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ORIGINAL RESEARCH

Molecular analysis of markers associated with chloroquine and sulfadoxine/pyrimethamine resistance in *Plasmodium falciparum* malaria parasites from southeastern Côte-d'Ivoire by the time of Artemisinin-based Combination Therapy adoption in 2005

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Purpose: Artemisin-based combination therapies became the recommended therapy in Côte-d'Ivoire in 2005, but both chloroquine (CQ) and sulfadoxine/pyrimethamine (SP) have been heavily used for many decades. Despite this long history, little is known about the geographical distribution of drug resistance–conferring genotypes outside the capital city of Abidjan. In this work, we compared the prevalence of drug-resistant genotypes in Bonoua, an urban area, and Samo, a rural agricultural area, in southeastern Côte-d'Ivoire, about 59 km from Abidjan.

Patients and methods: Samples were collected from symptomatic patients in both sites during the rainy season in 2005. Genomic DNA was isolated and codons associated with resistance to CQ and SP were analyzed: *pfcrt* codons Cys-72-Ser, Val-73-Val, Met-74-Ile, Arg-75-Glu, Lys-76-Thr; *pfdhfr* codons Ala-16-Val, Arg-51-Ile, Cys-59-Arg, Ser-108-Arg/Thr, and Ile-164-Leu; *pfdhps* codons Ser-436-Ala, Ala-437-Gly, Lys-540-Glu, Ala-581-Gly, and Ala-613-Thr/Ser.

Results: A limited number of genotypes were found in Bonoua compared with Samo. In both sites, the triple-mutant allele CV**IET** of *pfcrt* predominated: 100% in Bonoua and 86.2% in Samo. The wild-type allele, NCSI of *pfdhfr*, was common – 50% in Bonoua and 38.7% in Samo – but the triple-mutant **IRN**I and double-mutant NRNI were also frequent (**IRN**I, 32.6% in Bonoua and 19.4% in Samo; N**RN**I, 15.2% in Bonoua and 9.7% in Samo). In Samo, a wide range of different genotypes of *Pfdhps* was observed, with alleles carrying the Gly-437 codon fixed in Bonoua and comprising 73% of the isolates in Samo.

Conclusion: Although these two sites are only 8 km apart, they belonged to very different ecological environments. The overall prevalence of alleles of single-nucleotide polymorphisms associated with resistance to CQ and SP in both locations was among the highest of the region by 2005, although the more rural site showed a more diverse set of alleles and mixed infections. Continued surveillance of these markers will be a useful tool for drug policy, as both CQ and SP are still frequently used years after withdrawal, and SP is recommended by the World Health Organization for intermittent preventive therapy for pregnant women and infants. Data analyzed herein are among the first to be generated during the year of artemisin-based combination-therapy introduction in Côte-d'Ivoire and could be of some interest for malaria policy-makers.

Keywords: Côte-d'Ivoire, malaria resistance, chloroquine, sulfadoxine/pyrimethamine, *pfdhfr*, *pfdhps*, *pfcrt*

Introduction

Côte-d'Ivoire was among the African countries to recommended chloroquine (CQ) as the main antimalarial treatment more than 40 years ago.[1] However, resistance to CQ was detected from returning travelers as early as 1986[2] and confirmed locally in 1987.[3] Since then, there has been an upward trend in the prevalence and degree of CQ resistance.[1,4,5] In 2003, CQ was replaced by amodiaquine (AQ) as the first-line therapy.

Few sulfadoxine/pyrimethamine (SP) trials were implemented in the country before 2003. SP was officially maintained as second-line treatment for uncomplicated *falciparum* malaria until 2005, when both AQ and SP were withdrawn in favor of artemisin-based combination therapies (ACTs), as recommended by the World Health Organization: artesunate/amodiaquine as first-line treatment and artemether/lumefantrine as second-line.[6] However, as is often the case,[7,8] there is still extensive access to SP, CQ, and AQ, and their much lower cost has encouraged continued use of these monotherapies.[9] In addition, preference of patients for SP over AQ also meant that SP use was frequent; CQ withdrawal from the market was effectively realized 4 years after the official decision, upon a ministerial decree published in 2007.

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.[10] Similarly, alleles of *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene with mutant codons at positions 72–76 have been closely associated with resistance to CQ and AQ.[10]

In light of the heavy use of SP and CQ, it is not surprising that a high prevalence of mutant genotypes of *pfdhfr, pfdhps*, and *pfcrt* were reported in Abidjan, where drug resistance has been studied for years.[10,11] However, there is still almost no information on the frequency of markers of drug resistance in settings other than Abidjan. Moreover, there are few data examining the dynamics of genes conferring drug resistance between urban and rural settings in the country. The purpose of this work was to compare the genetic diversity of antimalarial drug resistance–conferring genotypes between neighboring urban and rural sites 59 km removed from Abidjan, by the year of ACTs introduction. Data analyzed herein are among the first generated during the year of implementation of ACT in Côte-d'Ivoire and provide information for policy decisions on drug policy and use in the country.

Material and methods

Study sites, participants, and sample collection

Samples were collected from symptomatic children (6–59 months of age) in Bonoua and Samo from June to December, 2005.[12] Bonoua (5°16′16.24 N; 3°35′38.13 W) is an urban site 59 km from Abidjan, and Samo, a rural area, is 8 km from Bonoua. Both sites belong to the sanitary district of Grand-Bassam (region of Sud-Comoe). Samples were collected on the day of inclusion from patients admitted with acute uncomplicated *P. falciparum* infection either at the local General Hospital in Bonoua or at the Health Care Center in Samo. Thick and thin blood smears were prepared for microscopic identification of *P. falciparum.* Monoinfection with *P. falciparum* was detected from Giemsa-stained blood smears, as described elsewhere.[12]

The study protocol received approval from the National Ethics and Research Committee prior to the implementation and was conducted in compliance with International Conference on Harmonisation Good Clinical Practice guidelines according to the Declaration of Helsinki (2000, amended in Tokyo 2004). The parents or guardians of the children provided participants' informed consent prior to inclusion.

Extraction of *Plasmodium falciparum* DNA

Whole blood from *P. falciparum*–positive persons was blotted on sterile Whatman (Maidstone, United Kingdom) 3 MM filter paper, stored dry at room temperature, protected with a silica-gel desiccant, and archived for later *P. falciparum* DNA isolation. DNA was extracted from blood spotted on filter paper using the QIAamp DNA Micro Kit (Qiagen, Valencia, CA), as per manufacturer's protocol. Genomic DNA (gDNA) was isolated from samples diagnosed as *P. falciparum*–positive persons.

PCR amplifications

Primers used for amplifications are described in Table 1. Extracted gDNA was amplified in two rounds of nested polymerase chain reaction (PCR). The first-round PCR amplifications were performed in a final volume of 20 μL, comprising 7 μL of pure water, 10 μL of 1 × PCR PreMix A (Epicentre Biotechnologies, Madison, WI), 0.4 μL of each primer 10 mM, 0.2 μL of FailSafe enzyme (Epicentre Biotechnologies), and 2 μL of template DNA. The second-round PCR amplification reactions were performed similarly to the first, with 0.5 μL of PCR products as DNA template and 8.5 μL of pure water. All reactions were performed

Table 1 Primers used for polymerase chain reaction amplifications and amplification for sequencing

Genes	Round	Primers	Sequences (5'–3')	Size (bp)
PCR amplifications				
pfcrt	Primary	S1236 (forward)	GGT GGA GGT TCT TGT CTT GG	21
		S1237 (reverse)	ATT AAA GTT GTG AGT TTC GGA TG	24
pfdhfr	Primary	S217 (forward)	CTC CTT TTT ATG ATG GAA CAA GTC TGC GAC GTT TTC G	37
		S218 (reverse)	TCA TAT GAC ATG TAT CTT TGT CAT CAT TCT TTA AAG GC	38
	Secondary	S1226 (forward)	AAC CTA AAC GTG CTG TTC AA	20
		S1227 (reverse)	AAT TGT GTG ATT TGT CCA CAA	21
pfdhps	Primary	S219 (forward)	GTC TGC GAC GTT TTC GAT ATT TAT GCC	27
		S229 (reverse)	GGC ATA TCA TTA TTT TTT TCT TCT CCT TTT TAT AC	34
	Secondary	S1224 (forward)	GAT TCT TTT TCA GAT GGA GG	20
		S1225 (reverse)	TTC CTC ATG TAA TTC ATC TGA	21
Amplifications for sequencing				
pfcrt		S1236 (forward)	GGT GGA GGT TCT TGT CTT GG	21
		S1237 (reverse)	ATT AAA GTT GTG AGT TTC GGA TG	24
pfdhfr		S1226 (forward)	AAC CTA AAC GTG CTG TTC AA	20
		S1227 (reverse)	AAT TGT GTG ATT TGT CCA CAA	21
pfdhps		S1224 (forward)	GAT TCT TTT TCA GAT GGA GG	20
		S1225 (reverse)	TTC CTC ATG TAA TTC ATC TGA	21

Notes: A premix for amplification of sequencing comprised only the one relevant primer used for the nested polymerase chain reaction (PCR) amplification. Products were incubated at 15°C for 5 minutes.

using 96-well plates on an MJ Research Tetrad PTC-225 Thermocycler (Bio-Rad, Hercules, CA).

Cycling conditions for the first- and second-round PCR cycles for *pfdhfr* were as follows: 94°C for 3 minutes; 30 cycles of 94°C for 30 seconds, 50°C for 45 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 10 minutes. For *pfdhps*, the amplification conditions were 94°C for 3 minutes; 34 cycles of 94°C for 45 seconds, 51°C for 45 seconds, and 60°C for 45 seconds; and a final extension at 60°C for 5 minutes. And for *pfcrt*, the amplification conditions were 94°C for 3 minutes; 34 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 62°C for 60 seconds; and a final extension at 62°C for 5 minutes.

Amplifications for sequencing reactions

The cycling sequencing reactions consisted of a linear amplification of extension products with BigDye Terminator (Applied Biosystems, Foster City, CA). Two master mixes were prepared for amplification, with one primer per mixture. Each master mix contained a total volume of 10 µL, using 2 µL of nested 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 Terminator.

Sequence analysis

Sequencing was run in both directions; products of amplification for sequencing were separated and analyzed on an ABI 3100 (Applied Biosystems). The DNA sequence assembly software Sequencher 4.1.4 (Gene Codes, Ann Arbor, MI), was used for base calling, trimming, display, and editing of sequences automatically read on the capillary machine. Control sequences of *pfdhfr* and *pfdhps* genes from *P. falciparum* 3D7 strain were uploaded from GenBank for SNPs detection. 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, and exon 2 of *pfcrt* C72S, V73V, M74I, N75E, K76T. Analysis was repeated twice, mainly for uncommon data. More than one chromatogram peak at the same codon locus was indicative of a mixed infection only if the height of the additional peak was more than 33% the height of the major peak. Mixed mutant (Mu) genotypes are detected when two or more populations of parasites with distinct genotypes at particular codons of interest are present in a single infection.[13]

Statistical analysis

The statistical analyses were performed with XLSTAT software, version 2010.6 (Addinsoft, Paris, France). Proportions were compared with a one-sided Z-test of comparison for two proportions. A 95% confidence interval around the difference of proportions was determined. A *P*-value lower than 0.05 was taken to indicate statistically significant differences. Frequencies of mutant (Mu), mixed (Mi), and wild-type (WT) genotypes were determined separately by the following relations as suggested by Sridaran et al.[14] Mutants = Mu/(Mu + Mi + WT); mixed alleles = Mi/(Mu + Mi + WT); wild type = WT/(Mu + Mi + WT).

Results

Success rates of molecular analysis conducted with samples collected from Bonoua and Samo are shown in Table 2.

Frequencies of genotypes associated with chloroquine resistance

Table 3 displays differences between Bonoua and Samo in the diversity and prevalence of CQ resistance–conferring genotypes, and Table 4 details these at the level of SNPs. The mutant genotype *pfcrt* Ile-74/Glu-75/Thr-76 (CV**IET**) reached fixation in Bonoua; neither mixed infections nor any other genotypes were detected there. In contrast, 8 km apart, in Samo, fixation of CV**IET** was not fully reached (38/44 [86.2%]). The triple-mutant haplotype was predominant, but six other genotypes were also detected, among which were the less common haplotype, *pfcrt* Met-74/Glu-75/Thr-76 (CVM**ET**) and four mixed genotypes. The frequency of the triple-mutant CV**IET** was significantly higher in Bonoua ($P = 0.004$).

Frequencies of individual mutant codons are compared in Table 4. The individual amino acids Cys-72, Ile-74, and Glu-75 comprising the triple-mutant haplotype CV**IET** were found in all the 48 isolates from Bonoua. In Samo, 38/47 (81%) of the isolates carried the mutation that encodes Ile-74, and 40/47 (85%) carried the mutant Glu-75. Most important, the prevalence of the key amino acid Thr-76 was higher than 90% in both sites (Table 4). No mutations were found in codon Val-73 in either site.

Frequencies of pyrimethamine resistance–conferring genotypes

There were no significant differences in frequencies for *pfdhfr* pure and mutant genotypes in Bonoua and Samo. The diversity of these genotypes was much lower in Bonoua than in Samo, where more mixed genotypes were detected. Three pure genotypes were detected in both sites (Table 4). The wild-type allele Asn-51/Cys-59/Ser-108 (NCSI) predominated in both sites (23/46, 50% in Bonoua, and 12/31, 38.7% from Samo); the triple-mutant Ile-51/Arg-59/Asn-108 (**IRN**I) was also common, (15/46, 32.6% in Bonoua, and 6/31, 19.4% in Samo). The double-mutant Asn-51/Arg-59/Asn-108 (N**RN**I) was observed in 7/46 (15.2%) of isolates in Bonoua and 3/31 (9.7%) in Samo. Five mixed genotypes, most of them with rare frequencies, were found in Samo but only one mixed genotype in Bonoua N-C/**R**-SI. The frequency of the mutant codon Asn-108 was almost 50% for both sites. All of the isolates also carried the wild-type Ile-164. Difference in the frequencies of individual mutation alleles and genotypes between Bonoua and Samo were not significant (Table 4).

Distribution and frequencies of sulfadoxine resistance–conferring genotypes

For both the *pfcrt* and *pfdhfr* loci, the number of mixed genotypes was lower than the number of pure genotypes for each of the analyzed genes (Table 3). In contrast for *pfdhps*, the frequency of isolates that carried mixed genotypes was higher in Bonoua, whereas in Samo, purely mutant genotypes predominated significantly ($P = 0.001$, not shown here). Again, the diversity of genotypes for the *pfdhps* gene was much lower in Bonoua than in Samo (Table 3). The single-mutant S**G**KAA was the unique pure genotype found in isolates from Bonoua. It was carried by four isolates (9.8%) among the 41 obtained in Bonoua and was the only allele that both sites had in common. In Samo, six additional pure genotypes were detected, among which were the uncommon double mutants **F**AKA**S** and **Y**AKA**S**, and the triple mutants **AG**KA**S**. Each of these genotypes, as well as the wild-type SAKAA were found in only one (3.3%) among 30 isolates from Samo. The double-mutant **AG**KAA and the single-mutant **A**AKAA were also detected in eight (26.8%) and three (10%) isolates out of the 30, respectively (Table 3).

Two mixed genotypes (S/**A**-**G**KAA and S/**L**-**G**KAA) among five were found in Bonoua. Particular attention has been paid to the unusual S/**L**-**G**KAA genotype.

Table 2 Success rates of molecular analysis of samples collected from Bonoua and Samo by the time of artemisin-based combination-therapy introduction

Sites	Filter-paper samples collected	Samples DNA-extracted	Samples successfully amplified (%)	Samples successfully sequenced (%)		
				pfcrt	*pfdhfr*	*pfdhps*
Bonoua	69	66	48 (72.7)	48 (72.7)	46 (69.7)	41 (62.1)
Samo	63	52	49 (94.2)	44 (84.6)	31 (59.6)	30 (57.7)
Total	132	118	97 (82.2)	92 (78.0)	77 (65.3)	71 (60.2)

Notes: Both success rate of polymerase chain reaction (PCR) amplification and sequencing reaction were calculated based on the number of DNA-extracted samples per site. For each site, the number of successfully amplified isolates was n = 48 out of 69 in Bonoua and n = 49 out of 63 in Samo. Failure to amplify fragments from a fraction of the patient samples was due to quality of the filter paper used, and extraction efficiency from the paper. All PCR amplification failures were excluded from further analysis.

Table 3 Distribution of genotypes conferring resistance to chloroquine and sulfadoxine/pyrimethamine in Bonoua and Samo in 2005 by the time of artemisin-based combination-therapy adoption

Genes	Genotypes	Bonoua		Samo		Significant *P*-value
		n	(%)	n	(%)	
pfcrt	Haplotypes					
	CV**IET**	48	(100.0)	38	(86.2)	0.004
	CVM**ET**	0	(0.0)	1	(2.3)	
	CVMNK	0	(0.0)	1	(2.3)	
	Mixed					
	CV-M/**I**-N/**E**-**T**	0	(0.0)	1	(2.3)	
	CV-M/**I**-N/**E**-K/**T**	0	(0.0)	1	(2.3)	
	CVM**E**-K/**T**	0	(0.0)	1	(2.3)	
	CV-M/N-**E**-K/**T**	0	(0.0)	1	(2.3)	
	Total	48	(100.0)	44	(100.0)	
pfdhfr	Haplotypes					
	NCSI	23	(50.0)	12	(38.7)	
	IRNI	15	(32.6)	6	(19.4)	
	N**RN**I	7	(15.2)	3	(9.7)	
	Mixed					
	N/**I**-C/**R**-S/**N**-I	0	(0.0)	6	(19.4)	
	N/**I**-C/**R**-SI	0	(0.0)	1	(3.2)	
	N/**I**-CSI	0	(0.0)	1	(3.2)	
	N/**I**-**RN**I	0	(0.0)	1	(3.2)	
	N-C/**R**-S/**N**-I	0	(0.0)	1	(3.2)	
	N-C/**R**-SI	1	(2.2)	0	(0.0)	
	Total	46	(100.0)	31	(100.0)	
pfdhps	Haplotypes					
	AGKAA	0	(0.0)	8	(26.8)	<0.0001
	S**G**KAA	4	(9.8)	7	(23.3)	
	AAKAA	0	(0.0)	3	(10.0)	
	AGKAS	0	(0.0)	1	(3.3)	
	FAKAS	0	(0.0)	1	(3.3)	
	S**A**KAA	0	(0.0)	1	(3.3)	
	YAKA**S**	0	(0.0)	1	(3.3)	
	Mixed					
	S/**A**-**G**KAA	13	(31.7)	4	(13.4)	0.027
	S/**A**-**G**KA-A/**S**	0	(0.0)	2	(6.7)	
	A-**G**/**A**-KAA	0	(0.0)	1	(3.3)	
	S/**A**-**G**/**A**-KAA	0	(0.0)	1	(3.3)	
	S/**L**-**G**KAA	24	(58.5)	0	(0.0)	<0.0001
	Total	41	(100.0)	30	(100.0)	

Notes: Capital letters in the genotypes column represent the single-letter codes of amino acids. Mutant amino acids are in bold and underlined. The total represents the number of successfully sequenced samples from each site.

Sequencing was repeated twice, and results were validated by an experienced technician. As a mixed genotype, its frequency was unexpectedly high in that setting: it was carried by 24 isolates (58.5%) out of 41 (Table 3).

The codon Ser-436-Ala appeared to be trimorphic with two mutant alleles, Phe-436 and Tyr-436, and the wild-type allele Ser-436 identified in Samo. The frequency of the mutant allele Gly-437 was significantly higher in Bonoua ($P = 0.0005$), where it was a component of the three genotypes found there (Table 4). No change was identified in the locus Lys-540 or Ala-581, positions in which mutants have been observed in the East African region.[10] Five alleles carried the Ser-613 mutation (Table 4).

Discussion

The purpose of the study was to compare the genetic diversity of molecular markers conferring CQ and SP resistance in isolates from symptomatic patients from two different settings: urban Bonoua and rural Samo, in southeastern Côte-d'Ivoire. In both sites, the CV**IET** allele of *pfcrt* was predominant, and in fact was fixed in Bonoua. Resistant alleles of *pfdhfr* and *pfdhps* were at a much lower level, although the triple-mutant allele **IRN**I associated with high-level resistance to pyrimethamine was common, but not predominant, in both locations.

A wide range of *pfdhps* genotypes was observed in both locations, but alleles carrying the single-mutant codon Gly-437 predominated. Differences in the diversity of alleles in the two settings was striking as well. In the rural Samo site, mixed infections were far more common than in Bonoua, and a number of unusual alleles of *pfdhps* were observed. For example, the simple mutant S**G**KAA was the only pure mutant among three *pfdhps* genotypes determined from Bonoua, while in Samo up to 11 genotypes were found. Our data suggest differences in the dynamics of distribution of drug resistance–conferring genotypes.

A complex interaction among several factors could explain the broader range of genetic variability in Samo in 2005: urbanization and rural exodus, agricultural activities and anopheline density, and migration in human populations. Differences in the distribution of resistant genotypes may reflect the different levels of urbanization of Bonoua and Samo. Our results confirm those of Soulama et al,[13] who found it significant that in Burkina Faso, urbanization affects the distribution of genotypes and allelic polymorphism of *MSP1* and *MSP2* between rural and urban areas. In the same country, differences in the prevalence of drug-resistance markers may be explained by a higher drug pressure in the urban area compared to the rural study area.[15]

An average *P. falciparum* entomological infection rate of 314.7 infective bites/person-year was recently reported for the whole country,[16] but not yet for Bonoua nor Samo. However a higher mixed-infection rate in Samo suggests higher malaria transmission in this agricultural area.[17] Many crops, like maize, vegetables, irrigated rice, and pineapple, a major crop in Samo, present excellent breeding foci for mosquitoes, lending support to this idea.[18–20]

Table 4 Spread of individual codon alleles between Bonoua and Samo in 2005 by the time of artemisin-based combination therapy adoption

Genes	Locus	Alleles	Bonoua		Samo		Significant *P*-value
			Proportions	(%)	Proportions	(%)	
pfcrt	M74I	**I**	48/48	(100.0)	38/47	(81.0)	<0.0001
		M/**I**	00/48	(0.0)	02/47	(4.0)	
	N75E	**E**	48/48	(100.0)	40/47	(85.0)	0.002
		N/**E**	00/48	(0.0)	01/47	(2.0)	
	K76T	**T**	48/48	(100.0)	40/42	(95.0)	
		K/**T**	00/48	(0.0)	01/42	(2.0)	
pfdhfr	N51I	**I**	15/46	(33.0)	06/31	(19.0)	
		N/**I**	31/46	(67.0)	16/31	(52.0)	
	C59R	**R**	23/46	(50.0)	13/31	(42.0)	
		C/**R**	01/46	(2.0)	08/31	(26.0)	0.002
	S108N/T	**N**	24/46	(52.0)	14/31	(45.0)	
		S/**N**	00/46	(0.0)	07/31	(23.0)	0.001
pfdhps	S436A	**F**	00/41	(0.0)	01/30	(3.0)	
		Y	00/41	(0.0)	01/30	(3.0)	
		S/**A**	13/41	(32.0)	07/30	(23.0)	
		S/**L**	24/41	(59.0)	00/30	(0.0)	<0.0001
	A437G	**G**	41/41	(100.0)	22/30	(73.0)	<0.0001
		A/**G**	00/41	(0.0)	01/30	(3.0)	
	A581G	A	42/42	(100.0)	30/30	(100.0)	
	A613S	**S**	00/42	(0.0)	03/30	(10.0)	0.034
		A/**S**	00/42	(0.0)	02/30	(7.0)	

Notes: Capital letters in the alleles column represent the single-letter codes for amino acids. Mutant amino acids are in bold and underlined. The denominator of each fraction represents the total number of gene fragments analyzed successfully for that locus. Only significant *P*-values < 0.05 have been reported. Some missing data were found that varied according to the locus or combination of loci analyzed. As a result, the number of successfully sequenced fragments also varied according to the locus or combination of loci analyzed.

Bogreau et al asserted that genetic diversity is more intense in urban areas due to an influx of people from surrounding rural areas bringing parasites to new locations.[21] In fact, in our study, it is likely that the rural Samo area experienced an influx of people from neighboring countries and within Côte-d'Ivoire, attracted by a major increase in pineapple cultivation. Migrants were attracted to the southern region of Côte-d'Ivoire in search of employment. The largest pineapple farms from southern country and industrial activities were settled nearby Samo.[19–22]

Pearce et al emphasized that haplotypes **AG**K and S**G**K, such as those reported here in Samo, are commonly encountered in West Africa.[23] For example, these were found in returning travelers from Nigeria, Ghana, Mali, and Guinea but also from Côte-d'Ivoire, as shown by Sutherland and colleagues.[24] These observations support the idea that the heterogeneity of the human population in Samo could be an important element in the greater parasite diversity as well. Thus, a determinant is not necessarily whether a site is urban or rural, but rather the heterogeneity of the parasites brought by people as they migrate from other areas into the site being studied.[2]

The very high level of the triple-mutant genotype *pfcrt* CV**IET** in both sites and its 100% frequency in Bonoua are consistent with other sites in West Africa and consistent with other measures of CQ resistance in Côte-d'Ivoire. Measures of clinical resistance were observed as early as 1986,[2] and increased progressively from 2%–28.9% between 1986 and 1988[1] to 43% in the western part of the country in 2001.[25] In vitro tests on isolates in Abidjan, in 2000 and later in 2004, confirmed the reduced sensitivity of parasites in this region to CQ, as well. The average IC_{50} value (the concentration of CQ required to inhibit growth by 50%) for CQ-resistant isolates was 196 nM compared to less than 100 nM for sensitive parasites in 2000, and 225 nM compared to 47 nM in 2004.[26,27] All of these data underscore the intense CQ drug pressure in this region, resulting in the virtual fixation of the triple-mutant genotype CV**IET** observed in 2005 in Bonoua.

By the time of ACTs adoption, CQ and SP were still available from unofficial retailers,[9] thus allowing self-medication,[8] or from pharmacies, since a ministerial decree to implement effective CQ withdrawal from the local drug market only came late in 2007. This context was certainly exacerbated by the political unrest in the country at that time, a situation that compromised the ability of the malaria-control program to monitor compliance with the new malaria-treatment recommendations.[28] In any case, our findings could seriously

compromise the use, if any, of new antimalarial therapeutic combinations with CQ for intermittent preventive treatment in pregnancy (IPTp) or further IPT for infants in either of these sites.[29,30]

Prevalence of the mutation Gly-437 was significantly higher in Bonoua, where it reached 100%, detected in 41/41 isolates, but 22/30 (73%) of samples in Samo, and this codon was associated with polymorphism in Ala-436.[10] Consistent with this observation, the mutant allele Gly-437 was also a component of all the three mutant haplotypes detected in the area. In fact, it is unusual to observe a high prevalence of mutant alleles of *pfdhps* in parasite populations where the prevalence of the triple-mutant *pfdhfr* allele **IRN**I is low, as it is here. This suggests that the usage of sulfa drugs in Bonoua and Samo may have exerted strong drug pressure on *falciparum* populations from Bonoua and Samo.[31] Thus the monitoring of sulfadoxine and related drugs is required, especially since trimethoprim–sulfamethoxazole is in use as prophylaxis for people living with HIV.[32,33]

There was no significant difference in the prevalence of Asn-108 or Arg-59 between the two sites. The prevalence of the mutant allele Asn-108 is of the same order of magnitude, with 45.5% and 46.4% of prevalence determined for the same allele in 2001 and 2006, respectively, in Abidjan.[26,34,35] In contrast, the higher rate of NCSI in both sites (50% in Bonoua and 38.7% in Samo) compared to **IRN**I (32.6% in Bonoua and 19.4% in Samo), suggests that pyrimethamine was likely still clinically effective by the time of SP withdrawal, but this may certainly have changed in the subsequent 7 years. Fortunately, in a recent paper,[36] Ouattara reported a lower prevalence of 35% of **IRN**I in Adzopé in 2007, located inland 108 km from Abidjan. Spatiotemporal studies would confirm whether the prevalence of this key mutation, along with SP pressure, decreases as one samples further inland. The prevalence of molecular markers associated with CQ and SP resistance could greatly increase in and around foci like big cities and decrease when moving away from them, depending on the local drug pressure.[37]

Conclusion

In 2005, by the time of ACT adoption, and 2 years after the replacement of CQ, noticeable differences appeared in Bonoua and Samo in the distribution of genotypes conferring resistance to CQ and SP. The spread of mutated genotypes differed according to the settings and the demographics of each site. In the long run, high-level prevalence of resistant genotypes could compromise the efficacy of SP for IPTp in Bonoua and Samo, but also threaten the use of any other alternative association with CQ as a partner drug for IPTp. Roughly 7–9 years after drug-policy changes, these findings need to be updated with a larger sample size, and more recent data need to be provided. Our results give a broad outline of the level of resistance to SP and CQ by the time of ACT introduction in Côte-d'Ivoire and are of interest for decision-makers or for global monitoring of malaria drug resistance via such networks as the Worldwide Antimalarial Resistance Network.[38]

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Disclosure

The authors report no conflicts of interest in this work.

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