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Analysis of *Plasmodium falciparum* Resistance to Chloroquine in Côte d'Ivoire after 20 Years: High Prevalence of Wild Strains

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A B S T R A C T

Malaria is a major public health problem worldwide, especially in Africa, where antimalarial drug-resistant strains are spreading. In Côte d'Ivoire, Dihydro-artemisinine Piperaquine (DHA-PPQ) has been included in the policy for simple malaria management, but key mutations of the *pfcrt* gene are being studied to determine the mutation points that modulate *Plasmodium falciparum* resistance to Piperaquine. Genomic DNA from 158 patients from the five study sites with *P.falciparum* malaria was extracted using Chelex 5% tween. Conventional PCR amplification of the *pfcrt* gene was followed by Sanger sequencing of the amplificates on the Sanger sequencing platform of the CRCHU of Quebec (Canada). Comparison of different proportions was carried out using Rstudio software version 4.1.3. Analysis of individual alleles showed a prevalence of wild strain alleles over mutant alleles at all study sites. The K76T mutation was found at relatively low prevalences at the sites: 4.5% (1/22) in Bouaké; 10% (2/20) in Yamoussoukro; 18.5% (4/20) in Ayame; 20% (5/27) in Man and 34.8% (24/69) in Anonkoua-koute. Analysis of the distribution of the wild strain haplotype (CVMNK) of the *pfcr*t gene showed a high proportion (55-80% prevalence) compared to mutant strain haplotypes (1-20% prevalence). The high prevalence of the CVMNK haplotype across all sentinel sites supports the potential for regained sensitivity to CQ in the treatment of simple malaria. Therefore, continued molecular surveillance and in vitro analysis of *pfcrt* gene polymorphism mutations is highly recommended.

Introduction

Malaria is a major public health problem in the world. In 2020, 241 million cases were recorded, 95% of which were in Africa, and the number of deaths reached 627,000. Malaria caused by *Plasmodium falciparum* is responsible for severe and potentially fatal cases (WHO, 2021). Early and reliable diagnosis (WHO, 2021) followed by effective treatment is one key to combating this disease.

The fight against malaria is currently facing the spread of antimalarial drug-resistant strains. In Côte d'Ivoire, chloroquine (CQ), considered to be low-toxic and affordable, was used as first-line in the therapeutic scheme in Côte d'Ivoire. It was removed due to the resistance of *Plasmodium falciparum* that has been demonstrated. The prevalence of chloroquine-resistant parasites has increased in Côte d'Ivoire since 1987. Cases of K76T mutation, linked to *Plasmodium falciparum* resistance to CQ, have been reported in different regions of Côte d'Ivoire with high rates ranging from 65 to 100% (Ako *et al.*, 2012; Dagnogo *et al.*, 2018).

In 2018, a ministerial decree (Arrêté n° 190 NSHP/CAB of 27 Nov. 2018) was taken to include Dihydro-artemisinine Piperaquine (DHA-PPQ) (Anonyme1), which is a derivative of artemisinine, in the simple malaria treatment policy in Côte d'Ivoire. In this molecule, Piperaquine (PPQ), a partner molecule with a long half-life, is a bis-quinoline compound composed of two 4-aminiquinoline halves with a structure similar to that of CQ and linked together by a central bond (Zhu *et al.*, 2019; Foguim *et al.*, 2020).

The structural and mechanism similarities with CQ predicted a similar mechanism of resistance and involved key mutations of the *pfcrt* gene (*P. falciparum* chloroquine resistance transporter). The *pfcrt* gene (PF3D7_0709000) was studied to determine mutation points that could affect *Plasmodium falciparum* resistance to Piperaquine. Exons 2, 3, and part of exon 4 covering codons 72-76, 93, 97, 101, 145, 146, 158 and 159, as well as exon 10 covering positions 343, 350, 353, and 356 were examined. New mutations in the *pfcrt* gene (T93S, H97Y, C101F, F145I, M343L, C350R, G353V) (Foguim *et al.*, 2020) recently found in parasites from Southeast Asia appear to be also involved in *P. falciparum* resistance to PPQ (Agrawal *et al.*, 2017).

This study aims to study the K76T mutation and new mutations in codons 2 and 3 that would be involved in *P. falciparum* resistance to piperaquine two decades after the removal of chloroquine in Côte d'Ivoire.

Materials and Methods

Study site

This is a retrospective study that took place in five (05) different locations spread across three (3) different regions of Ivory Coast in various ecological contexts: in the south: Anonkoua-koute (5°25’55.90"N;4°2’45.27"W) and Ayame (5°36’12,43" N ; 3°09’19,36" W); in the center: Bouake (7° 40' 59.999" N ; 5° 1' 0.001" W) and Yamoussoukro (6° 49' 0.001" N ; 5° 16' 59.999" W); in the west: Man (7°24’N, 7°24’W) (**Error! Reference source not found.**).

Study population and sample collection

This study utilized dried blood spot (DBS) samples collected on Whatman 3 MM filter paper that were archived at the Unit of Paludology previously collected over a seven-year period from 2013 to 2019 as part of a clinical trial. There were 852 J0 DBS samples from patients with confirmed *P. falciparum* malaria, for which demographic and parasitological data were available. For each patient with confirmed malaria through microscopic examination (thick blood smear and blood smear), approximately 2-5 mL of venous blood were taken in an EDTA tube. Approximately 50 µL of total blood was deposited on Whatman 3 MM filter paper disks. The blood-stained papers were dried at room

temperature away from dust.

Extraction of *Plasmodium falciparum* genomic DNA

The extraction of Plasmodial DNA from the DBS was carried out using the Tween-Chelex® 5% method of Simon *et al.*, (2020) with some modifications briefly described below. The DBS samples were lysed with a 0.5% Tween 20 solution diluted with PBS 1X and incubated at 4°C for 24 hours to release the parasite's genomic DNA.

The lysis solution was removed and replaced with PBS 1X to minimize the presence of hemoglobin, which can inhibit Taq polymerase activity. A preheated 5% Chelex solution was added and the mixture was incubated at 95°C for 15 minutes. The extracted DNA was centrifuged several times to eliminate residual Chelex and was stored at -80°C.

Conventional PCR amplification of the *pfcrt* gene

The amplification was carried out in a thermocycler (Eppendorf Mastercycler Gradient) under the following conditions: 25 µL final volume containing 5 µL of DNA, 0.25 µM of primer (SecIF: 5'-GGTAAATGTGCTCATGTGTTTAAACTTATT-3' /SecIR:5'-TTACTTTTGAATTTCCCTTTTTATTTCCA- 3'), 1X FIREPOL Master (Solis Biodyne) consisting of 10X buffer, 25 mM MgCl2, 5 mM dNTP, and stabilized polymerase, and milliQ water. The amplification conditions were as follows: 15 min at 95°C, 30 sec at 95°C, and 1 min at 60°C; 1 min at 72°C for 40 cycles, and a final extension step at 72°C for 10 min.

The resulting PCR products contained codons 72, 73, 74, 75, and 76. All fragments were subjected to 2% agarose gel electrophoresis containing Syber Safe as the intercalant. The bands were observed using the Bio-Rad Gel Doc TM Imager, and the size of the *pfcrt* gene was 241 bp.

The purified amplification products were cleaned up using the Charge Switch®-Pro PCR Clean-up kit (Invitrogen) according to the manufacturer's procedure. The purified amplicons were sequenced using the Sanger method by the Quebec Sanger Sequencing Platform (Canada).

Sequencing data analysis and mutation identification

The sequences were cleaned using MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura, Stecher, and Kumar 2021). They were then aligned using the BioEdit 7.2.5 software under the default Clustral program using the reference sequence of the *pfcrt* gene, PF3D7_0709000.1, available on PlasmoDB. In the wild strain, the positions of interest inducing chloroquine resistance are codons Cys-72, Val-73, Met-74, Asn-75, and Lys-76, corresponding to the wild-type allele combination of CVMNK. Any point mutations observed at these codons would be of interest as they would indicate chloroquine resistance of the strain in question.

Statistical analysis of data

All data was organized using Microsoft Excel 2016. The Geographic Information System software QGIS version 3.26.2 was used to create maps and determine prevalence. The statistical software RStudio version 4.1.3 (R Core Team, 2020) was used to perform statistical tests such as proportion tests (z-test), Fisher's test, and chi-squared test as appropriate. This tool also allowed for creating graphs using the ggplot2 package. A statistical difference was considered significant when the p-value was less than the $\alpha = 0.05$ threshold.

Results and Discussion

In total, 852 archived dried blood spots (DBS) for which demographic and parasitological data were available were selected for the study. 18.54% (158/852) were randomly selected from the DBS for *pfcrt* gene amplification and Sanger sequencing. These samples were distributed as follows: Anonkoua-kouté 43.7% (69/158), Ayame 17.1% (27/158), Bouake 14% (22/158), Man and Yamoussoukro each 13% (20/158). 100% of the sequenced samples were compared to the reference

sequence PF3D7_0709000.1 (Figure 2).

Frequency of individual *pfcrt* gene alleles

The frequency analysis of the alleles at exons 2 and 3 of the *pfcrt* gene did not reveal any mutations at codons 93, 97, and 101. The analysis of the alleles of individuals at amino acid positions 72 to 76 showed a predominance of the prevalence of wild-type alleles (60.9 to 100%) over mutant alleles across all five (5) study sites. In fact, the prevalence of wild-type alleles at different positions was between 90 and 100% (72-Cys); 75 and 100% (73-Val); 19.9 and 100% (74-Met); 72.5 and 95.5% (75-Asn); 60.9 and 86.4% (76-Lys) (Table 2).

While a low prevalence of mutant strain alleles was observed (3.7 to 34.8%). At the CRT-72 position, the 72-Phe and 72-Ser alleles were respectively observed in Ayame 7.4% (2/27) and Man 10% (2/20). At the CRT-73 position, the 73-Glu and 73-Pro alleles were respectively observed in Ayame 7.4% (2/27) and Man 10% (2/20).

At the CRT-74 position, the 74-Ile allele was observed in Anonkoua-kouté 26.1% (18/68); Ayame 11.1% (3/27); Man 15% (3/20) and Yamoussoukro 10% (2/20). At the CRT-75 position, the 75-Glu allele was observed in Anonkoua-kouté 21.7% (15/69); Ayame 11.1% (3/27); Yamoussoukro 20% (4/20) and the 75-Lys allele in Man 10% (2/20).

At the CRT-76 position, the 76-Thr allele was observed in Anonkoua-kouté 34.8% (24/69); Ayame 18.5% (5/27); Man 20% (4/20); Yamoussoukro 10% (2/20). At the same position, the 76-Asn and 76-Pro alleles were respectively observed in Bouake 9.1% (2/22) and Man 15% (3/20) (Table 2).

Furthermore, the chi-squared independence and Fisher tests showed no statistically significant difference (p value < 0.05) between the presence of wild and mutant alleles on the different sites in this study (Table 2).

Distribution of phenotypes and haplotypes in the study

The analysis of the distribution of the phenotypes of the *pfcrt* gene isolates showed a high proportion of wild-type isolates (76-Lys) compared to resistant isolate phenotypes (76-Thr) (Figure 3).

A statistically significant difference between wild-type and resistant isolate phenotypes was observed, with a p-value of 8.019e-16 (Anonkoua-kouté); 1.723e-06 (Ayame); 4.438e-05 (Bouake); 5.81e-05 (Man), and 0.0002 (Yamoussoukro).

Regarding the analysis of the distribution of haplotypes of the *pfcrt* gene in this study, the wild-type haplotype CVMNK was predominant across all sites with a prevalence of 60.9% (Anonkoua-kouté); 63% (Ayame); 77.3% (Bouake); 55% (Man) and 80% (Yamoussoukro)(Figure 4). In the study, the CVIET haplotype was observed in Anonkoua-kouté (20.3%); Ayame (11.1%); Man (5%) and Yamoussoukro (10%). In addition to these two haplotypes, nineteen (19) other haplotypes carrying mutated amino acid positions were identified with relatively low proportions of 1.4 to 10% as prevalence at each site. These are the haplotypes: CAMNP (0 - 5%), CEMNK (0 - 7.4%), CEMNT (0 -5%), CLMKK (0 - 5%), CQMNK (0 - 3.7%), CQMNT (0 - 4.5%), CVIDT (0 - 4.3%), CVIEN (0 - 1.4%), CVMDS (0 - 1.4%), CVMEK (0 - 5%), CVMKK (0 - 3.7%), CVMNI (0 - 1.4%), CVMNN (0 - 9.1%), CVMNT (0 -10.1%) FLMNT (0 -3.7%), FPLDP (0 -3.7%), SKIKP (0 - 5%), SPIDP (0 - 5%) etWEKEG(0 - 5%) (Figure 4).

The analysis of the distribution of these haplotypes showed a great variety of haplotypes from 2013 to 2019. Three of these haplotypes had a high prevalence during this study period. They were: CVMNK, CVIET, and CVMNT. Firstly, in 2013, the respective prevalence's were observed at 80%, 10%, and 10%. Additionally, in 2016, the prevalence's were 61.1%, 11.1%, and 5.6% respectively. Finally, in 2019, the prevalences were 66.0%, 13.8%, and 6.4% respectively (Figure 4).

In Côte d'Ivoire, with the official ban of Chloroquine (CQ) in 2003. CQ has not been used in Côte d'Ivoire for nearly 20 years although self-medication may have continued during these two

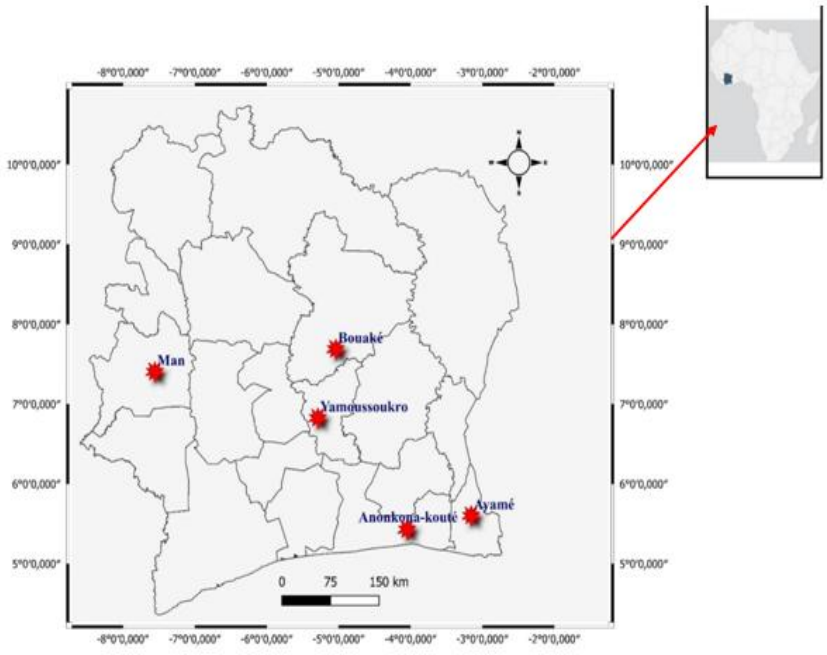
decades.

Table.1 Prevalence of individual SNP of *pfcrt* gene in study sites.

Amino acid position	Alleles	Anonkoua (N=69)		Ayame (N=27)		Bouake (N=22)		Man (N=20)		Yamoussoukro (N=20)		p – value χ2or Fisher
		n	%	n	%	n	%	n	%	n	%	-
CRT-72	Cys*	69	**100.0**	25	**92.6**	22	**100.0**	18	**90.0**	19	**95.0**	0.2414
	Phe	0	0.0	2	**7.4**	0	0.0	0	0.0	0	0.0	0.6
	Ser	0	0.0	0	0.0	0	0.0	2	**10.0**	0	0.0	1
	Trp	0	0.0	0	0.0	0	0.0	0	0.0	1	5.0	1
CRT-73	Ala	0	0.0	0	0.0	0	0.0	1	5.0	0	0.0	1
	Glu	0	0.0	2	**7.4**	1	4.5	1	5.0	1	5.0	0.3
	Lys	0	0.0	0	0.0	0	0.0	1	5.0	0	0.0	1
	Leu	0	0.0	1	3.7	1	4.5	0	0.0	0	0.0	0.4
	Pro	0	0.0	1	3.7	0	0.0	2	**10.0**	0	0.0	1
	Gln	0	0.0	1	3.7	1	4.5	0	0.0	0	0.0	0.4
	Val*	69	**100.0**	22	**81.5**	19	**86.4**	15	**75.0**	19	**95.0**	0.259
CRT-74	Ile	18	**26.1**	3	**11.1**	0	0.0	3	**15.0**	2	**10.0**	1
	Lys	0	0.0	0	0.0	0	0.0	0	0.0	1	5.0	1
	Leu	0	0.0	1	3.7	0	0.0	0	0.0	0	0.0	1
	Met*	51	**73.9**	23	**85.2**	22	**100.0**	17	**85.0**	17	**85.0**	0.09094
CRT-75	Asp	4	5.8	1	3.7	0	0.0	1	5.0	0	0.0	1
	Glu	15	**21.7**	3	**11.1**	0	0.0	1	5.0	4	**20.0**	1
	Lys	0	0.0	1	3.7	1	4.5	2	**10.0**	0	0.0	1
	Asn*	50	**72.5**	22	**81.5**	21	**95.5**	16	**80.0**	16	**80.0**	0.09094
CRT-76	Gly	0	0.0	0	0.0	0	0.0	0	0.0	1	5.0	1
	Ile	1	1.4	0	0.0	0	0.0	0	0.0	0	0.0	1
	Lys*	42	**60.9**	21	**77.8**	19	**86.4**	13	**65.0**	17	**85.0**	0.2414
	Asn	1	1.4	0	0.0	2	**9.1**	0	0.0	0	0.0	0.3
	Pro	0	0.0	1	3.7	0	0.0	3	**15.0**	0	0.0	1
	Ser	1	1.4	0	0.0	0	0.0	0	0.0	0	0.0	1
	Thr	24	**34.8**	5	**18.5**	1	4.5	4	**20.0**	2	**10.0**	1

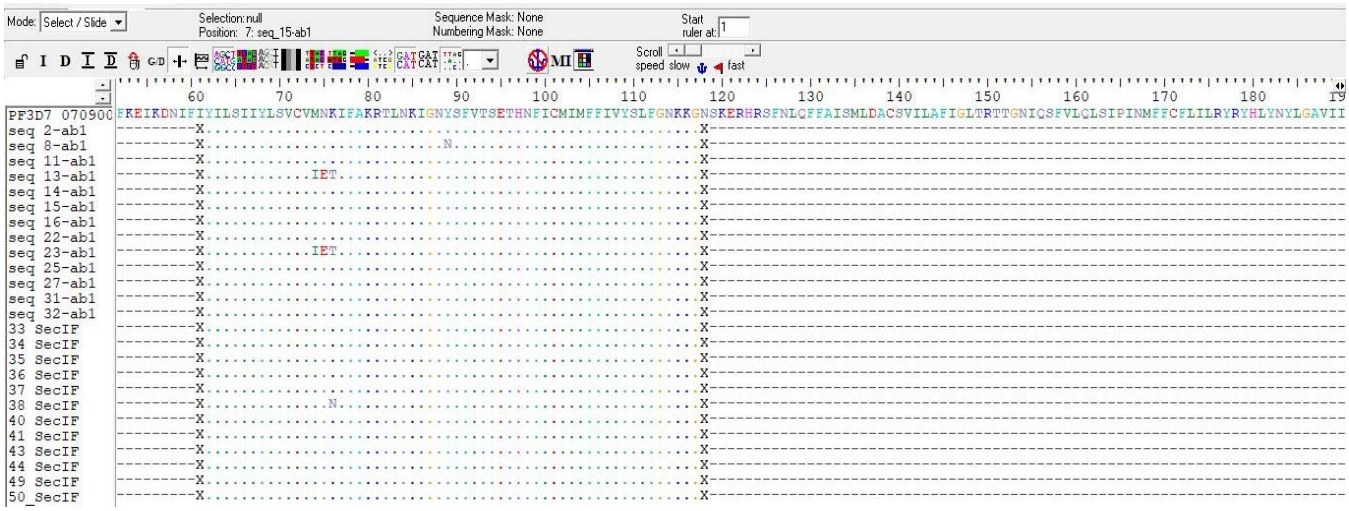
N: represents the total number of sequenced isolates n: represents the number of isolates successfully sequenced by codon *: denotes the amino acid of wild strain PF3D7-CRT

Fig.1 Study sites



Realized by: Kouman KouaméBouatiniAngélo, in December 2022.

Fig.2 Sequence alignments of isolates with reference strain PF3D7 0709000.1



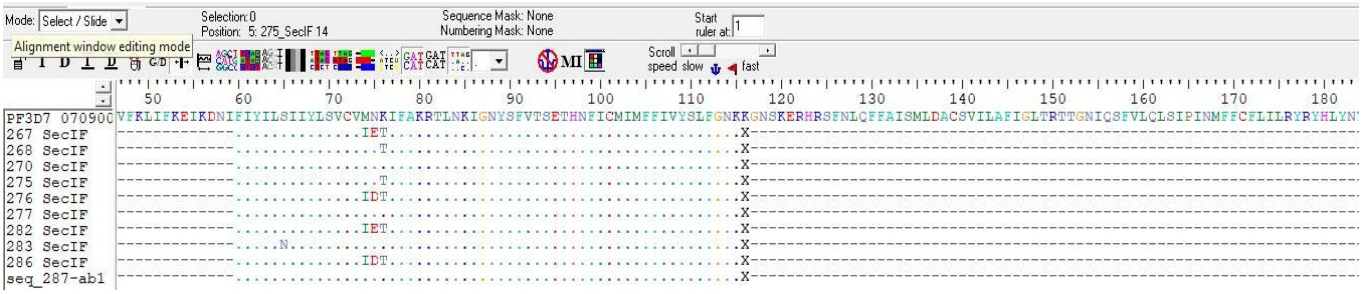
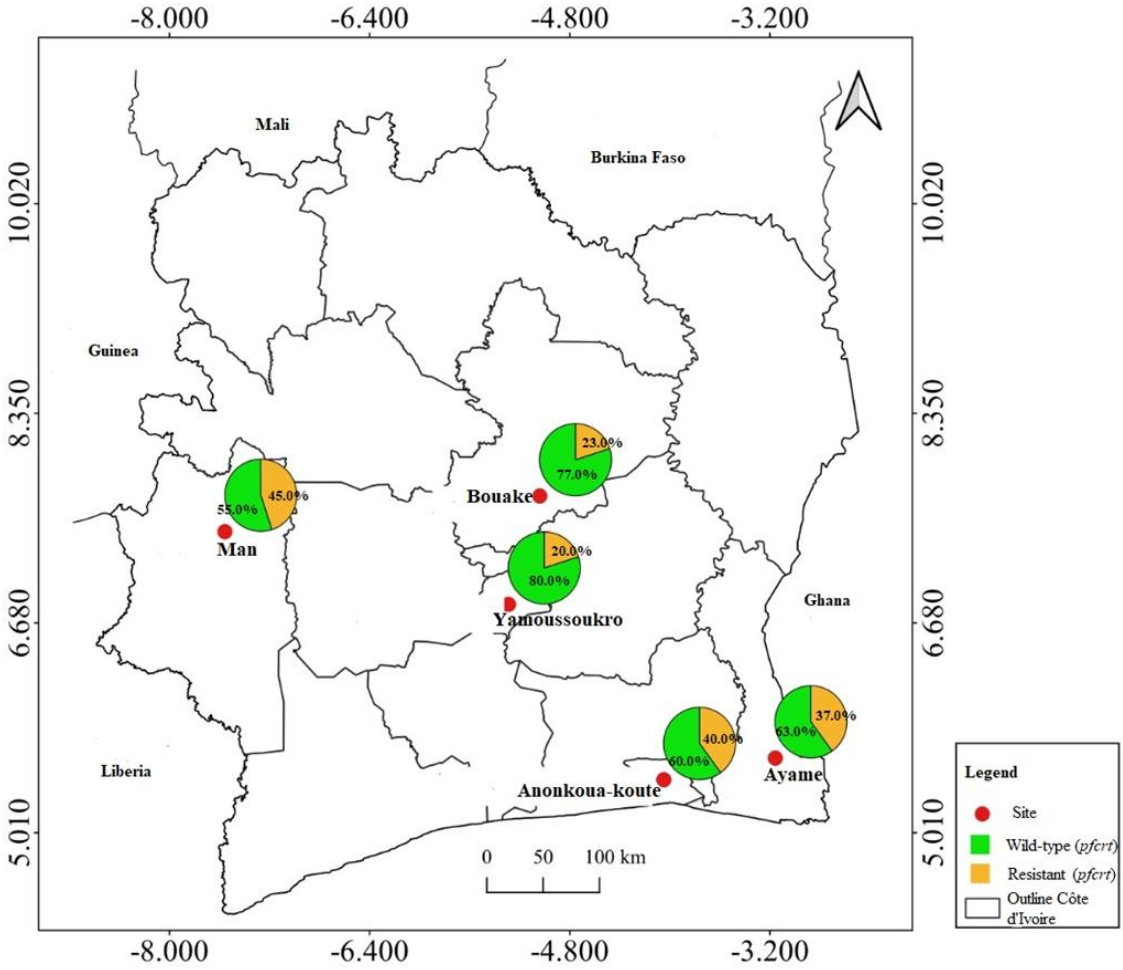
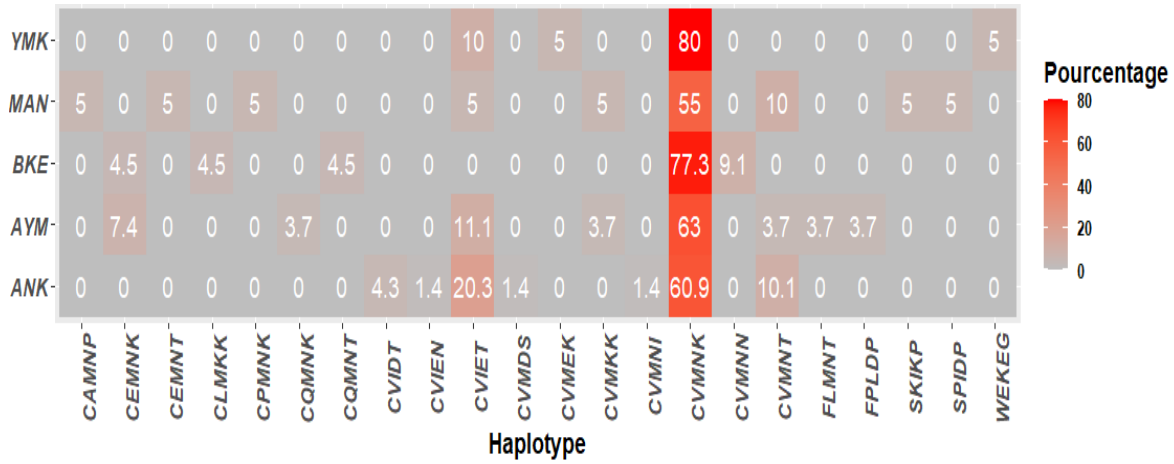


Fig.3 Distribution of phenotypes in Côte d'Ivoire



Realized by**:** Kouman KouaméBouatiniAngélo, in January 2023

Fig.4 Distribution of *pfcrt* haplotypes in Côte d'Ivoire from 2013 -2019



ANK: Anonkoua-koute; AYM: Ayame; BKE: Bouake; MAN: MAN; YMK: Yamoussoukro

In the five study sites, the predominance of the wild allele 76-Lys with a prevalence of 60.9 to 86.4% highlighted is in agreement with Dagnogo *et al.*, (2018) which highlighted a similar prevalence of 60% in Anonkoua-kouté. Then a prevalence of 62 and 65% respectively in Port-Bouët and Ayame which are localities of Côte d'Ivoire.

And this would be due to the low drug pressure exerted on the parasites. Also, this reduced pressure can be explained by the withdrawal of chloroquine since 2003, which would have favored an emergence of parasites sensitive to chloroquine.

Studies have shown that chloroquine withdrawal has resulted in a return of CQ-sensitive *P. falciparum* strains (Vatomandry *et al.*, 2022; Sitali *et al.*, 2019). In the Comoros archipelagos, for example, a significant increase in the frequency of wild strains of the *pfcrt* gene, up to 76%, was observed between 2006 and 2014 (Huang *et al.*, 2016). However, in some malaria-endemic countries, despite changes in treatment policy, mutant alleles associated with CQ resistance persist (Ocan *et al.*, 2019), hence the interest of monitoring.

The point mutation on codon 76 of the *pfcrt* gene allows *P. falciparum* to limit the accumulation of CQ in its digestive vacuole, where it exerts its inhibitory action. This gene is also involved in the decreased susceptibility of *P. falciparum* to Amodiaquine and quinine (Hassen *et al.*, 2022). Unlike the sites of Anonkoua-koute, Ayame, Bouake, Man and Yamousoukro, we observed relatively low prevalences of the 76-Thr allele. Indeed, recovery of the K76 allele at the expense of T-76 has been documented, especially in sub-Saharan African countries such as Malawi (Ménard *et al.*, 2008), Tanzanie (Bwire *et al.*, 2020), Kenya (Chebore *et al.*, 2020) and Ouganda (Balikagala *et al.*, 2020), where the AL is implemented. The use of artemisinin derivatives such as AL, ASAQ, DHA-PPQ would be the cause of this resurgence of wild-strain isolates (CVMNK). However, our results contrast with (Hassen *et al.*, 2022). Although AL is used as first-line treatment for *P. falciparum* in Ethiopia, poor recovery of parasites carrying the wild K76 allele has been detected. Meanwhile, in Nigeria and Chad where the proportion of the T76 allele was 94.5% and 95% respectively, although chloroquine has been withdrawn from the treatment regimen since 2004 in favor of ASAQ and AL (Moussa Hassane Taïsso *et al.*, 2022; Ikegbunam *et al.*, 2019). This high prevalence was due to an unrecommended circulation of chloroquine from Nigeria. Indeed, Massakory is a crossroads where the supply of general goods and pharmaceuticals comes from Nigeria.

In the present study, mutations at exons 3 and 4 could not be studied because the primer pair used covered only exon 2 and one potion from exon 3. For perspectives it would be necessary to design primers that can cover all exons in order to explore the maximum number of codons. Indeed, some codons useful in the mechanism of modulation of

the resistance of *Plasmodium falciparum* to piperaquine have not been observed (Boonyalai *et al.,* 2020; Ross *et al.,* 2018)

The high prevalence of the CVMNK haplotype in all sentinel sites supports a potential increase in sensitivity to CQ in the treatment of single-line malaria. Therefore, continuous and in vitro molecular monitoring of *pfcrt* polymorphism mutations is strongly recommended.

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