High Frequency of *Pfcr*-76T Allele in *Plasmodium falciparum* Isolates From Gabon and Ivory Coast After the Withdrawal of Chloroquine

Fréquence élevée de l'allèle *Pfcr*-76T dans les isolats de *Plasmodium falciparum* provenant du Gabon et de la Côte d'Ivoire après le retrait de la chloroquine

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Abstract The aim of this study was to assess the proportion of *Pfcr*-76T mutant allele four years after the adoption of new malaria control guidelines in Gabon and Ivory Coast. Frequency of K76T mutation of *Pfcr* gene was compared between *Plasmodium falciparum* isolates from Gabon and Ivory Coast. Samples were collected in 2008 in Gabon and in 2009 in Ivory Coast. In total, 151 isolates were selected and analysed by nested-PCR-RFLP for *Pfcr*-76 allele identification: 63 in Abobo (Ivory Coast) and 78 in Oyem (Gabon). The proportion of *Pfcr*-76T mutant allele was higher in Oyem (70%) compared to Abobo (46%) ($p=0.005$). This allele was more frequently detected in patients less than 5 years old in Oyem (75 %) compared to Abobo (52%) ($p<0.01$). The present work showed different prevalence of *Pfcr*-76T allele between both sites probably due to a different drug pressure on *P. falciparum* strains circulating in these areas. Moreover, a decrease of the mutation frequency is observed compared to data obtained in 2005. Therefore, a continuous monitoring of this allele and other drug resistance molecular markers is required in these countries.

Keywords Malaria · *Plasmodium falciparum* · Resistance · Chloroquine · *Pfcr* gene · Ivory Coast · Gabon · Sub-Saharan Africa

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Résumé Le but de cette étude était d'évaluer la proportion de l'allèle mutant *Pfcr*-76T quatre ans après l'adoption de nouvelles stratégies de contrôle du paludisme au Gabon et en Côte d'Ivoire. La fréquence de la mutation K76T du gène *Pfcr* a été comparée entre les isolats de *P. falciparum* du Gabon et de Côte d'Ivoire. Des échantillons ont été prélevés en 2008 au Gabon et en 2009 en Côte d'Ivoire. Au total, 151 isolats ont été sélectionnés et analysés par PCR-nichée-RFLP pour l'identification de l'allèle *Pfcr*-76: 63 à Abobo (Côte d'Ivoire) et 78 à Oyem (Gabon). La proportion de l'allèle mutant *Pfcr*-76T était plus élevée à Oyem (70 %) comparée à Abobo (46 %) ($p = 0,005$). Cet allèle était plus fréquemment détecté chez les patients de moins de 5 ans à Oyem (75 %) comparé à Abobo (52 %) ($p < 0,01$). Le présent travail a montré une prévalence différente de l'allèle *Pfcr*-76T entre les deux sites, probablement en raison d'une pression médicamenteuse différente sur les souches de *P. falciparum* circulant dans ces zones. Une diminution de la fréquence de cette mutation est observée par rapport aux résultats de 2005. Par conséquent, un suivi continu de cet allèle et d'autres marqueurs moléculaires de la résistance aux antipaludiques est recommandé dans ces pays.

Mots clés Paludisme · *Plasmodium falciparum* · Résistance · Chloroquine · Gène *Pfcr* · Côte d'Ivoire · Gabon · Afrique intertropicale

Background

Chloroquine (CQ) was a highly effective antimalarial drug during decades in malaria endemic areas. It has conducted to a reduction of malaria morbidity [9]. However, the emergence of CQ resistant *Plasmodium falciparum* (*P. falciparum*) strains, in the 1950's in Southeast Asia and Latina America and in the 1980's in Africa, conducted to the abandonment of

this drug. CQ therapeutic failures have been attributed to parasites carrying mutant forms of *Pfcr* gene [8].

The substitution of lysine (K) by tyrosine (T) at codon 76 of the PfCRT protein was linked to *P. falciparum* resistance to CQ [2]. This mutation was found in all samples collected in 2005 in Ivory Coast and in those collected between 1995 and 2002 in Gabon [1,3]. In the beginning of 2000's most of malaria endemic countries have replaced CQ by artemisinin-based combination therapies (ACTs) [9]. Since 2005, in Ivory Coast and Gabon, artesunate-amodiaquine combination was used as first-line treatment and artemether-lumefantrine as second line for the treatment of uncomplicated malaria. Nine years after, both combinations are used as first-line treatment, artesunate-mefloquine or dihydroartemisin-phosphate piperaquine being the second line treatment. Many surveys reported that withdrawal of CQ is followed by a significant decrease of *Pfcr*-76T frequency [5]. The aim of this study was to compare *Pfcr*-76T allele distribution in *P. falciparum* isolates collected in Abobo (Ivory Coast) and Oyem (Gabon) after withdrawal of CQ and the deployment of ACTs in these countries.

Material and Methods

Cross-sectional studies were conducted from September 2008 to February 2009 in General Hospital of Abobo and from February 2008 to January 2009 in the Regional Hospital of Oyem (RHO) in the north of Gabon.

Malaria transmission is perennial in both sites. In Oyem and in Abobo, malaria prevalence was 39% and 61.9%, respectively at the time of samples collection. RHO is one of the five sentinel sites selected for malaria survey by the Malaria National Control Programme (MNCP) in Gabon. The General Hospital of Abobo is a study site selected by the team of Malaria Research and Control Center of the National Institute of Health of Abidjan, to assess the prevalence of malaria among febrile patients.

Peripheral blood samples were collected from febrile children aged less than 11 years in Oyem and from patients aged less than 54 years in Abobo. The oral consent of adults patients and of the parents or legal guardians of children was obtained.

Age, sex, temperature, and others demographic data were recorded.

Blood samples were collected for malaria parasite diagnosis based on microscopic examination of Giemsa-stained thick film in both sites. Moreover, approximately, 50 µL of blood were spotted onto filter papers (WatmanTM). Each was dried and stored with two desiccants in plastic bags at room temperature (20°C) until DNA extraction. Before molecular analysis, 10% of *P. falciparum* infected samples were randomly selected. DNA was extracted from dried blood spot

(DBS) using Chelex 100 at 5% according to manufacturer's instructions (Bio-Rad, Laboratories, Hercules, CA). Nested-Polymerase Chain Reaction Restriction Fragment Length Polymerase (Nested PCR-RFLP) was used for mutant (*Pfcr*-76T) and wild-type (*Pfcr*-76K) allele's detection [8]. The amplicons and digested products were revealed by electrophoresis on 2% agarose gel. The transilluminator (UV) was used for the visualization of fragments.

Molecular analysis performed on samples collected in 2008 in Gabon is described elsewhere [7]. Similar methods have been used for all selected samples.

Data were analysed using Epi InfoTM7. All variables were compared using Chi 2 or Fisher test exact to compare the proportion. P-value <0.05 was considered statistically significant. Mixed infection concerned the isolates carrying wild type and mutant type alleles at codon 76.

Ethical Consideration

The study performed under the direction of Ministry of Health of Ivory Coast was approved by the National Ethics Committee. In Gabon, the Department of Parasitology-Mycology and Tropical Medicine of the Faculty of Medicine of Libreville is committed by the Gabonese Ministry of Health represented by MNCP, to perform the evaluation of malaria prevalence and antimalarial drug resistance monitoring throughout the country.

Results

A total of 689 patients from Oyem and 634 patients from Abobo were screened. Among these patients, 63 have been selected in Abobo and 78 in Oyem. The median age of screened patients from Oyem is 2 [1-4] years old and that of selected patients 2 [1.8-4] years old were comparable ($p=0.1$). In this site, the frequency of female patients was also similar between screened patients and selected ones: 46.4% (320/689) vs 54% (42/78) respectively ($p=0.2$).

In Abobo, the median age of screened patients, 6 [5.4-7] years old, was comparable to that of selected patients (6 [5-7] years old) ($p=0.2$). The frequency of female patients was lower in the group of screened patients: 24.7% (157/634) vs 51% (27/63) in selected group ($p=0.003$).

Among selected patients ($n=141$) female represented 48.9% ($n=69/141$) of the patients.

Patients aged more than 10 years old ($n=15$) were all from Abobo. The proportion of children less than 5 years old was 61.7% ($n=87/141$). They were 76.9% ($n=60/78$) in Oyem and 42.8% ($n=27/63$) in Abobo ($p<0.01$). Among patients, 27.7% ($n=39/141$) were older children ([5-10] years). They

were 33.3% (n=21/63) in Abobo and 23.1% (n=18/78) in Oyem ($p=0.2$).

The median parasite density was 19280 [19000-20400] p/μL; and was 1.6 fold higher in patients from Oyem: 21 618 [21000-22360] p/μL vs 13400 [12360-16000] p/μL in Abobo.

Overall, *Pfcr*t-76T mutant allele was detected in 59.5% (n=84/141) of the isolates (Table I). This proportion was significantly higher in Oyem compared to Abobo: 70% (n=55/78) vs 4% (n=30/63), respectively ($p<0.01$) (Table I). None isolate carried a mixed infection in Oyem while three were found in Abobo (Table 1). The median age of patients infected by mutant parasites was 3 [2-4] years comparable to that of patients harbouring wild type parasites: 4 [3-5] years. The proportion of K76T mutation was comparable between the patients less than 5 years old (63%; n=55/87) and patients aged between 5 and 10 years (54%; n=21/39) ($p=0.4$). In Oyem, the frequency of mutant allele was significantly different according to age being the highest among the youngest patients: 75% vs 55% ($p=0.02$). In Abobo, the proportion of this allele was similar between both groups: 52% vs 52% ($p=0.6$) (Table 2). Comparing patients less than five years old from both sites, those infected by mutant parasites (*Pfcr*t-76T allele) were less frequent in Abobo (52%; n=14/27) than those from Oyem (75%; n=45/60) ($p<0.01$) (Table II). In contrast, the propor-

tion of mutant isolates was comparable in patients aged between 5 and 10 years from Oyem (n=10/18) and from Abobo (n=11/21) ($p=0.5$) (Table II). Among adult patients in Abobo; 8/15 carried mutant alleles and one patient harboured a mixed infection.

Discussion

Monitoring the occurrence of malaria parasite resistance to antimalarial drugs and its spread is essential in all malaria-endemic areas notably after introduction of new antimalarial drugs. For this purpose, molecular markers are good tools. Prior to the change of the national policy of malaria treatment, the prevalence of the mutant *Pfcr*t allele 76T was around 97% in the centre of Gabon and 100% at Bonoua, at 59 km from Abobo in Ivory Coast in 2005 [1,3]. In the present work, lower prevalence of mutant alleles (*Pfcr*t-76T) is found in Abobo (48 %) and in Oyem Gabon (70%) 3 to 4 years after CQ withdrawal. These data corroborate with those reported in Congo (52.1%) and Sierra Leone (54.0%) where a decreasing frequency of mutant allele was observed [10]. Nevertheless, the decline of *Pfcr*t-76T allele frequency was different according to each endemic area. This decline is lower in Abobo than in Oyem suggesting a different antimalarial drug pressure between sites. It has also been reported

Table 1 Comparison of prevalence of the mutation K76T of *Pfcr*t gene at Oyem and Abobo according to sites / *Comparison de la prévalence de la mutation K76T du gène Pfcr*t à Oyem et Abobo en fonction des sites

<i>Pfcr</i> t K76T	Oyem-2008 n=78	Abobo-2009 n=63	P	OR (95% CI)	All N=141
Mutant single 76T, n (%)	55 (70.5)	27 (42.8)	<0.01	3.40 (1.6-6.8)	57.4 (81)
Wild single 76K, n (%)	23 (29.5)	33 (52.3)	<0.01	0.38 (0.1-0.7)	39.7 (56)
MI** 76K/T, n (%)	0 (0.0)	3 (4.7)	-	-	2.1 (3)
Total mutant 76T, n (%)	55 (70.5)	30 (47.6)	0.005	2.8 (1.3-5.6)	59.5 (84)
Total wild 76K, n (%)	23 (29.5)	36 (57.1)	<0.01	0.3 (0.1-0.6)	41.8 (59)

** MI (mixed infection): isolate carrier mutant 76T and wild 76K allele's type / *IM (Infection Mixte)* : isolat porteur d'allèle mutant 76T et d'allèle sauvage 76K

Table 2 Proportion of *Pfcr*t-76T and *Pfcr*t-76K alleles according to age and sites / *Proportion des allèles Pfcr*t-76T et *Pfcr*t-76K en fonction de l'âge et des sites

	Mutant	Wild	p	OR (95% CI)	MI
Median age (years)	3 [2.5-3.5]	4 [3-5]	-	-	3 [1-28]
Abobo-2009					
<5 years, % (n)	51.8 (14/27)	62.9 (17/27)	0.5	0.6 (0.2 -1.8)	7.4 (2/27)
≥5 years, % (n)*	52.3 (11/21)	47.6 (10/21)	0.5	1.2 (0.3 - 4.0)	0.0
Oyem-2008					
<5 years, % (n)	75.0 (45/60)	25.0 (15/60)	<0.001	9.0 (3.9 - 20.5)	0.0
≥5 years, % (n)	55.5 (10/18)	44.4 (8/18)	0.1	1.5 (0.4 - 5.8)	0.0

*This group concerned only the children aged from 5 to 10 years / *Ce groupe concernait seulement les enfants âgés de 5 à 10 ans*

that CQ resistance has a fitness cost in terms of parasite reproduction; thus sensitive parasites have a survival advantage in the absence of drug pressure [4].

Otherwise, antimalarial drug for self-medication that constitutes a key component of antimalarial drug resistance development and spread, is frequent in Oyem and may explain the slow decrease of mutant allele frequency in this area [10]. The prevalence of self-medication was near 30% in Oyem and lower in Abobo, Ivory Coast, 11.1% [6]. Moreover, although CQ has been withdrawn, other 4-aminoquinoleines such as amodiaquine are still used in combination with artemisinin derivatives. ACTs and other antimalarial drugs are frequently sold in pharmacies for self-medication use.

According to age, a predominance of *Pfcr*-76T allele was observed in isolates from patients less than 5 years old in urban (100%) and rural areas (86.2%) in 2005 in Ivory Coast [1]. In the present study, the prevalence of this allele (*Pfcr*-76T) is still high in this group, notably in Oyem. *Pfcr*-76T allele was detected in a study carried out in Angola in more than 80% of asymptomatic adults [2]. In the present study, this allele was found in 54% of adults.

All codons of *Pfcr* gene related to antimalarial drug resistance have not been analysed in this study and this may constitute the main limit of this investigation. Therefore, the detection of additional mutations in this gene may be of importance in isolates from Gabon and Ivory Coast [8].

Conclusion

This study shows a different distribution of *Pfcr*-76T allele between Abobo (Ivory Coast) and Oyem (Gabon) after withdrawal of chloroquine. Moreover, a relation between the prevalence of the K76T mutation and the age was found in Oyem. The high frequency of this mutation underlines the importance of monitoring *P. falciparum* strains genetic profile to protect the antimalarial molecule partners used with artemisinin.

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Conflict of interest : The authors do not have any conflict of interest to declare

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