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# Re-Emerging Chloroquine Sensitivity of *Plasmodium falciparum* in Côte d'Ivoire: Analysis of *pfcrt* Gene Polymorphism in Two Health Districts

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## ABSTRACT

**Background and Objective:** Malaria is one of the main causes of morbidity and mortality in the world, particularly in endemic areas. Its management is hampered by the resistance of *Plasmodium falciparum* to almost all anti-malarial drugs. Mutations in the *pfcrt* (*Plasmodium falciparum* Chloroquine Resistance Transporter) gene are associated with *Plasmodium falciparum* resistance to chloroquine (CQ), and more specifically, mutations at loci 72 to 76 located in the second exon of the gene. This study aims to explore the return of *Plasmodium falciparum*'s sensitivity to chloroquine in two health districts of Southern Côte d'Ivoire through *pfcrt* gene polymorphisms. **Materials and Methods:** After obtaining informed consent, blood samples were collected from patients of all sexes and ages with uncomplicated *P. falciparum* malaria at Dabou General Hospital and Anonkoua-Kouté Health Centre, located in the South of Côte d'Ivoire, in the Dabou and Abidjan 1 health districts, respectively. *Plamodium falciparum* genomic DNA was extracted and amplified by nested-PCR using primers specific for the *pfcrt* gene. Amplification products were sequenced using the Sanger method at Eurofins Genomics. Key codons (72, 73, 74, 75, and 76), molecular markers of *P. falciparum* resistance to chloroquine, were analyzed. Data were analyzed in R using the $\chi^2$ test to compare *pfcrt* allele and genotype prevalences, with significance set at $p<0.05$. **Results:** A total of 112 out of 140 DNA fragments (80%) were successfully sequenced from two sites in Southern Côte d'Ivoire. Analysis of *pfcrt* codons 72-76 revealed high prevalences of wild-type alleles, with the CVMNK haplotype (chloroquine-sensitive) present in 78.6% of samples. Mutant alleles were rare, and no significant differences were found between the two sites ($p>0.05$). **Conclusion:** Nearly twenty years after chloroquine was abandoned as a treatment for uncomplicated malaria in Côte d'Ivoire, the proportion of parasites sensitive to this antimalarial appears to be increasing in the south of the country, particularly in the health districts of Dabou and Abidjan 1.

## KEYWORDS

*Plasmodium falciparum*, chloroquine, sensitivity, *pfcrt*, polymorphism, Côte d'Ivoire



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## INTRODUCTION

Malaria is a global public health problem. In 2022, 247 mL cases were recorded, 95% of them in Africa, and the number of deaths was 619,000, 96% of them in children under the age of 5[1]. *Plasmodium falciparum* is responsible for serious and potentially fatal cases[2]. Early diagnosis, accompanied by effective medical follow-up, is one of the ways of combating the disease[3]. However, the management of malaria is made difficult by the resistance of *P. falciparum* to most antimalarial drugs, including artemisinin-based combination therapies (ACTs)[4].

Faced with the emergence of ACT-resistant *Plasmodium falciparum*, several studies have suggested a return to the use of chloroquine, the first antimalarial drug used as first-line treatment for acute uncomplicated *P. falciparum*, until reports of chloroquine resistance led to its withdrawal. Indeed, it has been suggested that an effective and sustained withdrawal of chloroquine could lead to the re-emergence of chloroquine-sensitive *P. falciparum*[5]. Mutations in the *Plasmodium. falciparum* Chloroquine Resistance Transporter (*pfcrt*) at several codons, including codon 76, have been identified as being linked to chloroquine resistance in *P. falciparum*. The study of these molecular markers is therefore essential for monitoring the emergence, spread, and evolution of antimalarial drug resistance[6].

In Côte d'Ivoire, studies have shown a high prevalence of the wild strain haplotype (CVMNK) of the *pfcrt* gene in various sentinel sites, giving rise to the possibility of sensitivity to chloroquine in the treatment of uncomplicated malaria[7]. This study aimed to investigate the prevalence of the K76T mutation in the health districts of Dabou and Abidjan 1, twenty years after the official withdrawal of chloroquine for the treatment of uncomplicated malaria in Côte d'Ivoire.

## MATERIALS AND METHODS

**Study site:** This prospective study was conducted from May to August, 2024 at the Dabou General Hospital and the Anonkoua-Kouté Health Centre, located in the Dabou and Abidjan 1 health districts, respectively. These two districts are located in the South of Côte d'Ivoire, where the climate is equatorial, with annual rainfall in excess of 1,700 mm and temperatures ranging from 27 to 33°C[8]. Malaria is seasonal, predominating during the rainy season from June to September, with peaks in prevalence in October-November. According to the Multiple Indicator Cluster Survey (MICS) carried out by the Côte d'Ivoire Ministry of Planning and Development, *P. falciparum* is dominant, for more than 90% of malaria parasites identifie[9,10]. The main malaria vectors in this study area (the forested south of Côte d'Ivoire) are members of the *An. Gambiae s.l.* and *An. Funestus s.l.* complexes[10]. The Anonkoua-kouté health centre (Abidjan 1 health district) and the Dabou general hospital (Dabou health district) were selected on the basis of their geographical location and a high annual incidence of malaria cases[10].

**Study population and sample collection:** The study population consisted of subjects of any sex and age suffering from uncomplicated *P. falciparum* malaria. From each patient diagnosed as a malaria carrier by microscopic examination, approximately 2-5 mL of venous blood was collected in an EDTA tube. Blood spots were produced by depositing approximately 50 µL of whole blood onto Whatman 3 MM paper discs using a micropipette with filter cones. The papers containing the blood spots (confetti) were dried for approximately 60 to 120 min at room temperature in a dust-free environment. The unused blood in the EDTA tube was stored in microtubes at -20°C for possible future use.

**Extraction of *Plasmodium falciparum* DNA:** *Plasmodium falciparum* genomic DNA was extracted from blood confetti using the QIAamp DNA Blood kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions. Samples were eluted in a volume of 60 µL with nuclease-free water and stored at -20°C for future use.

**PCR amplification of the *pfcrt* gene:** Exons 2, 3, and part of exon 4 covering positions 72 to 76, 93, 97, 101, 145, 146, 158, and 159 of the *pfcrt* gene (PF3D7_0709000) were amplified by conventional PCR using the primer pairs: Forward: 5'-GGT AAA TGT GCT CAT GTG TTT AAA CTT ATT-3' and Reverse: 5'-TTA CTT TTG AAT TTC CCT TTT TAT TTC CA-3. The PCR reaction mixture contained 5 µL of DNA, 10 µM of each primer, 5 µL of 5X HotFirepol MasterMix enzyme consisting of 10X buffer, 25 mM $MgCl_2$, 5 mM dNTP and stabilised polymerase, and 13 µL of molecular biology water in a final volume of 25 µL. PCR was performed in the Mastercycler type PTC100TM thermal cycler (Eppendorf Mastercycler, PTC-100 Peltier Thermal Cycler) using the following amplification programme: 95°C for 5 min, 31 cycles of 95°C for 15 sec, 60°C hybridisation temperature for 2 min, polymerisation at 72°C for 2 min and a final extension step of 10 min at 72°C. The amplification products were migrated onto a 2% agarose gel containing diluted SybrSafe (1/1000). The gel was recovered and observed under a UV lamp using the UV transluminator (Gel DocTM EZ Imager). The presence or absence of bands at 241 bp was used to judge PCR efficiency.

**Sequencing of the *pfcrt* gene:** To cover mutations, positive samples were purified using the Charge Switch®-Pro PCR kit (Invitrogen) according to the manufacturer's protocol and sequenced in the forward direction. The 20 µL of each purified PCR product was packaged in a 1.5 mL microtube, and 5 pmol/µL of forward primer was submitted to the Beijing Genomics Institute (BGI) genomics platform in China for Sanger sequencing. After the sequencing reaction, the DNA sequences obtained were recovered in FASTA and ABI formats. The ABI sequences were used for cleaning using MEGA11: Molecular Evolutionary Genetics Analysis version 11[11]. Next, the sequences were aligned using BioEdit 7.2.5 software under the default ClustalW program with the reference sequence of the *pfcrt* gene (PF3D7_0709000.1) available on PlasmoDB. In the wild-type strain, the positions of interest inducing chloroquine resistance are codons Cys-72, Val-73, Met-74, Asn-75, and Lys-76, corresponding to the CVMNK wild-type allelic combination.

**Analysis of sequencing data and identification of mutations:** After the sequencing reaction, the DNA sequences were recovered as FASTA files. The sequences were cleaned up and aligned using BioEdit 7.2.5 under the default Clustal program using the reference sequence of the *pfcrt* gene, PF3D7_0709000.1, available on PlasmoDB. In the wild-type strain, the positions of interest inducing chloroquine resistance are codons Cys-72, Val-73, Met-74, Asn-75, and Lys-76, corresponding to the CVMNK wild-type allelic combination. The loci of interest, i.e., codons at positions 72, 73, 74, 75, and 76 of the *pfcrt* polypeptide or nucleotides at positions 216, 219, 222, 225, and 228 of the *pfcrt* gene, were searched for.

**Statistical analysis:** Data were organized using Microsoft Excel 2016. Then, the $\chi^2$ test of the R software was used to compare the prevalences of individual alleles and genotypes of the *pfcrt* gene of the study sites[12]. A statistical difference and/or association was considered significant if p of the $\chi^2$ test <0.05.

## RESULTS

**Patient profile and sequencing results:** A total of 140 patients with uncomplicated *Plasmodium falciparum* malaria were included in the study, 94 (67.14%) of whom were female and 46 (32.86%) male. The age of the patients ranged from 2 to 59 years, with a mean age in Anonkoua-Kouté and Dabou of 21±17 and 11±7.8 years, respectively. A total of 140 blood samples were collected at the two sites, with one sample per person (Table 1).

Of the 140 samples collected at the 2 study sites, 80 (80/140, i.e., 57.14%) samples came from patients at the Anonkoua Kouté site and 60 (60/140, i.e., 42.86%) from the Dabou General Hospital site.

Of the 140 DNA fragments sent for sequencing, 112 (112/140; i.e., 80%) were successfully sequenced, including 60 (60/80; i.e., 75%) from Anonkoua Kouté and 52 (52/60; i.e., 86.67%) from Dabou General Hospital (Table 1).

Table 1: Samples used for molecular analysis and sequencing results

| Sites | Collection period | Age groups (years) | Average age (years) | Confetti collected | Fragments successfully sequenced n (%) |
|---|---|---|---|---|---|
| Anonkoua-Kouté | May to June, 2024 | 4 to 59 | 21±17 | 40 | 30 (75) |
| Dabou | July to August, 2024 | 2 to 32 | 11±7.8 | 30 | 26 (86.7) |
| Total | | | | 70 | 56 (80) |

Table 2: Prevalence of the individual SNP of the *pfcrt* gene in the study sites

| Codons | Alleles | Anonkoua Kouté (N = 60) | Dabou (N = 52) | p-test $\chi^2$ | All sites (N = 112) |
|---|---|---|---|---|---|
| CRT-72 | Cys* | 60 (100%) | 52 (100%) | - | 112 (100%) |
| | Val* | 56 (93.3%) | 52 (100%) | 0,288 | 106 (94.6%) |
| CRT-73 | Glu | 4 (6.7%) | 0 (0%) | 0.5361 | 4 (3.6%) |
| | Cys | 2 (3.3%) | 0 (0%) | 1 | 2 (1.7%) |
| | Met* | 48 (80%) | 46 (88.5%) | 0.6206 | 94 (84%) |
| CRT-74 | Island | 10 (17.7%) | 6 (11.5%) | 0.8697 | 16 (14.3%) |
| | Arg | 2 (3.3%) | 0 (0%) | 1 | 2 (1.8%) |
| | Asn* | 50 (83.3%) | 44 (84.6%) | 1 | 94 (84%) |
| CRT-75 | Glu | 10 (17.7%) | 6 (11.5%) | 0.8697 | 16 (14.3%) |
| | Lilies | 0 (0%) | 2 (3.85%) | 0.9424 | 2 (1.8%) |
| CRT-76 | Lys* | 50 (83.3%) | 46 (88.5%) | 0.8697 | 96 (85.7%) |
| | Thr | 10 (17.7%) | 3 (11.5%) | 0,8697 | 16 (14.3%) |

N: Represents the total number of isolates successfully sequenced and *Refers to the amino acid in the wild-type strain PF3D7-CRT

Table 3: Prevalence of haplotypes in the study sites

| | Prevalences by site | | | |
|---|---|---|---|---|
| Phenotypes | Anonkoua kouté (N = 60) n (%) | Dabou (N = 52) n (%) | p-test $\chi^2$ | All sites (N = 112) n (%) |
| **Sauvage** | | | | |
| CVMNK | 44 (73.3%) | 44 (84.6%) | 0.4841 | 88 (78.6%) |
| **Simple mutant** | | | | |
| CEMNK | 4 (6.67%) | 0 (0%) | 0.5361 | 4 (3.6%) |
| CVRNK | 2 (3.3%) | 0 (0%) | 1 | 2 (1.8%) |
| CVMKK | 0 (0%) | 2 (3.85%) | 0.9424 | 2 (1.8%) |
| **Triple mutant** | | | | |
| CVIET | 10 (16.7%) | 6 (11.54%) | 0.8697 | 16 (14.3%) |

N: Represents the total number of isolates successfully sequenced and N: Number of haplotypes observed

**Prevalence of individual alleles of the *pfcrt* gene and distribution of haplotypes in the study sites:** Analysis of the alleles of individuals at amino acid positions 72 to 76 showed a predominance of wild-type alleles (83 to 100%) in the 2 sites (Table 2).

The wild-type alleles Cys-72, Val-73, Met-74, Asn-75, and Lys-76 were observed at prevalences of 100, 100, 88.5, 84.6, and 88.5%, respectively, in Dabou, compared with 100, 93.3, 80, 83.3, and 83.3% in Anonkoua Kouté. No mutations were observed at codons 72 and 73 in Dabou. The Anonkoua Kouté site contained the most mutant alleles, with the highest percentage (17.7%) at codons 74, 75 and 76 (Table 2).

At codon 76, the mutation frequency was 17.7% in Anonkoua-Kouté compared with 11.5% in Dabou (Table 2). No significant difference was observed between the prevalences of individual alleles of the *pfcrt* gene determined in Anonkoua-Kouté and Dabou ($p>0.05$).

With regard to the analysis of the distribution of haplotypes of the *pfcrt* gene, the CVMNK wild-type haplotype was predominant at the 2 sites with prevalences of 73.3 and 84.6%, respectively, at Anonkoua Kouté and Dabou (Table 3). The CVIET haplotype corresponding to the triple mutant haplotype was observed at prevalences of 16.7 and 11.54% at Anonkoua Kouté and Dabou, respectively (Table 3). No significant difference was observed between the prevalences of the haplotypes (wild and mutant) of the *pfcrt* gene determined in Anonkoua-Kouté and Dabou ($p>0.05$).

## DISCUSSION

Today, one of the major challenges in the fight against malaria is the evolution of *P. falciparum* resistance to antimalarial drugs. Surveillance aimed at detecting molecular markers of drug resistance is a rapid and effective means of assessing parasite resistance in the field[13]. This study assesses the prevalence of the K76T mutation in the *Plasmodium falciparum pfcrt* gene in patients with uncomplicated malaria in the Dabou and Abidjan 1 health districts of Southern Côte d'Ivoire. Previous studies carried out in West Africa, and particularly in Côte d'Ivoire, have shown a marked correlation between the Thr-76 mutation and treatment failures on the one hand, and between the Thr-76 mutation and *in vitro* chemoresistance of *P. falciparum* isolates to chloroquine on the other[14-16].

Results indicate that no mutations were observed on codons *pfcrt* 72 and *pfcrt* 73 for the Dabou site, whereas for the Anonkoua Kouté site, only codon *pfcrt* 72 was wild-type. These results are in agreement with the work of Angélo *et al.*[7] identified wild-type alleles at these 2 codons in patient samples from Anonkoua Kouté and Bouaké[7].

With regard to codon *pfcrt* 76 of the *pfcrt* gene, our results indicate that the prevalence of the *pfcrt* K76T mutant allele was 11.5 and 17.7% in Dabou and Anonkoua Kouté, respectively. These data confirm the decline in the prevalence of K76T mutations compared with previous data reported in the country. Indeed, previous reported a progressive decrease in the *pfcrt* K76T mutant allele in Anonkoua Kouté (34.8%), Ayamé (25 to 18.5%), and Yopougon (16%)[7,17,18]. No significant difference was observed between the prevalences of this mutant allele (*pfcrt* K76T) in the two health districts. This could be explained by the fact that these districts are very close to each other (less than 100 km). Better still, the two districts are part of the greater Abidjan area, with the same malaria epidemiological facies and the same climatic conditions[19].

These observations are consistent with data reported elsewhere in Nigeria, Ethiopia, Saudi Arabia, and the DRC[14,20-22]. This increase in the prevalence of the wild *pfcrt* K76 allele suggests a re-emergence of chloroquine-sensitive strains in these two districts of Côte d'Ivoire. The use of chloroquine for the treatment of malaria has led to a high prevalence of mutant strains in Côte d'Ivoire[15,23,24]. Its replacement by artemisinin-based combination therapies in 2005 was accompanied by a drastic reduction in the prevalence of these mutant strains, with a gradual return of wild strains of *pfcrt* that are chloroquino-sensitive[7,17,18]. Indeed, the *pfcrt* K76T single-nucleotide polymorphism (SNP) is widely used as a molecular marker of chloroquine resistance, while additional variants within the *pfcrt* protein modulate resistance levels to chloroquine and other quinoline-based drugs[13]. The low frequency observed for isolates carrying the Thr-76 mutation could also be explained by several factors, including the effectiveness of the CQ abandonment policy in Côte d'Ivoire and the efficacy of artemisinin-based combination therapies.

The wild-type haplotype (CVMNK) was predominant at the 2 sites, with a prevalence of 73% at Anonkoua-Kouté and 85% at Dabou. These prevalences are higher than those reported by Angélo *et al.*[7], who reported prevalences ranging from 55 to 80% for the sites in Anonkoua-Kouté, Ayamé, Man, Bouaké, and Yamoussoukro in Côte d'Ivoire[7]. These high prevalences of the CVMNK haplotype suggest a potential increase in the number of chloroquine-susceptible parasites circulating in the two health districts (Dabou and Abidjan1). Furthermore, chloroquine-resistant strains *of P. falciparum* are characterised by CVIET (mainly in Africa) and SVMNT (Asia and South America) haplotypes covering codons 72 to 76 of the *pfcrt* gene[25]. Study results indicate that the prevalence of the CVIET haplotypes in Anonkoua-Kouté and Dabou was 17 and 12%, respectively. These prevalences are similar to those observed in other localities in Côte d'Ivoire and in Turkey[7,17,26]. However, they are higher than those observed in man in Côte d'Ivoire (5%), Congo (5.2%), Mozambique (5.2%), and Burkina Faso (5.8%)[7,27,28]. These low prevalence rates are evidence of the circulation of parasites sensitive to chloroquine, several years after the withdrawal of this antimalarial drug as a first-line treatment for uncomplicated malaria. Indeed, several studies suggest that an effective and sustained withdrawal of chloroquine could lead to the reappearance of chloroquine-sensitive *P. falciparum*[5,14].

## CONCLUSION

Twenty years after chloroquine was withdrawn as a first-line treatment for uncomplicated malaria, the prevalence of the *pfcrt* K76T mutant allele and the CVIET haplotype both linked to chloroquine resistance has significantly declined in the health districts of Abidjan 1 and Dabou. This reduction suggests a limited circulation of chloroquine-resistant *P. falciparum* strains in these areas. The findings indicate that the discontinuation of chloroquine, combined with the introduction of artemisinin-based combination therapies (ACTs), may have contributed to the resurgence of chloroquine-sensitive parasites in Southern Côte d'Ivoire, particularly in Anonkoua-Kouté and Dabou.

## ETHICAL CONSIDERATION

The study was conducted under the Declaration of Helsinki and approval was received (CNER) in Life Sciences and Health of Côte d'Ivoire. After appropriate information and explanations, the adult participants and the parents or legal guardians of all children wishing to participate in the study gave their written consent before sampling.

## SIGNIFICANCE STATEMENT

This study discovered the re-emergence of chloroquine-sensitive *Plasmodium falciparum* strains in Southern Côte d'Ivoire, marked by a high prevalence of the wild-type CVMNK haplotype in the *pfcrt* gene. This finding can be beneficial for malaria treatment strategies, particularly in areas where drug resistance had previously rendered chloroquine ineffective. By providing genetic evidence of declining chloroquine resistance, the study highlights the potential for reintroducing this cost-effective drug under strict surveillance. It also underscores the importance of continuous molecular monitoring to guide therapeutic policies. This study will help researchers to uncover the critical areas of antimalarial drug resistance reversal and parasite evolution that many researchers were not able to explore. Thus, a new theory on resistance restoration dynamics may be arrived at.

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