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Sulfadoxine-Pyrimethamine Resistant Haplotypes in Asymptomatically and Symptomatically Malaria Infected Individuals in Côte d'Ivoire

Berenger Aristide Ako Ako[1,2*], Marnie Johansson[2], Rokia Traore[1], Toure André Offianan[1], Eric Adji Gbessi[1], Coulibaly Mangoa Yahya[3], Simon-Pierre Assanvo Nguetta[3], Louis Koné Penali[1] and Carol Hopkins Sibley[2]

[1]*Department of Malariology, Institut Pasteur de Côte d'Ivoire, Abidjan, Côte d'Ivoire*

[2]*Department of Genome Sciences, University of Washington, Seattle, WA, USA*

[3]*Laboratoire de Génétique, UFR Biosciences, Université Félix Houphouët-Boigny, Abidjan, Côte d'Ivoire*

[*]**Corresponding author:** Berenger Aristide Ako Ako, Malariology Department of Institut Pasteur de Côte d'Ivoire BP 490 Abidjan, Côte d'Ivoire, Tel: +225 05 63 67; E-mail: ako.aab@gmail.com

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Abstract

Background: Most studies that assess efficacy of antimalarial drugs focus on the outcome of clinical treatment. However, community surveys of surrogate indicators are often more practical and can provide a wider view of possible changes in drug response, but it has not been clear whether assessment of parasite isolates from patients and asymptomatic individuals are directly comparable. In the present work, we have compared the prevalence of molecular markers associated with resistance to sulfadoxine-pyrimethamine in parasites isolated from asymptomatic and symptomatic individuals.

Methods: The study was conducted during April and May 2008 in Anonkoua-Kouté (Abobo-Abidjan) in southern Côte d'Ivoire, an area where SP has been intensively used for more than 20 years. *Plasmodium falciparum* monospecific infection was detected by blood smears, followed by a genomic DNA extraction from blood spots on filter paper. Extracted DNA was amplified by nested-PCR, and *pfdhfr* and *pfdhps* sequences analyzed.

Results: Ninety six of 107 asymptomatic schoolchildren sampled were positive for *P. falciparum*; 48 of these isolates were used for molecular analysis. A subset of 67 samples from malaria patients of school age was analyzed in parallel. For pfdhfr, the wild-type NCSI and the triple mutant IRNI alleles were both present in about 30 and 50% of the isolates from asymptomatic children and symptomatic malaria patients, respectively. For pfdhps, the symptomatic children mostly carried the single mutant genotype SGKAA although the double mutant AGKAA was the predominant allele in both populations.

Conclusions: Direct comparison of molecular markers of SP resistance demonstrates that the prevalence of these alleles is comparable in isolates derived from asymptomatic and symptomatic individuals. The results from this study support the possibility of using cross sectional surveys of surrogate molecular markers of SP efficacy to inform decisions about choice of drugs for intermittent preventive treatment of pregnant women or seasonal malaria chemoprophylaxis.

Keywords: Asymptomatic; Symptomatic; Children; pfdhfr; pfdhps; Côte d'Ivoire

Background

Transmission of malaria has declined in several regions of the world and renewed the interest in malaria elimination. This relative success relied mostly on the scale-up of interventions; the improvement of diagnostic testing by the use of parasitological confirmation of a case and a willingness to improve burden estimates by better defining the populations at risk were both important [1]. In high transmission settings, interventions most often focus on children under 5 who have not yet developed immunity, and pregnant women. However, the burden of infection is most often highest in asymptomatic children between 5-15 [2]. In fact, the majority of infections are asymptomatic or sub-clinical in all transmission settings [3,4] and due to the production of gametocytes; parasites from asymptomatic people may be more infectious to mosquitoes than are parasites from symptomatic individuals [5,6]. Thus, individuals with asymptomatic *P. falciparum* infection play a significant role as an infection reservoir. They are more likely to remain untreated and carry parasites and gametocytes for a longer period.

Various strategies have been proposed to address treatment of the reservoir of asymptomatic infections, and in Côte d'Ivoire, most strategies use sulfadoxine-pyrimethamine (SP) either alone, or in combination with amodiaquine [7]. SP is still commonly used in the community without reference to formal healthcare systems, which is particularly problematic because dosage may be inadequate and the quality of drugs substandard [1]. Parasite resistance to the SP component is a threat to these strategies.

Resistance to pyrimethamine has been correlated with parasites that carry mutations at codons 51, 59, 108, and 164 in the *Plasmodium falciparum dihydrofolate reductase* (*pfdhfr*) gene, and resistance to sulfadoxine with mutations at codons 436, 437, 540, 581, and 613 in the *P. falciparum dihydropteroate synthase* (*pfdhps*) gene [8].

Côte d'Ivoire is a West African country which belongs to the countries of high malaria endemicity setting with regions of perennial malaria transmission. Prior studies have revealed a high prevalence of SP resistance genotypes among symptomatic individuals in the country [9,10]. A recent nation-wide health demographic survey and multiple indicators indicated a parasite prevalence of 18.1% among symptomatic children less than 5 years old and 7% in pregnant women in Côte d'Ivoire [11]. However, limited studies have examined asymptomatic carriage as well as SP resistance genotypes among isolates from school children [12-14].

With the advent of molecular markers correlated with resistance to SP, community surveys of surrogate indicators are often more practical and can provide a wider view of possible changes in drug response, but it has not been clear whether assessment of parasite isolates from patients and asymptomatic individuals are directly comparable. In addition, baseline molecular data are needed before a proposed implementation of chemoprophylaxis among school children in Côte d'Ivoire.

We hypothesized that a long-term usage of SP has exerted drug pressure on the *P. falciparum* population increasing the prevalence of resistant alleles, and that measurements in isolates derived from asymptomatically and symptomatically infected participants would be comparable. This study sought to compare the prevalence of SP resistance molecular markers between asymptomatic *P. falciparum* infected schoolchildren and individuals of comparable age with acute uncomplicated malaria, living in suburban area in Anonkoua-kouté, in southern Côte d'Ivoire, where the risk of malaria transmission is stable.

Methods

Study sites

The study took place in Anonkoua-kouté (5°25 ' 55.90" N; 4°02 ' 45.27" W) a suburban village in the vicinity of Abobo, a municipality in northern Abidjan, Côte d'Ivoire. The country is an endemic area for *P. falciparum* malaria, with perennial transmission in the southern forest and seasonal transmission in the northern savanna. Southern Côte d'Ivoire has two rainy seasons, a long one which runs from March to July, and a short one from September to November. Anonkoua-kouté has a health care center which served a population of 61,249 inhabitants in 2008 with malaria cases representing 54.20% of the medical visits.

Study design and population

The present study made use of two sets of blood samples. (i) Cross sectional survey including schoolchildren of 4 to 15 years of age from three of the village primary schools collected from May to June 2008. Upon receiving informed consent a questionnaire was completed for each participant, a rapid diagnostic test was performed and a venous blood sample was collected for laboratory analysis. Samples from patients with axillary temperature below 37.5°C were included after confirmation of a parasite positive blood slide by light microscopy. (ii) A set of 67 *P. falciparum* positive samples taken on day of inclusion were selected from two clinical trials in children between 5 and 12 years. These children will be called the symptomatic group in this comparison. In both clinical subsets, samples were collected in Anonkoua-kouté in year 2008. Eligible subjects were of either sex, with no evidence of severe malnutrition, a reported history of fever at inclusion or within the previous 24 h, and microscopically-confirmed uncomplicated *P. falciparum* mono-infection. Patients with signs and symptoms of simple malaria were enrolled after confirmation by light microscopy and written informed consent.

Capillary blood was obtained by fingerprick. Thick and thin films were stained with 10% Giemsa (pH 7.2) for 15 minutes. Asexual parasitemia was quantified against 200 leukocytes, assuming a white blood cell count of 8000/mL. Presence of gametocytes was recorded through this same method. All slides were re-read and any discrepancy was resolved by a third reader.

Collection of blood samples: Blood samples for PCR analysis were collected on Whatman 3MM filter paper. A total of 3 drops of blood were spotted on filter paper. Dried at room temperature (25°C) and wrapped in desiccant-containing plastic bags, the filter paper was stored dry for genomic DNA extraction. Genotyping analysis was performed in the Department of Genome Sciences, University of Washington School of Medicine, Seattle (WA, USA).

DNA extraction and amplifications: Genomic DNA was extracted from dried blood spots with the QIAamp DNA Micro Kit (Qiagen, Valencia, CA) as per manufacturer's protocol. Briefly, filter paper was cut, and then denatured with Proteinase K. The lysate was washed twice with buffers through a silicate-containing membrane. Lastly, DNA was eluted with ultra-pure water. Extracted DNA was amplified in a two round PCR. For the primary PCR, the primer pairs used were S217 (5'- CTC CTT TTT ATG GAA CAA GTC TGC GAC GTT TTC G -3') and S218 (5'- TCA TAT GAC ATG TAT CTT TGT CAT CAT TCT TTA AAG GC -3') for pfdhfr and S219 (5'- GTC TGC GAC GTT TTC GAT ATT TAT GCC -3') and S229 (5'- GGC ATA TCA TTA TTT TTT TCT TCT CCT TTT ATA C -3') for pfdhps. For PCR amplification, a 20 µL reaction volume was used which contained 1X PCR PreMix A (Epicentre), 0.2-0.5 µmol/L of each primer, 0.5 U of FailSafe® enzyme mix (Epicentre) and 0.5-2 µL of template DNA. Cycling conditions set for the MJ Research Tetrad PTC-225 thermal cycler (BioRad) for pfdhfr, were as follows: 94°C for 3 min; 30 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 60 s; and 72°C for 10 min. For pfdhps : 94°C for 3 min; 30 cycles of 94°C for 45 s, 51°C for 45 s, and 60°C for 45 s; and 60°C for 5 min. A secondary amplification was then performed, using the primer pairs S1226 (5'- AAC CTA AAC GTG CTG TTC AA- 3') and S1227 (5'- AAT TGT GTG ATT TGT CCA CAA -3') for pfdhfr (giving a 793 pb fragment), and S1224 (5'- GAT TCT TTT TCA GAT GGA GG -3') and S1225 (5'- TTC CTC ATG TAA TTC ATC TGA -3') for pfdhps giving a 753 -bp fragment.

Gene sequencing

Products of the second round PCR were used for the cycle sequencing reaction which consisted of a linear amplification of extension products with BigDye® Terminator. The sequencing was performed in a 10 µL volume, using 1-3 µL of PCR product, 1.7 µL of 5× sequencing buffer for BigDye® (400 mmol/L of Tris-HCl and 10 mmol/L of $MgCl_2$; pH 9.0), 0.8 µmol/L of primer, and 1 µL of BigDye® (Applied Biosystems). Then an Applied Biosystems capillary electrophoresis-based genetic analysis followed. The DNA Sequence Assembly Software Sequencher® 4.1.4 (Gene Codes Corporation, Ann Arbor, MI), was used for base calling, trimming, display and editing of sequences automatically read on the capillary machine. The following codon positions were screened to detect point mutations associated with SP resistance: pfdhfr N51I, C59R, S108N/T, I164L, *pfdhps* S436A, A437G, K540E, A581G, A613T/S.

Statistical analysis

The prevalence of a particular mutation was calculated as the proportion of the samples that carried the specific mutant codon among the total number of samples successfully analyzed for this mutation. Likewise, the prevalences of triple, quadruple and quintuple mutants were determined as the proportion of subjects with the three, four and five mutations among the total numbers of samples tested for each. Mixed infections were detected when two or more distinct genotypes at particular codons of interest were present in a single infection. Prevalence of individual mutations as well as genotypes were determined and compared between asymptomatic children and symptomatic patients with a z test. A 95% confident interval around the difference of proportions was determined. Proportions of categorical variables (haplotypes) were compared using Pearson's Chi square test run with Xlstat Pro. Comparison of age, parasite density, and body weight was performed between the two groups with an unpaired Student *t* test. A Spearman correlation test was also performed between parasite density and participant's age. These statistical analyses were run with Graph.pad Prism 5.0.

Ethical considerations

The study protocol received approval from the National Ethics and Research Committee prior to implementation and was conducted in compliance with ICH-GCP guidelines according to the declaration of Helsinki (Version of 2000 amended in Tokyo 2004). Participant's informed consent was provided by the parents or legal guardians of the children prior to the inclusion.

Results

Baseline characteristics of the study population

The cross-sectional survey including a total of 808 schoolchildren detected 111 individuals (14%) with parasite positive blood smears of which 107 had an axillary temperature less than 37.5°C. The 4 schoolchildren with fever (≥ 37.5°C) were excluded from the study and sent to the village health center for appropriate care according to the national malaria program policy. The light microscopy detected and confirmed 96 (89.7%) mono-infections with *Plasmodium falciparum* among the remaining non feverish schoolchildren with positive blood smears. To avoid inconsistency in PCR product yields, filter paper samples from 51 children with parasitemia greater than or equal to 500 asexual stages per µL were analyzed for the pfdhfr and pfdhps point mutations [15]. From the subset of symptomatic children with malaria, 67 samples were analyzed. The flow chart of Figure 1 indicates the details of the study.

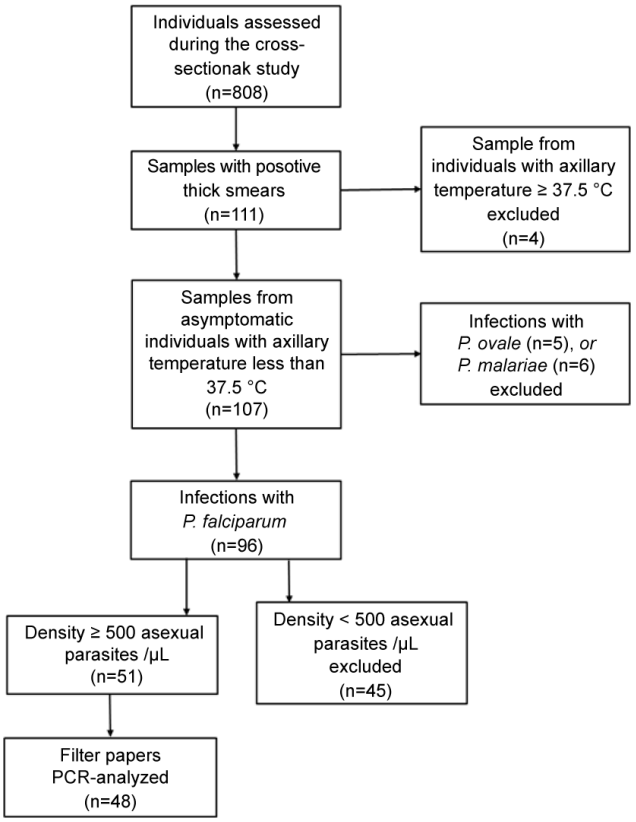

Figure 1: Study protocol for the cross sectional study in school children.

Table 1 details the differences between the two groups. The symptomatic children were significantly younger and weighed less than the asymptomatic group (z-test; p<0.0001). As expected, the mean parasite density and axillary temperature were also significantly higher in symptomatic than asymptomatic children (z-test; p <0.0001), a reflection of their different clinical status.

P. falciparum infection	Asymptomatic individuals (n=96)	Symptomatic participants (n=67)	p
Male, n (%)	46 (47.9%)	34 (52.3%)	0.171
Mean age, years (SD) [range]	9.4 (2.4) [4-15]	7.1 (2.9) [4-13]	<0.0001
<5 year, n (%)	1 (1.0%)	20 (30.8%)	
5– <10 years, n (%)	53 (55.2%)	29 (44.6%)	
10–15 years, n (%)	42 (43.8%)	16 (24.6%)	
Mean weight, kg (SD) [range]	27.2 (5.8) [16-48]	21.4 (7.7) [8-42.7]	<0.0001
Axillary temperature, °C (SD) [range]	36.6 (0.3) [35.6-37.4]	38.7 (1.3) [36.5-41.5]	<0.0001

Asexual parasites per µL			
< 500, n (%)	45 (46.9%)	0	
500– <2000, n (%)	30 (31.3%)	0	
≥ 2000, n (%)	21 (21.9%)	67 (100%)	
Geometric mean, [95% CI]	631.8 [472-846]	33477 [24704-45365]	<0.0001
Patients with gametocytes, n (%)	4 (3.7%)	0	

Table 1: Baseline Characteristics Of The Included Individuals From Anonkoua-Koutél; Note: No Significant Difference Of Variances Of Participants' Age Was Found Between The Two Groups (F=1.5; P=0.072) In Anonkoua-Kouté. In Contrast, Difference Of Variances Of The Weight Was Significant (F=1.75; P=0.012).

Prevalence of pfdhfr or pfdhps mutations

Table 2 displays the prevalence of mutant alleles in isolates collected from asymptomatically and symptomatically infected children from Anonkoua-kouté. On the whole, the two groups have similar prevalence of individual alleles for the pfdhfr gene. The prevalence of the mutant alleles at the three key loci N51I, C59R and S108N was more than 40% of the apparently monoclonal isolates from both symptomatic and asymptomatic individuals. There were no statistically significant differences in the proportion of alleles at any of the three codons in isolates from the school children and the group of young malaria patients compared. No mutant alleles appeared in locus I164L.

Codon	Mutant and	Asymptomatic		Symptomatic		p	95% CI
	mixed allele	n	(%)	n	(%)		
DHFR-51	Ile (I)	19	-45.2	26	-40	0.593	-0.14 – 0.24
	Asn (N)/Ile (I)	6	-14.3	8	-12.3	0.767	-0.11 – 0.15
	Asn (N)	17	-40.5	31	-47.7	0.461	-0.26 – 0.12
DHFR-59	Arg (R)	18	-40.9	34	-52.3	0.238	-0.30 – 0.08
	Cys (C)/Arg (R)	7	-15.9	11	-16.9	0.888	-0.15 – 0.13
	Cys (C)	19	-43.2	20	-30.8	0.187	-0.06 – 0.31
DHFR-108	Asn (N)	18	-40.9	23	-52.3	0.561	-0.13 – 0.24
	Ser (S)/Asn (N)	7	-15.9	8	-12.3	0.599	-0.10 – 0.17
	Ser (S)	19	-43.2	34	-34.4	0.347	-0.28 – 0.10
DHPS-436	Ala (A)	13	-43.3	29	-44.6	0.907	-0.23 – 0.20
	Ser (S)/Ala (A)	10	-33.3	13	-20	0.18	-0.06 – 0.33
	Tyr (Y)/Ala (A)	2	-6.8	0	0	0.143	-0.02 – 0.16
	Tyr (Y)/Ser (S)	1	-3.3	0	0	0.309	-0.03 – 0.10
	Ser (S)	4	-13.3	23	-35.4	0.01	-0.39 – -0.05
DHPS-437	Gly (G)	8	-27.6	37	-56.9	0.004	-0.50 – -0.09
	Ala (A)/Gly (G)	8	-27.6	17	-26.1	0.885	-0.18 – 0.21
	Ala (A)	13	-44.8	11	-16.9	0.007	0.08 – 0.48
DHPS-540	Glu (E)	1	-2.2	0	0	0.312	-0.02 – 0.07
	Lys (K)	44	-97.8	65	-100	0.312	-0.07 – 0.02
DHPS-581	Gly (G)	0	0	1	-1.5	0.314	-0.05 – 0.02

	Ala (A)	45	-100	64	-98.5	0.314	-0.02 – 0.05
DHPS-613	Ser (S)	0	0	2	-3.1	0.151	-0.07 – 0.01
	Ala (A)/Ser (S)	4	-8.9	0	0	0.036	0.01 – 0.17
	Ala (A)	41	-91.1	63	-96.9	0.221	-0.15 – 0.04

Table 2: Prevalence of individual allele in isolates collected from Anonkoua-kouté; Note : Significant p-values are in bold. CI: Confident Interval.

The picture was a bit more complex for the pfdhps gene. For instance, mixed isolates were quite common: 33.3% of prevalence for the asymptomatic schoolchildren and 20% in the symptomatic group at locus S436A. The symptomatic children were also significantly more likely to carry the wild type S436 codon 35.4% compared with 14.3% (z-test; p=0.010). Conversely at locus A437G, the prevalence of the mutant allele predominated significantly in the malaria infected group, 56.9% (z-test; p=0.004) whereas isolates from the asymptomatically infected carried mostly the wild type allele (44.8%; p=0.007). In addition, three highly resistant rare mutants not commonly found in western Africa, were also observed at very low levels in asymptomatic participants (Glu-540 at 2.2%) and symptomatic patients (Gly-581 at 1.5% and Ser-613 at 3.1%).

Prevalence of pfdhfr and pfdhps genotypes

In order to use molecular markers as a reflection of changes that correlate with SP efficacy, the haplotypes of alleles of pfdhfr and pfdhps needed to be inferred. Table 3 displays these haplotypes. In both populations, about 50% of the isolates carried a triple mutant genotype IRNI and 30% carried the wild type NCSI genotype. The prevalence of these genotypes predominated over the remaining lower proportion genotypes; the single mutant ICSI and the double mutant ICNI appeared solely in isolates from the asymptomatic schoolchildren (Table 2). No relationship was found between genotype prevalence and the two groups (Chi-square=0.389, df=2; p=0.823).

In the study population, of the nine genotypes detected for the pfdhps gene, the double mutant haplotype AGKAA predominated both in the asymptomatic schoolchildren (39.3%) and in the patients' group (47.7%), and these were not significantly different (z-test; p= 0.449). Among the less common haplotypes, the mutant genotype AAKAA was significantly (z-test; p=0.001) associated with the asymptomatically infected schoolchildren (35.7%) while SGKAA genotype was mostly (z-test; p=0.018) carried by the malaria patients with 32.3% (Table 3). The locus S436A appeared tetramorphic in Anonkoua-kouté with the emergence of S436Y mutant allele. The isolates analyzed carried no mutant codons at K540E, so common in many parts of East Africa [16].

Gene	Genotypes	Asymptomatic		Symptomatic		p	95% CI
		n	Prevalence (%)	n	Prevalence (%)		
pfdhfr	IRNI	21	-50	31	-47.7	0.816	-0.17 – 0.22
	NCSI	14	-33.3	20	-30.8	0.782	-016 – 0.21
	NRNI	3	-7.1	11	-16.9	0.11	-0.22 – 0.02
	ICNI	2	-4.8	0	0	0.147	-0.02 – 0.11
	IRSI	1	-2.4	3	-4.6	0.524	-0.09 – 0.05
	ICSI	1	-2.4	0	0	0.311	-0.02 – 0.07
pfdhps	AGKAA	11	-39.3	31	-47.7	0.449	-0.30 – 0.13
	AAKAA	10	-35.7	8	-12.3	0.018	-0.04 – 0.43
	SGKAA	2	-7.1	21	-32.3	0.001	-0.40 – -0.10
	SAKAA	2	-7.1	2	-3.1	0.444	-0.06 – 0.15
	YGKAS	1	-3.6	0	0	0.309	-0.03 – 0.10
	YAKAA	1	-3.6	0	0	0.309	-0.03 – 0.10
	A/Y GKAS	1	-3.6	0	0	0.309	-0.03 – 0.10
	AAKGA	0	0	1	-1.5	0.314	-0.05 – 0.02

	AGKAS	0	0	2	-3.1	0.151	-0.07 – 0.01

Table 3: Prevalence of genotypes carried by isolates from asymptomatically and symptomatically infected individuals from anonkoua-kouté.; Note : Significant P-Values are in bold. CI: Confident Interval.

Combined haplotypes by type of participants

Among the 18 combined pfdhps and pfdhfr haplotypes observed in Anonkoua-kouté, a combination of the triple mutant pfdhfr IRNI and double mutant pfdhps SGEAA, the so called "quintuple" genotype as strongly predictive of SP inefficacy [8], was not observed in our data set (Table 4). Where present, the wild type haplotype NCS/SAKAA was at very low prevalence (4.6%). In contrast, the quintuple mutant haplotype pfdhfr IRNI and pfdhps AGKAA predominated in the two groups. No significant differences in the proportions of these haplotypes from the two groups were found (Chi-square=0.110, df=2; p=0.947).

Mutant	**dhps and dhfr combined**								**Asymptomatic**		Symptomatic		p	95% CI
	N51I	C59R	S108N	S436A	A437G	K540E	A581G	A613S	n	Prevalence (%)	n	Prevalence (%)		
Quintuple									7	(31,8)	23	(35,3)	0.758	-0.26 – 0.19
	I	R	N	A	G	K	A	A	6	(27,3)	22	(33,8)		
	I	R	N	A	G	K	A	S	0	0	1	(1,5)		
	I	R	N	Y	G	K	A	S	1	(4,5)	0	0		
Quadruple									6	(27,3)	16	(24,6)	0.807	-0.19 – 0.24
	I	R	N	A	A	K	A	A	4	(18,2)	3	(4,6)		
	I	R	N	S	G	K	A	A	0	0	7	(10,8)		
	I	R	S	A	G	K	A	A	0	0	1	(1,5)		
	N	R	N	A	G	K	A	A	2	(9,1)	5	(7,7)		
Three or less mutations									9	(40,8)	26	(39,9)	0.94	0.10 – 0.56
	N	C	S	A	A	K	A	A	3	(13,6)	0	0		
	N	C	S	S	A	K	A	A	0	0	3	(4,6)		
	N	C	S	A	A	K	G	A	0	0	1	(1,5)		
	N	C	S	A	G	K	A	A	2	(9,1)	2	(3,1)		
	N	C	S	A	G	K	A	S	0	0	1	(1,5)		
	N	C	S	S	G	K	A	A	0	0	13	-20		
	N	R	N	A	A	K	A	A	0	0	3	(4,6)		
	N	R	N	S	G	K	A	A	0	0	2	(3,1)		
	N	R	N	S	A	K	A	A	0	0	1	(1,5)		
	I	R	N	S	A	K	A	A	3	(13,6)	0	0		
	I	C	N	S	A	K	A	A	1	(4,5)	0	0		

Table 4: Prevalence of combined pfdhfr and pfdhps alleles in anonkoua-kouté; Note : Significant p-values are in bold. CI: confident interval.

Combined haplotypes by age or sex

In terms of age, the prevalence of the haplotype comprising the triple mutant pfdhfr IRNI and any pfdhps single mutant was significantly more prevalent in individuals between 4-8 years than those older than 8 in the studied population. No relationship was found between the prevalence of a particular haplotype and sex (Table 5).

dhps and dhfr combined	8-13 years vs. 4-8 years	Feminine vs. Masculine
	n=36 vs. n=53	n=45 vs. n=44
Quintuple mutant: IRNI-AGKAA	22.20% vs. 18.90%	31.1% vs. 31.1%
	(p=0.671 ; -0.13 – 0.21)	(p=0.943 ; -0.20 – 0.19)
Quintuple mutant: IRNI-AGKAS	2.80% vs. 1.90%	2.2% vs. 0.0%
	(p=0.779 ; -0.06 – 0.07)	(p=0.312 ; - 0.02 – 0.07)
Quadruple mutant: IRSI-AGKAA	5.60% vs. 3.80%	0.0% vs. 0.0%
	(p=0.687 ; -0.07 – 0.11)	
Quadruple mutant: NRNI-AGKAA	8.30% vs. 7.40%	0.0% vs. 0.0%
	(p=0.874; -0.11 – 0.12)	
Quadruple mutant: IRNI-AAKAA	13.90% vs. 34.00%	15.6% vs. 13.3%
IRNI-SGKAA	(p=0.024; -0.36 – -0.03)	(p=0.798; -0.13; 0.17)
Three or less	47.20% vs. 34.00%	51.1% vs. 53.3%
mutations	(p=0.186; -0.07 – 0.35)	(p=0.745; -0.24 – 0.17)
NCSI-AAKAA		
NCSI-SAKAA		
NCSI-AAKGA		
NCSI-AGKAA		
NCSI-AGKAS		
NCSI-SGKAA		
NRNI-AAKAA		
NRNI-SGKAA		
NRNI-SAKAA		
IRNI-SAKAA		
ICNI-SAKAA		

Table 5: Combined pfdhfr/pfdhps haplotypes detected in Anonkoua-kouté in year 2008 in Côte d'Ivoire; Note : Significant p-values are in bold. CI: confident interval.

Discussion

The goal of our study was to compare the prevalence of mutant alleles in pfdhfr and pfdhps in asymptomatic school children and malaria patients of comparable age.

In the study village, the mean age of asymptomatically infected schoolchildren surveyed (9.4 ± 2.4 year) was close to 9.8 ± 2.5 years old as reported by Rohner et al. [14] in Toumodi, in the center part of Côte d'Ivoire. We determined a gametocytic index of 3.7% solely in samples from the asymptomatic schoolchildren. Increased partial immunity allows not only asymptomatic carriage of falciparum parasites but favors the production of gametocytes in older children [6]. This observation underscores the importance of assessing the proportion of parasites that carry alleles associated with SP resistance in this large group of asymptomatic children.

In Côte d'Ivoire, sulfadoxine-pyrimethamine (SP) was replaced as the recommended antimalarial drug for *P. falciparum* malaria treatment in 2005, but the drug is still widely available in the country [17]. Since it is recommended for the intermittent preventive treatment in pregnant women [18], SP is still accessible for acute malaria treatment especially from the informal sector [19,20].

The present study identified 8 Single Nucleotide Polymorphisms associated with SP resistance in subsets of samples from asymptomatic schoolchildren and symptomatic patients from Anonkoua-kouté. At the molecular scale, the proportions of mutant alleles and genotypes of the pfdhfr gene did not differ significantly in the two groups. Moreover, mutant alleles at C59R and S108N loci showed the same proportion in each group with 40.9% and 52.3% in isolates from the asymptomatically and symptomatically infected respectively. The lower prevalence of 22% (23/104) of the mutant allele S108N previously reported in samples from asymptomatic individuals in 1998 [12] has almost doubled within 10 years in Abidjan. In the symptomatic group, the key mutant allele reached 50% (27/54) of a collection of isolates from malaria patients in 2001 [21]. In 2006, a similar prevalence of 46.4% was reported in Yopougon, a municipality of Abidjan [22]. The trend continued even after SP was no longer recommended to treat uncomplicated malaria in the country, increasing up to 52.3% in 2008.

The proportions of the triple mutant genotype IRNI in Anonkoua-kouté was consistent with the above prevalences of key mutant alleles. For example, the genotype was carried by 44.8 and 50% of parasites infecting asymptomatic school children and malaria patients, respectively while parasites with the wild type NCSI genotype represented about 30%. Compared to the 17% (20/118) reported by Djaman et al. [10] in Abidjan, the prevalence of the triple mutant has increased while the prevalence of the wild type allele decreased from 61% (72/118) to 30.8%. The increase in the number of mutations was associated with an increase in *ex vivo* resistance to pyrimethamine as previously shown in Sénégal [23], Côte d'Ivoire [10], Gabon [24], and the Central African Republic [25].

For the dhps gene, the isolates from the asymptomatic schoolchildren carried about 45% of the wild type allele at locus A437G whereas the symptomatic patients mostly carried mutant alleles at loci S436A (35%) and A437G (59.6%). It has been shown [26] that the proportion of the mutant S436A allele is associated with the use of sulfadoxine and *in vivo* resistance to this molecule. Likewise, Vasconcelos et al. [27] in Brazil noted that the use of sulfa drugs in the treatment of bacterial infections raised the level of both alleles S436A and A437G. In line with this, the single mutant genotype AAKAA (35.7%) was associated with asymptomatic carriage while AGKAA (32.3%) genotype was distinctive of the malaria patients.

Rare mutations, A613S and A581G were also found in the symptomatic group and appeared in genotypes associated with the pfdhps gene. The A581G or S436Y alleles with the A613S/ or A613T allele are associated with a loss of sensitivity to sulfadoxine [28-30]. The emergence of these rare mutations in Anonkoua-kouté suggests that monitoring SP resistance during SP/IPTp implementation will be important. In line with previous work in Mali and Ghana [15,20,31] the increased number of mutant alleles at key loci of the pfdhfr and pfdhps genes in Anonkoua-kouté reflects a long history of the usage of sulpha drugs including SP consistent with the level of SP in West Africa [15,20,31]. This provides evidence for an ongoing selection of resistant parasites [32] due to the intensive use of sulpha drugs such as Trimethoprim-Sulfadoxine in the treatment of fever associated with respiratory affections in children less than 15 years or against opportunistic infections occurring in individuals infected with HIV/AIDS [33].

The virtual absence of the mutant allele K540E in Anonkoua-kouté is consistent with previous data in West Africa [34-36] and is indicative of a lower level of resistance to SP in Anonkoua-Kouté than in eastern Africa were it prevails [37,38]. Mutant alleles S108T and A16V associated with resistance to cycloguanil were absent in Anonkoua-Kouté, consistent with previous findings from Burkina-Faso and Mali [34,35].

Conclusions

These studies strongly support the use of molecular markers as an important part of surveillance as policies on IPTp, IPTi and SMC are evaluated. Overall, the absence of pfdhps 540E and the predominance of pfdhps AGKAA and the single mutant haplotype SGKAA alleles suggest that, despite the presence of the IRNI allele of pfdhfr, SP resistance has probably not evolved to high levels in Anonkoua-kouté as it has in Eastern Africa. In addition, this region of Côte d'Ivoire is similar to other areas of West Africa, where the use of SP for intermittent preventive treatment of malaria is likely to be practical.

Authors' contributions

BAAA carried out the molecular genetic studies, participated in the sequence analysis, performed the statistical analysis of results and participated in the preparation of the first draft of the manuscript in collaboration with other authors. OAT conceived the study, was responsible for planning and implementation of the study, provided ongoing supervision on behalf of LKP and helped to draft the manuscript. MJ provided training for BAAA, participated in the sequence alignment and sequence analysis. RT had primary involvement in the implementation of field and realized thick and thin blood smears and provided material for field aspect. EAG was involved in the implementation of field aspects and provided help for thick and thin blood smears. LKP provided overall supervision of the Department of Malariology staff and was involved in planning the project, participated in the design of the study. CHS procured funding for, hosted and supervised the molecular biology aspects of the project, and had input in the manuscript. All authors read and approved the final manuscript.

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