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Full Length Research Paper

Prevalence of *Plasmodium falciparum pfcrt*-K76T mutation in asymptomatic vs symptomatic school children in three epidemiological areas of Côte D'Ivoire

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Plasmodium **asymptomatic carriers are considered as parasite reservoir in humans. Therefore, they can be at the origin of the expansion of chemoresistant plasmodial strains. In order to be effective, new strategies for malaria elimination must take into account these individuals. This cross-sectional study aimed to determine the prevalence of *Plasmodium falciparum pfcrt*76T allele in asymptomatic versus symptomatic children in different epidemiological areas of Côte d'Ivoire. It was conducted from May 2015 to April 2016 in the rural and urban areas of Grand-Bassam, Abengourou and San Pedro during the rainy and the dry seasons. A total of 2361 blood samples were collected from schoolchildren. The plasmodial prevalence was 40%. *P. falciparum* was the majority species with 95.7%. Fifteen percent or 360 blood samples were drawn for setting Nested PCR. The overall prevalence of *pfcrt*76T allele was 15.8%. This prevalence of *pfcrt*76T was 17% and 15% in asymptomatic and symptomatic patients respectively (*p* = 0.7098). The prevalence of *pfcrt*76T allele was not associated with the geographical area and the clinical status of the carrier. However, it was linked to the intensity of *P. falciparum* transmission.**

Keywords: *Plasmodium falciparum*, Côte d'Ivoire, *pfcrt*, asymptomatic, malaria.

BACKGROUND

Plasmodium falciparum is responsible for 90% of malaria deaths, according to the World Health Organization (WHO) (World Health Organization, 2016). Children under five years old and pregnant women are the most affected population. In Côte d'Ivoire, malaria is endemic in most parts of the country with much higher transmission during the rainy season (Mara et al., 2013; Yavo et al., 2016). To face this concern, two strategies have been adopted: the elimination of the vectors with insecticides, insecticide-treated bednets and the use of antimalarial drugs in both curative treatment and particular prophylaxis in pregnant women (Misbahi, 2013). However, the emergence of insecticide-resistant Anopheles and the emergence of *Plasmodium* resistance to antimalarial drugs have made the fight against malaria more difficult. Chloroquine (CQ) was one of the first molecules used for the malaria treatment. Unfortunately, in the early 1960s, the first chloroquine-resistant *Plasmodium* strains were detected in Southeast Asia and Latin America, and then chloroquine resistance was expanded to affect Africa (Djaman et al., 2004; IH Menan et al., 2007; Mengesha and Makonnen, 1999; Misbahi, 2013; Wernsdorfer, 1991). To cope with this resistance, since 2007 the Ministry of Health of Côte d'Ivoire, like other countries in sub-Saharan Africa, has recomme-

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nded the withdrawal of the CQ from the antimalarial drugs market and the use of Artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria. Nine years after the withdrawal of CQ, we conducted this study to assess the prevalence of *P. falciparum* codon 76 mutation (*pfcrt*-K76T), a molecular marker of chloroquine resistance, in asymptomatic versus symptomatic children in three different epidemiological areas.

MATERIALS AND METHODS

Study sites and sample collection

It was a cross-sectional study conducted from May 2015 to April 2016 in the rural and urban areas of Grand-Bassam, Abengourou and San-Pedro in the rainy season and in the dry season (Figure 1). Abengourou and San-Pedro are sentinel sites of the National Malaria Control Program in Côte d'Ivoire (PNLP / CI). The city of Grand-Bassam is home to a regular population in Abidjan (economic capital and sentinel site of the PNLP) because of the proximity between these two localities. It is also close to Bonoua where a previous study reported high frequencies of resistance molecular marker to chloroquine and sulfadoxine-pyrimethamine (Ako et al., 2012). The study areas were selected taking into account the epidemiology of malaria throughout the country. Indeed, in Côte d'Ivoire, the epidemiology of malaria is linked to the climatic and phytogeographic areas of the territory, which varies from north to south.
The town of Grand-Bassam is located southeast of Côte d'Ivoire on the coast about 45 km east of the city of Abidjan. Grand-Bassam enjoys an equatorial transition regime. The coastal area is very humid with an average annual rainfall exceeding 1500 mm. Temperatures remain high and constant throughout the year, ranging from 24°C (August) to 29°C (March). In this city, there are four seasons including the long rainy season (April to June), the short dry season (July to September), the short rainy season (October to November) and the long dry season (December to March).
Located at 210 kilometers from Abidjan, Abengourou is a city in the east of Côte d'Ivoire. The climate of the region is an equatorial type with constant temperatures and very heavy rains. There are four seasons including the long rainy season (mid-March to mid-July), the short dry season (mid-July to August), the short rainy season (September to mid-November) and the long dry season (mid-November to mid-March). It is a forest area with dense forests in places and some plots. There are many exploited shallows for rice farming.
San Pedro is a south-west city of Côte d'Ivoire, about 350 km from Abidjan, the economic capital. It is the third largest city in the country. The climate is a humid tropical type with a littoral area, characterized by four seasons, including a large rainy season (April to mid-July) and a small (September to November), a large dry season (December to March) and a small (mid-July to September). It's characterized by a relatively high average rainfall, ranging from 1203.6 mm to 1392 mm of rain per year, and an average temperature around 26°C per month. Two major environmental phenomena characterize this city: the presence of many large swampy areas and the presence of a cordillera of hills separating the city from the seacoast.
In each area, 3 primary schools and 11 pupils per class were randomly selected. After getting the written and informed consent from parents or legal guardians, a questionnaire was dispensed to each schoolchildren and schoolchildren's parent to collect socio-demographic and clinical data. The physical examination was done and it is possible to note the absence or the presence of clinical signs of malaria. Then a blood sample was collected in a tube containing EDTA to perform the bloods smear and dried blood spots (DBS). For the determination of parasitaemia, asexual forms of *Plasmodium* were counted for 500 leucocytes and expressed in number of trophozoites / µl of blood, assuming that the average number of leukocytes is 8000 / µL. The parasitemia was obtained by averaging the results of two independent readers. In case of discordance of the parasite density of more than 50% between the first two readings, a third reading was made by an experienced biologist.

Molecular analysis

Plasmodial DNA extraction was made from dried blood spots on DBS paper using the "Quick-DNA Universal Kit" kit (lot No. ZRC 186993, Zymo research, California, USA) according to the instructions from the manufacturer and stored at -20°C.
The fragment of interest of *pfcrt* gene was amplified by nested PCR. The reaction mixture consisted of 15.875 µL of water quality molecular biology, 2.5 µL of 10X Standard Taq Reaction Buffer (New England Biolabs, Inc® (NEB)), 0.5 µL of dNTP (10 mM), 0.5 µL of each forward and reverse primer (10 µM), 0.125 µL of Taq DNA Polymerase (NEB) and 5 µL of DNA (DNA extract or PCR products depending on the cycle). The primer sequences used to carry out the PCR are shown in Table 1. All manipulations were performed in a class II Biological Safety Cabinet (BSC). Amplifications were performed using the SimpliAmp ™ 96-well Thermocycler (Thermo Fischer Scientific, Waltham, MA, USA).
The conditions for the first and second PCR were previously described. The amplicons of the second PCR were digested with 1 µL of endonuclease *Apo*INEB and its 10X buffer NEBuffer 3.1 in a reaction volume of 50 µL then incubated at 50°C for 1 hour. The DNA bands were revealed by electrophoresis (kuroGEL Midi 13 VWR®) on agarose gel prepared at 1.5%, containing 3 µL of ethidium bromide and visualized under UV light.

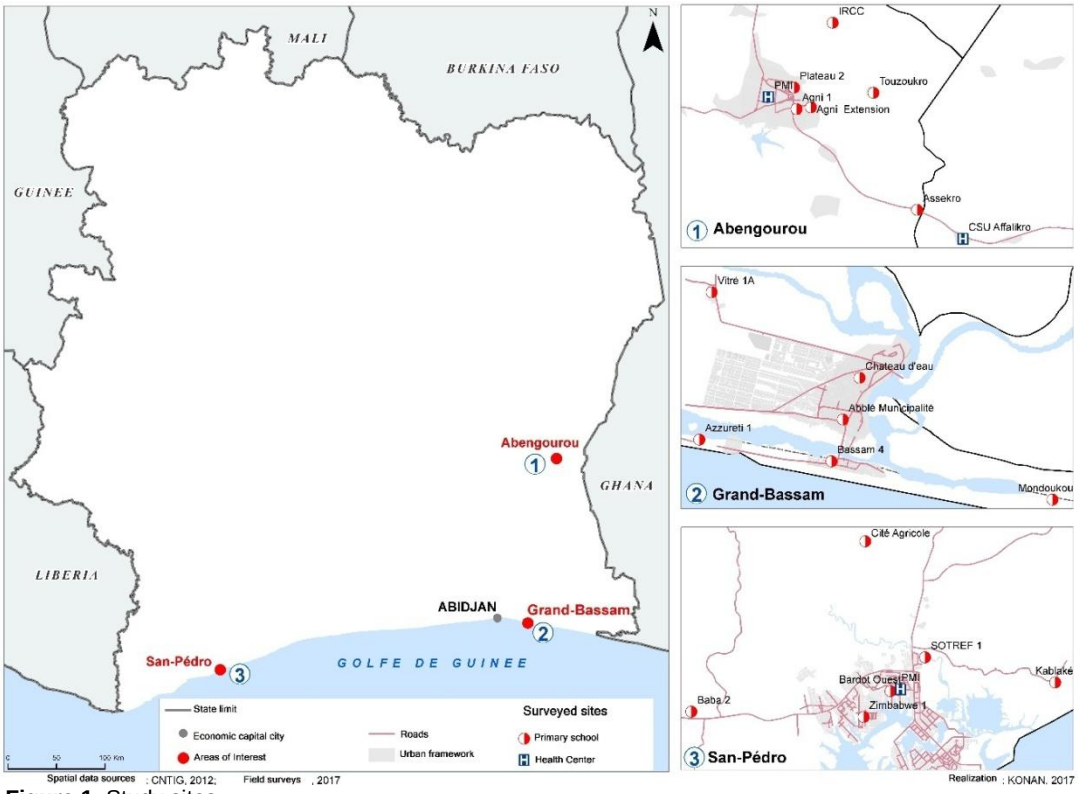

Figure 1. Study sites.

Table 1.Nucleotide sequences of primers.

Step	Primers	Nucleotide sequences	products size(bp)
First PCR	*pfcrt* 76 forward 76-A	5'GCGCGCGCATGGCTCACGTTTAGGTGGAG3'	206
	*pfcrt*76 reverse 76-B	5'GGGCCCGGCGGATGTTACAAAACTATAGTTACC3'	
Second PCR	*pfcrt* 76 forward 76-D1	5'TGTGCTCATGTGTTTAAACTT3'	145
	pfcrt 76 reverse 76-D2	5'CAAAACTATAGTTACCAATTTTG3'	

The K76T mutation results in the loss of an *ApoI* recognition site so that the mutated DNA samples (76T) remain undigested (145bp) while those in which the mutation is absent produce two fragments of 98 and 47bp. DNA sequencing of undigested samples was used to confirm the results of the PCR-RFLP.

Ethics

This study was approved by the National Committee for Research Ethics (CNER) under number 020 / MSLS / CNER-dkn. It was conducted in accordance with the Helsinki declaration adopted by the 18th World Medical Assembly in 1964 and its amendments, the ICH recommendations for clinical studies and the national laws and regulations of Côte d'Ivoire.

Statistical analysis

The data was entered into the Epidata Version 3.1 software and analyzed by GraphPad Prism 5 for windows (version 5.01, August 7, 2007). Differences between wild and mutant alleles were assessed using Fischer's exact test or Exact chi-square test. The level of significance for the statistical tests was set at the threshold α equal to 0.05. The nucleotide sequences were analyzed with BioEdit Sequence Alignment Editor (version 7.2.6

Table 2. Demographic and parasitological characteristics of the study population.

		Values n (%)	Rainy season n (%)	Dry season n (%)	Abengourou n (%)	Grand-Bassam n (%)	San-Pedro n (%)
Sex							
Male		1143(48,4)	575	568	404	370	369
Female		1218(51,6)	615	603	378	417	423
Age (years)							
Mean (SD)	9,3(2,5)						
Min-Max	4-16						
< 5		126(5,3)	75(6,3)	51(4,4)	48 (6,1)	48 (6,1)	30 (3,8)
[6-10]		1439(60,9)	720(60,5)	719(61,4)	495 (63,3)	488 (62)	456 (57,6)
>10		796(33,7)	395(33,2)	401(34,2)	239 (30,6)	251(31,9)	306 (38,6)
Parasitemia (trophozoite/µL)							
Mean (SD)	635,8(2911,6)						
Min-Max	15-79520						
<10 000		2339 (99,1)	1179 (99,1)	1160 (99,1)	770 (98,5)	786 (99,9)	783 (98,9)
>10 000		22(0,9)	11 (0,9)	11 (0,9)	12(1,5)	1(0,1)	9 (1,1)
Rate of Plasmodium infection		943(40)	451(37,9)	492(42)	139(17,7)	393(50,3)	411 (51,9)
***Plasmodium* species rate**							
P. falciparum		902(95,7)	419(93)	483(98,2)	135(97,1)	366(93,1)	401(97,6)
P. malariae		1(0,1)	0	1(0,2)	1(0,7)	0	0
P. ovale		1(0,1)	0	1(0,2)	1(0,7)	0	0
P. falciparum + P. malaria		34(3,6)	28(6,2)	6(1,2)	2(1,5)	25(6,4)	7(1,7)
P. falciparum + P. ovale		3(0,3)	2(0,4)	1(0,2)	0	0	3(0,7)
P. falciparum + P. malaria + P. ovale		2(0,2)	2(0,4)	0	0	2(0,5)	0

(3/31/2017)).

RESULTS

The overall prevalence of asymptomatic *Plasmodium* carriers was 24.1% (n = 569/2361) and 17.4% for symptomatic malaria. Table 2 shows demographic and parasitological characteristics of recruited subjects. Average age of the children was 9.3 years (SD =2.5). We randomized 15% or 360 samples (244 asymptomatic subjects and 116 symptomatic subjects) of blood for the implementation of Nested PCR. The genotyping of *pfcrt* was carried out for 215 positive PCR products (59.7%). There was a significant difference between the prevalence of the *pfcrt*-76T allele in the rainy season (76.5%) compared to that observed in the dry season (23.5%), p = 0.0354 (Table 3). The prevalence of the *pfcrt*-76T allele was 17% and 15% for asymptomatic and

Table 3. Distribution of alleles according to the seasons.

	Rainy season	Dry season	p-value*
Wild type K76, n(%)	101(79.5)	80 (90.9)	
Mutant type 76T, n(%)	26(20.5)	8(9.1)	
Total	127	88	0.0354

*p-value based on Fisher testor Exact chi-squaretest.

Table 4. Distribution of alleles according to the subject's clinical status

	Asymptomatic	Symptomatic	p-value*
Wild type K76, n(%)	99(83.2)	82 (85.4)	
Mutant type76T, n(%)	20(16.8)	14 (14.6)	0.7098
Total	119	96	

*Fisher testor Exact chi-squaretest.

Table 5. Distribution of alleles by study site.

Study site	*pfcrt* Wild type K76, n (%)	*pfcrt* Mutant type 76T, n (%)	p-value*
Abengourou	67(80.7)	16(19.3)	
Grand-Bassam	30(73.2)	11(26.8)	
San-Pedro	81(89)	10(11)	0.0678
Total	178	37	

*Fisher testor Exact chi-squaretest

symptomatic patients, respectively, giving an overall prevalence of 15.8% (Table 4). The prevalence of the *pfcrt*-76T allele was 19.3, 26.8 and 11 for Abengourou, Grand-Bassam and San-Pedro. No statistically significant difference was observed (Table 5).

DISCUSSION

Chloroquine as, a previous highly efficacious and affordable antimalarial agent, suffered widespread loss of efficacy in most malaria-endemic countries globally. The high resistance to chloroquine and its subsequent withdrawal from use across most malaria-endemic countries was responsible for more than doubling of malaria-associated mortality especially in Sub-Saharan Africa (Ocan et al., 2018). In Côte d'Ivoire, Between its replacement in 2003 by AQ and its official withdrawal in 2007, studies have reported prevalence rates of CQ resistance from 56% to 100% (Ako et al., 2012; Bla et al., 2014; Diawara et al., 1996; Ouattarar et al., 2010). This situation may be explained by the fact that CQ was still available on the market and also by self-medication which was becoming more common as it was accessible to low-income families (Afoakwah et al., 2014; Granado et al., 2011).

Nine years later, the present study is the first one for the molecular monitoring of chloroquine resistance in Côte d'Ivoire with parasites collected from asymptomatic and symptomatic children. Overall, there is a significant decrease in the prevalence of isolates carrying the *pfcrt* 76T allele.

Thus, if the new policy of uncomplicated malaria treatment through the use of ACTs is followed correctly, a level of *P. falciparum* sensitivity to CQ could be led in

Côte d'Ivoire. This is consistent with previous studies that have shown a decline in the prevalence of the *pfcrt*76T allele in some African countries, such as Mali, Kenya, Uganda, Senegal, Nigeria, Burkina Faso, Zanzibar and Benin (Balogun et al., 2016; Conrad et al., 2014; Frosch et al., 2014; Kiarie et al., 2015; Morris et al., 2015; Ndiaye et al., 2012; Ogouyèmi-Hounto et al., 2013; Sondo et al., 2015; Tukwasibwe et al., 2017). However, to confirm this tendance a large scale study at national level is needed. This could combine both the molecular methods and the in vitro chemosensitivty test.

In Malawi, after 13 years of change in malaria treatment with Sulfadoxine-pyrimethamine since 1993, chloroquine-sensitivity was found at 100% (Frosch et al., 2014; Kublin et al., 2003; Laufer and Plowe, 2004). In Ghana, the total prevalence of pfcrt 76T was 8% in Accra, Kintampo and Navrongo in 2017 (Abugri et al., 2018). Before the change in use of CQ to ACTs in 2005, a prevalence range of 46% to 98% of mutant pfcrt 76T was reported at five sentinel sites (Duah et al., 2007). In Zambia, chloroquine was used as a first-line treatment for uncomplicated malaria until treatment failures led the Ministry of Health to replace it with artemether-lumefantrine in 2003. Ten years later chloroquine sensitivity has been recovered (Mwanza et al., 2016). In Mozambique, the prevalence of *pfcrt*K76 increased from less than 5% to 80% in just 5 years (Thomsen et al., 2013).

Our data also show that chloroquine resistance is unrelated to the clinical status of the subject, unlikely to what has already been demonstrated in Ghana (Afoakwah et al., 2014). However, in Uganda, prevalences of close chloroquine-resistant plasmodial isolates have been reported in symptomatic (98.77%) and asymptomatic (88.07%) cases. In our settings, the prevalence of the mutant allele was significantly different between the rainy season and the dry season. These results are similar to those found by other authors (Jovel et al., 2015; Ord et al., 2007). Indeed, the prevalence of the resistant allele would increase with the consumption of antimalarials which is more important in the rainy season during the high transmission of malaria in this period. On the other hand, the geographical area had no influence on the prevalence of the 76T mutant allele of *P. falciparum* during our study. This suggests a quasi-equivalent selection pressure in both urban and rural areas.

CONCLUSION

The low prevalence of the 76T allele may support the return of CQ in the treatment of malaria in combination with another antimalarial drug. This study also shows that asymptomatic carriers of *P .falciparum* are a reservoir of *P. falciparum* isolates potentially resistant to CQ. This profile should be taken into account in the strategies against malaria because of the use of ACTs as first line malaria treatment containing amodiaquine, a closely molecule to chloroquine.

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