/SP) a été adopté comme stratégie de chimio-prévention chez les groupes à risque. Cependant, l'utilisation à grande échelle de cette stratégie pourrait entraîner une augmentation de la résistance à la SP. Notre étude a examiné l'impact possible de la SP/IPT administrée aux nourrissons et aux enfants sur la prévalence des marqueurs moléculaires de résistance, en comparant les sites avec et sans TPI. Des échantillons de sang positifs à *P. falciparum* ($n = 325$) ont été collectés sur papier buvard au cours d'enquêtes transversales en 2009 et 2010 chez des enfants de moins de cinq ans vivant dans les districts de Vélingara et Saraya (zone TPI) et le district témoin de Tambacounda. Les mutations situées sur les gènes de résistance à la SP (*dhfr*/*dhps*) ont été déterminées par *Polymerase Chain Reaction-Sequence-Specific Oligonucleotide Probes* et *Enzyme-Linked ImmunoSorbent Assay* (PCR-SSOP-ELISA). Nos résultats ont montré que la prévalence de la double mutation *Pfdhfr* (CNRN et CICN) est restée stable (< 10 %) au cours du temps dans le groupe témoin ($p = 0{,}69$). Dans le groupe TPI+, cet haplotype double mutant a augmenté de façon significative : de 2 % en 2009 contre 20 % en 2010 ($p = 0{,}008$). La prévalence de l'haplotype *Pfdhfr* triple mutant (CIRN) a augmenté dans les deux groupes, mais seulement de façon significative dans le groupe TPI+ de 41 % à 65 % en 2011 ($p = 0{,}005$), respectivement. En revanche, la mutation *Pfdhps* 437G a diminué dans les deux groupes de 44,6 % à 28,6 % ($p = 0{,}07$) et de 66,7 % à 47,5 % ($p = 0{,}02$) entre 2009 et 2010 dans les groupes témoin et TPI, respectivement. Au cours de notre étude, des changements importants ont été notés dans les gènes associés à la résistance SP. Toutefois, ces changements sont survenus dans les deux groupes ; la stratégie TPI semble seulement avoir un impact limité sur le développement de la résistance à la SP. Cependant, une surveillance continue sera nécessaire lorsque cette stratégie sera mise en place à l'échelle nationale au Sénégal, conformément aux recommandations de l'Organisation mondiale de la santé (OMS).

1. Background

During the last decades, *Plasmodium falciparum* has become resistant to widely-used antimalarial drugs such as chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) in Africa [1]. Still, in the absence of an effective malaria vaccine, malaria chemoprevention using SP is currently one of the essential strategies to control *P. falciparum* infections in endemic countries. Intermittent preventive treatment (SP-IPT) for malaria is the administration of a therapeutic dose of SP to risk groups, regardless of their current infection status [2]. Since 2003, many African countries through their National Malaria Control Program (NMCP) advocated the use of SP-IPT for malaria chemoprevention in pregnant women (IPTp). In recent years, several pilot studies in Africa, including Senegal, have explored the use of SP for malaria prevention in infancy (IPTi) through the EPI vaccination programme. These studies showed that the use of IPTi provided a 30% overall protection against clinical malaria episodes (95%CI: 19.8%; 39.4%) as well as an overall reduction in anaemia (< 8 g/dL) of 21.3% (95%CI: 8.3%; 32.5%) in a pooled analysis of data from six published studies [3,4]. Following these and other findings, WHO has recommended the implementation of the IPTi strategy [3]. However, another study in Tanzania has conversely shown no protection at all using SP for IPTi, due to the high level of SP-resistance in the *P. falciparum* populations in the study site [5]. 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 [6,7]. In such areas, administration of IPT to children several times during the seasonal peak in malaria transmission (IPTc) has been investigated as a method for preventing malaria. Several studies undertaken in Sub-Saharan Africa, including Senegal, have demonstrated protective efficacies against clinical malaria from 31% to 93%, and a meta-analysis gave an overall protective efficacy of monthly administered IPTc of 82% (95%CI 75%–87%) during the malaria transmission season [8]. Following all these findings, WHO recommended IPTi [3], and IPTc, now named Seasonal Malaria Chemoprevention (SMC), is going to be adopted very soon in areas where malaria transmission is seasonal. SP alone remains the drug of choice for intermittent preventive treatment of pregnant women and infants, while SP plus amodiaquine (AQ) is used in children under five in Africa [9]. However, the efficiency of these IPT-strategies is highly dependent on the level of SP-resistance in the *P. falciparum* populations.

P. falciparum in vivo and in vitro resistance to pyrimethamine and sulfadoxine is associated with single nucleotide polymorphisms (SNPs) in the *P. falciparum* dihydrofolate reductase (*Pfdhfr*) gene and the *P. falciparum* dihydropteroate synthetase gene (*Pfdhps*), respectively. Resistance to pyrimethamine is caused by SNPs, resulting in amino acid changes mainly at positions N51I, C59R, S108N/T and I164L of the *Pfdhfr* gene [10,11]. In the *Pfdhps* gene, five SNPs, namely, S436A/F, A437G, K540E, A581G and A613S/T, have been reported to be linked to *P. falciparum* resistance to sulphadoxine [12,13]. An

accumulation of SNPs in both *Pfdhfr* and *Pfdhps* genes leads to an increased risk of clinical treatment failures. The predictive value of these markers varies geographically, depending on, e.g., baseline prevalence, age, and immunity [14–16]. 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 [17,18].

Regarding IPT, recent evidence suggests that high prevalence of highly resistant double mutant Pfdhps (A437G+K540K) mutant-essentially the quintuple *Pfdhfr*/*Pfdhps* mutant, may undermine the 20% protective efficacy of SP-IPTi. The impact of IPTi on a selection of *Pfdhfr*/*Pfdhps* mutations assessed in several places has showed that the observed period between the treatment and the first detection of *P. falciparum* infections as well as the incidence rate of infections with *Pfdhfr*/*Pfdhps* quadruple mutants was significantly shorter and twice as high in the SP treatment group when compared to placebo, respectively [19]. A similar trend has been noted when examining the impact of IPTc: The prevalence of *Pfdhfr*/*Pfdhps* quadruple mutants was significantly higher in the SP-AQ group compared to the control group [20]. In Senegal, post-intervention prevalence of triple *Pfdhfr* and *Pfdhps* A437G mutants was also significantly higher in the IPTc group using SP-AQ treatment than in the placebo arm [21].

However, the cumulative effects of the intervention remain unknown. This study was conducted in areas where both strategies IPTi and IPTc have been implemented since 2007 and 2009, respectively, and its aim was to investigate the possible impact of IPTi/c on the prevalence of SNPs in *Pfdhfr*/*Pfdhps* after a long-term follow-up in Senegal.

2. Methods

2.1. DNA extraction and Plasmodium falciparum dihydrofolate reductase/Plasmodium falciparum dihydropteroate synthetase single nucleotide polymorphisms analysis

P. falciparum DNA was extracted from positive finger prick blood spots by the Chelex-100 method of Wooden et al. [22], with some modifications described by Pearce et al. [23]. A nested PCR reported by Pearce et al. [23] was used to amplify fragments of the *Pfdhfr* and *Pfdhps* genes. 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 (*dhfr*) or N1/N2 (*dhps*), one unit of DNA HotStart polymerase (Ampliqon III; VWR-Bie Berntsen, Denmark), a buffer containing 1.5 mM $MgCl_2$, as recommended by the manufacturer, and 1 μL of extracted DNA. 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. The M9 and R/primers for the *dhfr*- and *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.

P. falciparum single nucleotide polymorphisms (SNPs) at *dhfr* (position 50/51, 59, and 108), *dhps* (positions 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 [24].

3. Results

In all, 1903 samples were collected in the health district of Tambacounda without IPTi/c intervention; e.g., the control zone (804 in 2009 and 1099 in 2010) and 2457 (1215 in 2009 and 1242 in 2010) in the health district of Vélingara 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% (81/804) in 2009 to 13.9% (153/1099) in 2010 ($\chi = 5$, $P = 0.02$). Similarly, in the IPTi/c zone, the prevalence increased from 3.7% (46/1215) in 2009 to 10.1% (125/1242) in 2010 ($\chi = 32.57$, $P = 10^{-3}$). In all, 234 samples were *P. falciparum* RDT positive in the control group and 176 in 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.

3.1. Prevalence of single nucleotide polymorphisms in the Plasmodium falciparum dihydrofolate reductase and Plasmodium falciparum dihydropteroate synthetase genes in samples from intermittent preventive treatment of infants and children and control zones between 2009 and 2010

For *Pfdhfr*, SNPs were examined at c51, c59, c108 and c164 in the selected RDT positive samples. In the control zone, the prevalence of N51I, C59R and S108N, including mixed infections, were 65.3% (47/72), 65.3% (47/72) and 72.6% (53/73), respectively, in 2009, while 75.0% (48/64), 76.6% (49/64) and 91.0% (69/76), respectively, in 2010. In the IPTi/c zone, the prevalences of N51I, C59R and S108N, including mixed infections, were 60.8% (48/79), 67.5% (54/80), and 73.0% (54/74), respectively, in 2009, while in 2010, they were 60.7% (51/84), 71.2% (52/73), and 79.7% (59/74), respectively. For both zones, only wild types (I164) were detected at c164.

For the *Pfdhps* gene, SNPs at c436, c437, c540, c581, and c613 were examined. A majority of infections carried mutations at A437G over a year, a few 613S [0.05(3/62)], and no 540E mutation was found. The prevalence of parasites harbouring the *Pfdhps* 437G mutation (as SGKAA, AGKAA and FGKAA) was 44.6% (25/56) in 2009 and 28.6% (16/56) in 2010 in the control group ($\chi^2 = 1.45$, $P = 0.22$), while a significant decrease of *Pfdhps* 437G containing haplotypes was observed in the IPT group from 66.7% (54/81) in 2009 to 47.5% (29/61) in 2010 ($P = 0.02$).

3.2. Prevalence of constructed Plasmodium falciparum dihydrofolate reductase/Plasmodium falciparum dihydropteroate synthetase haplotypes in samples from intermittent preventive treatment of infants and children and control zones between 2009 and 2010

In the control zone, the prevalence of parasites harbouring the double mutants haplotype (CICN, CNRN) including mixed haplotype infections was 9.3% (6/64) in

Table 1
Prevalence of *Pfdfr*/*Pfdhps* mutation before and after IPTi/c in Senegal.

	Control		*P*-value	IPT		*P*-value
	2009	2010		2009	2010	
Pfdhfr double	9.3% (6/64)	5.1% (2/39)	0.68	2.3% (1/43)	20.0% (10/50)	0.008
Triple (3M)	52.11% (37/71)	64.28% (36/56)	0.16	40.57% (28/69)	64.61% (42/65)	0.005
Pfdhps 437G	44.64% (25/56)	28.57% (16/56)	0.07	66.66% (54/81)	47.54% (29/61)	0.02
Quadruple mutant	36.84%(14/38)	20% (5/25)	0.15	35.59% (21/59)	27.08% (13/48)	0.34

Pfdhfr: *Plasmodium falciparum* dihydrofolate reductase gene; *Pfdhps*: *Plasmodium falciparum* dihydropteroate synthetase gene; IPT: intermittent preventive treatment; IPTi/c: intermittent preventive treatment of infants and children.

Table 2
Temporal distribution of *Pfdhfr*/*Pfdhps* SNPs mutant haplotype from 2006 to 2010.

Year	Haplotypes	Control group	IPTi/c group	*P*-value	
2006	*Pfdhfr 3M*	7.0% ($n = 27$)	17% ($n = 135$)	0.33	
	Pfdhps 437G	29.0% ($n = 27$)	45% ($n = 135$)	0.13	
	4M	3.7% ($n = 27$)	4.4% (135)	0.72	
2007	*Pfdhfr 3M*	52% ($n = 25$)	25% ($n = 95$)	0.01	IPTi
	Pfdhps 437G	32% ($n = 25$)	16% ($n = 95$)	0.12	
	4M	8% ($n = 25$)	7.4% ($n = 95$)	0.74	
2008	*Pfdhfr 3M*	10% ($n = 150$)	25% ($n = 250$)	$< 10^{-3}$	
	Pfdhps 437G	39% ($n = 150$)	61% ($n = 250$)	$< 10^{-3}$	
	4M	4% ($n = 150$)	7% ($n = 250$)	0.192	
2009	*Pfdhfr 3M*	52.1% ($n = 71$)	40.6% (69)	0.17	IPTi + IPTc
	Pfdhps 437G	44.6% (25/56)	66.6% (54/81)	0.01	
	4M	36.8% (14/38)	35.6% (21/59)	0.90	
2010	*Pfdhfr 3M*	64.3% ($n = 56$)	64.6% ($n = 65$)	0.96	
	Pfdhps 437G	28.6% (16/56)	27.1% (13/48)	0.86	
	4M	20.0% (5/25)	27.1% (13/48)	0.50	

Pfdhfr: *Plasmodium falciparum* dihydrofolate reductase gene; *Pfdhps*: *Plasmodium falciparum* dihydropteroate synthetase gene; IPTi/c: intermittent preventive treatment of infants and children; SNPs: single nucleotide polymorphisms.

2009 and 5.1% (2/39) in 2010 with no significant difference between the years ($P = 0.68$) (Table 1). In the IPTi/c zone, the prevalence of a double mutant haplotype including mixed haplotype infections was 2.3% (1/43) in 2009, while the prevalence increased significantly to 20.0% (10/50) in 2010 ($P = 0.008$). Regarding the triple mutant *Pfdhfr* haplotype (CIRN), there was an insignificant trend for an increase in prevalence in the control group from 52.1% (37/71) in 2009 to 64.3% (36/56) in 2010 ($P = 0.47$), while in the IPT group, a significant increase from 40.6% (28/69) in 2009 to 64.6% (42/65) in 2010 ($P = 0.005$) was observed. 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) decreased between 2009 and 2010 in the control group from 36.8% (14/38) to 20.0% (5/25), ($\chi = 2.03$, $P = 0.15$), while in the IPTi/c group, the prevalence in 2009 was 35.6% (21/59) and 27.1% (13/48) in 2010; the difference was not significant in the IPT group ($P = 0.15$) (Table 1). The quintuple mutation CIRN/SGEAA plus CIRN/AGEAA was not found in our study area over years.

3.3. Temporal distribution of Plasmodium falciparum dihydrofolate reductase/Plasmodium falciparum dihydropteroate synthetase single nucleotide polymorphisms/haplotype prevalence in samples from intermittent preventive treatment of infants and children and control zones between 2006 and 2010

Results of the *Pfdhfr*/*Pfdhps* SNPs/haplotypes in samples from 2009 and 2010 were compared with previous published results obtained in the years 2006, 2007 and 2008 from the same study area. Except for a sudden low prevalence of the *Pfdhfr* triple mutant haplotype (10.0%) observed in 2008 in the control zone primarily, a general trend toward an increase of *Pfdhfr* triple mutant (3M) parasites was noted from 2006 to 2010 in both groups: from a prevalence of 7.0% and 17.0% in 2006 to 64.3% and 64.6% in 2010 for control and IPTi/c zones, respectively (Table 2). For the *Pfdhps* 437G mutant, except for a significant difference noted between control and IPTi groups in 2008, comparable prevalence are noted over the years between both groups (Table 2), while we noted a significant decrease in the control zone from 66.7% in 2009 to 47.5% in 2010 ($P = 0.02$). The prevalence of the quadruple mutation was very low in the previous study ($< 10\%$).

4. Discussion

In malaria endemic settings, intermittent presumptive treatments (IPT) have shown high protective efficacy against malaria, anaemia and death in pregnant women (IPTp), in children (IPTc) and in infancy (IPTi). Despite the beneficial impact of these strategies, mass implementation of IPT raises overall concern on whether IPT impacts the development and spread of drug resistance. In pregnant women, the potential impact of IPTp on the selection of SP markers has been examined by comparing groups of women receiving SP-IPTp with controls that do not receive the treatment. In Ghana, the prevalence of the *Pfdhfr* triple mutant was nearly identical in both groups and did not increase with an increasing number of SP-IPT doses among

the group of delivering women [25]. Contrarily, in Tanzania, the prevalence of *Pfdhps* A581G was significantly higher in SP-IPTp group compared to those that do not receive the treatment [26]. Authors also reported an association between SP use and higher parasitaemia, suggesting that highly resistant parasites in pregnant women receiving SP-IPTp may not only survive, but also reach higher densities and perhaps spread more rapidly than their less resistant counterparts. This study evaluated the possible impact of IPTi/c one drug resistance marker after a long-term follow-up in an area where both strategies were conducted in Senegal.

This study has shown a general trend toward an increase in the prevalence of triple *Pfdhfr* haplotypes in both intervention and control areas over just 1 year, from 2009 to 2010. Contrarily, a high prevalence of the *Pfdhps* 437G mutant was observed in both groups in 2009; however, the prevalence seemed to decrease the next year. Finally, combined, a 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 to a baseline study conducted by Faye et al. [27] in the same area, a general increase in all mutant haplotypes was noted. Furthermore, the prevalence of parasites with quadruple mutants was high in the IPT group compared to the control group. In Ghana, during the IPTi strategy, a study showed that the observed period between the treatment and the first detection of *P. falciparum* infection with triple *Pfdhfr* mutant plus *Pfdhps* A437G was significantly shorter in the SP treatment group when compared to the placebo [28]. Similarly, in Mozambique, Mayor et al. observed that the prevalence of *Pfdhfr*/*Pfdhps* quintuple mutants nearly doubled in the IPTi-SP group compared to the placebo group [19]. In contrast, in Mali, a study showed that the prevalence of SP-resistance markers did not increase over a one-year period of SP–IPTi intervention [29]. For IPTc, Cissé et al. have shown that the post-intervention prevalence of *Pfdhfr* triple mutants plus *Pfdhps* A437G mutants was significantly higher in the SP-artesunate treatment arm than the placebo arm [21]. 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 in the placebo group [20].

Disclosure of interest

The authors have not supplied their declaration of conflict of interest.

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