International Scholars Journals

African Journal of Parasitology Research ISSN 2756-3391, Vol. 12 (1), pp. 001-010, January, 2025. Available Online at www.internationalscholarsjournals.com © International Scholars Journals

Research Article

High proportion of *pfpm-2* multiple-copies carrying isolates, a piperaquine resistance marker, prior dihydroartemisinie-piperaquine adoption in Cote d'Ivoire in 2018

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Received: 14-Aug-2023, Manuscript No. AJPROAJ-23-110353; Editor assigned: 17-Aug-2023, Pre QC No. AJPROAJ-23-110353 (PQ); Reviewed: 01-Sep-2023, QC No. AJPROAJ-23-110353; Revised: 23-Jan-2025, Manuscript No. AJPROAJ-23-110353 (R); Published: 30-Jan-2025

ABSTRACT

Introduction: In recent years, the therapeutic arsenal used to fight against malaria in Côte d'Ivoire has been enriched with several Artemisinin-Based Combinated Treatment (ATCs), all of which are well-tolerated. The sensitivity of *P. falciparum* to these molecules was even confirmed by molecular and phenotypic tests in force. However, these tests only evaluate the sensitivity of artemisinin derivatives and not that of the partner molecule. One of these ATCs, Dihydroartemisinin-Piperaquine (DHA-PPQ), was officially introduced in 2018 for the management of uncomplicated malaria and deployed throughout the country. This study aims to test the hypothesis that *pfpm2* multiple copies carrying isolates, one of the molecular markers of resistance to PPQ, were circulating well before its official introduction.

Methodology: *P. falciparum* DNA was obtained from archived Dried Blood Spot (DBS) cards. These DBS correspond to isolates collected at five sentinel sites where clinical trials were conducted between 2013 and 2019. The number of copies of the *pfpm2* gene was estimated by $N=2^{-\Delta\Delta Ct}$. The Cochran-Armitage trend test was used to compare annual proportions of isolates carrying more than one copy of *pfpm2* per site and per year.

Results: During the study period, 850 DBS were examined. On average, more than 50% of isolates carried multiple copies of *pfpm2* at each site studied. In Ayamé, the average proportion reached 78.6% (95% CI: 72.8-83.4%), while it was 94.6% (95% CI: 88.2-97.8%) in Bouaké and 74.9% (95% CI: 67.4-81.1%) in Man. This latter site experienced a significant decrease in this proportion ($p=0.0033$ and $z<0$), while it increased significantly in Yamoussoukro ($p=2.6\ e^{-12}$ and $z>0$).

Conclusion: Overall, the study sites revealed proportions of isolates carrying multiple copies of the *pfpm2* gene exceeding 50% during the period from 2013 to 2019, with a significant decrease in Man and a significant increase in Yamoussoukro. Prior evaluation would have been opportune before the deployment of the DHA-PPQ combination.

Keywords: Piperaquine, *pfpm2*, Copy number, Cote d'Ivoire

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INTRODUCTION

The resistance of *Plasmodium falciparum* to antimalarial drugs continues to pose a major threat to efforts that aimed at combating and eliminating malaria (Diawara et al., 2017).

Since the early 2000's, the World Health Organization has recommended the use of Artemisinin-Based Combination Therapies (ACTs) as the first-line treatment in sub-Saharan Africa (WHO, 2021).

This recommendation, combined with the use of insecticide-treated bed nets and environmental sanitation, has significantly contributed to reducing the morbidity and mortality associated with malaria (Whegang et al., 2019). In Cote d'Ivoire, where malaria is endemic year-round with seasonal peaks, several therapeutic regimens involving ACTs have been revised over the past two decades (PNLP, 2018). The most recent one (Arreté N°190 MSHP/CAB of November 27th, 2018) recommends, as first-line treatment, in addition to Artesunate-Amodiaquine (ASAQ) or Artemether-Lumefantrine (AL), Dihydroartemisinin -Piperaquine (DHA-PPQ) and other ACTs such as Artesunate-Pyronaridine or Artesunate-Mefloquine. The national malaria control program has established a dozen sentinel sites across the countries, which are considered as strategic channels for the nationwide deployment of currently effective antimalarial for the population (Tossea et al., 2018) .

In such a context, it is essential to regularly evaluate the sensitivity of *P. falciparum* strains to official antimalarial drugs. For this purpose, molecular and phenotypic tests are recommended, with the former allowing the analysis of the *pfK13* gene polymorphism, while the latter determine the percentage of mature forms after 72 hours of exposure to the active ingredient of artemisinin derivatives, Dihydroartemisinin (DHA).

However, the mentioned molecular tests only target the *pfkelch 13* gene, a marker of resistance to artemisinin derivatives (Witkowski et al., 2013; Gbessi et al., 2021). Similarly, the phenotypic RSA tests, which have been used recently, measure only the sensitivity to DHA (Menard et al., 2016; Gnondjui et al., 2022). Unlike what has been observed in Southeast Asia, until now, even when combined with phenotypic tests, molecular tests have not yet detected a local emergence of resistance to ACTs in Africa (Bastiaens et al., 2014; Tamma and Cosgrove, 2014; Meshnick et al., 2015). Indeed, DHA-PPQ has been identified as the most effective and safe ACT for treating uncomplicated *P. falciparum* malaria in this region (Tindana et al., 2022). However, it appears that the effectiveness of ACTs only applies to the artemisinin derivative and not the partner molecule. This observation raises questions about the very definition of the efficacy of a therapeutic combination, as only one of the two components has proven its efficacy (Benoit-Vical et al., 2016). Therefore, it is important to regularly assess the sensitivity of *P. falciparum* isolates to official antimalarials using recommended molecular and phenotypic tests.

In Côte d'Ivoire, the Piperaquine (PPQ), which serves as the partner molecule in the DHA-PPQ combination, has not been comprehensively studied, despite the availability of technical platforms capable of conducting the PSA test (Witkowski et al., 2017). The PSA test assesses the survival of parasites exposed to a single and relevant dose of piperaquine from a pharmacological standpoint (Thanh et al., 2017; Bopp et al., 2018; Ross et al., 2018; Boonyalai et al., 2020). Both in Southeast Asia (Amato et al., 2017; Witkowski et al., 2017) and Africa (Rasmussen et al., 2017; Russo et al., 2018), the elevated number of copies of the plasmepsin II gene (*pfmp2*; PF3D7_1408000) is considered a molecular marker of P. falciparum resistance to piperaquine. This gene, located on chromosome 14, encodes a protease that participates in the degradation of hemoglobin. The increased number of *pfmp2* copies enhances the survival of parasites exposed to piperaquine by increasing the production of amino acids to compensate for hemoglobin degradation, which is inhibited by PPQ (Witkowski et al., 2017; Tsombeng et al., 2019). In the absence of the phenotypic PSA test, molecular tests remain suitable surveillance tools due to their efficacy and accessibility.

Regular evaluation of the efficacy of artemisinin derivatives and their partner molecules is necessary due to the limited number of antimalarial drugs available in the current local therapeutic arsenal. The risk of resistant parasites emerging from Southeast Asia and spreading to the African continent is real and concerning. This scenario could resemble the emergence of Chloroquine (CQ) resistance and its dissemination in Africa (Leroy et al., 2019). In the context of Multiple First-Line Therapy (MFT), as implemented in Côte d'Ivoire, the simultaneous deployment of Artemether-Lumefantrine (AL) and Dihydroartemisinin-Piperaquine (DHA-PPQ) may facilitate the short-term emergence of drug resistance due to genotypes resistant to piperaquine (Zupko et al., 2022).

Considering all potentially effective therapeutic combinations without prior evaluation of *P. falciparum* sensitivity to partner molecules before their adoption as official antimalarial treatments could potentially limit their long-term efficacy (Tindana et al., 2022). The present study aims to test the hypothesis of the circulation of isolates carrying multiple copies of the *pfpm2* gene at the time of DHA-PPQ deployment, which was recently adopted as a first-line treatment in Côte d'Ivoire.

MATERIALS AND METHODS

Study design

Collection of samples and informed consent: This is a cross-sectional study in which Dried Blood Spot (DBS) samples were collected between 2013 and 2019. These samples were obtained simultaneously with therapeutic trials conducted by

the Paludology Unit of the Institut Pasteur of Cote d'Ivoire.

They were obtained from participants of all ages and genders who presented with uncomplicated *P. falciparum* malaria confirmed by microscopic examination (positive thick blood smear and blood film). Prior informed consent was obtained from all participants.

Approximately 50 µL of peripheral blood was collected by finger prick from the left ring finger and spotted onto Whatman 3 MM filter paper (Cytiva, CA, USA). The DBS samples were air-dried, protected from dust and humidity, and individually placed in ziplock bags containing silica gel. They were then stored at room temperature, shielded from moisture, until molecular analyses were conducted. Simultaneously, thick and thin blood smears were prepared for *Plasmodium* species identification and parasite counting. Blood films were stained with 10% concentrated Giemsa and examined under a microscope to determine the presence of *Plasmodium* sp.

Study sites

The studies were conducted in five localities distributed across three regions, each with distinct ecological and geographical contexts. In the south, we had Anonkoua-Koute and Ayame; in the west, Man; and in the central region, Bouaké and Yamoussoukro. Anonkoua-Kouté (5°25'55.90" N; 4°02'45.27" W) is a peri-urban village located in the Abobo-Nord health district in Abidjan. The village experiences annual precipitation exceeding 1700 mm and temperatures ranging from 27°C to 33°C. A community-based urban health facility (FSUCOM) serves an estimated population of 61,250 inhabitants as of 2008.

In that same year, 18,281 malaria cases were recorded out of a total of 33,727 consultations, representing 54.2% of medical consultations. FSUCOM Anonkoua-Kouté was selected for this study due to consistently high annual incidences of malaria cases. Ayamé (5°36'12.43" N; 3°09'19.36" W) was chosen as a study site due to its equatorial epidemiological profile, hydrography, and vegetation favoring a high density of anopheline mosquitoes. Precipitation in this region ranges from 1120 to 2306 mm (N'Diaye et al., 2015). In 2015, the General Hospital of Ayamé Mission Catholique recorded 1472 malaria cases out of a total of 5252 consultations, representing 28.02% of medical consultations.

Man (7°24'N; 7°24'W) is a city situated in a basin surrounded by a mountain range in western Côte d'Ivoire, approximately 570 km from Abidjan by road. It is the capital of the Western region, with a population of around 200,000 inhabitants. Man has a savannah climate, with an average annual temperature of 25°C and average precipitation of 1182.8 mm. The city of Bouaké, located in the central region of Côte d'Ivoire, is the main malaria transmission area in this region, hosting the transmission vectors Anopheles gambiae and *Anopheles funestus.* The annual incidence in this city exceeds 500% (PNLP, 2018). Yamoussoukro, covering an area of 9387 km² with a population of 700,282 inhabitants, is one of the twelve sentinel sites responsible for monitoring *Plasmodium falciparum* chemoresistance. It is located 242 km from Abidjan and is characterized by forest-type vegetation and a mountainous terrain. The region experiences a humid tropical climate with significant annual precipitation, averaging 1,775 mm (with peaks of 2,000 mm). The average annual temperature is 25°C, with a peak of 26.5°C in March. The municipality of Yamoussoukro has numerous malaria treatment centers, among which the Regional Hospital (CHR) holds a prominent position, with an annual incidence ranging from 400 to 499% (PNLP, 2018). Regarding Yamoussoukro and Bouaké, the available DBS samples date back to 2015/2016, as therapeutic trials were conducted from that period onwards.

Isolation of *Plasmodium falciparum* genomic DNA

Plasmodium genomic DNA was extracted from DBS using the 5% Tween-Chelex® method (Simon et al., 2020) with slight modifications. DBS pieces were fully immersed in 1 mL of 1X PBS solution diluted with 0.5% Tween 20. The tubes were vigorously agitated for 30 seconds using a Vortex (VWR Galaxy ministar®) and then incubated at +4°C for 24 hours. This lysis step ruptured the red blood cell membrane, releasing hemoglobin into the solution and allowing the parasite genomic DNA to be adsorbed onto the filter paper.

The following day, the lysis solution was discarded, and 1 mL of 1X PBS solution was added to the tube, which was then incubated at +4°C for 30 minutes to maximize washing and removal of hemoglobin. Hemoglobin is an inhibitor of Taq polymerase, an essential enzyme in the DNA polymerization process. Meanwhile, the 5% (w/v) Chelex solution was heated to 95°C to activate its protective properties against potential degradation by ionic or enzymatic attack (DNases). Next, the entire 1X PBS washing solution was removed, and 300 µL of preheated 5% Chelex solution was added. The mixture was homogenized for 30 seconds using the Vortex and then incubated at 95°C for 15 minutes at a moderate speed. The microtubes were opened every 5 minutes to release accumulated pressure.

Subsequently, the microtubes were centrifuged at 12,539 rpm for 3 minutes. The supernatant, containing 250 µL of genomic DNA, was carefully collected and transferred to a pre-labeled 1.5 mL microtube. The supernatant (220 µL) was further collected and transferred to a new pre-labeled 1.5 mL microtube, then centrifuged at 12,539 rpm for 3 minutes. These two centrifugation steps were crucial to ensure the absence of Chelex® carryover in the final eluate, as residual Chelex can bind to Mg^{2+} (an essential cofactor for DNase action), inhibiting subsequent PCR applications. Several aliquots of the extracted DNA were prepared and stored at -80°C at the Center for Biological Resources (CeReB) of the Institut Pasteur of Cote d'Ivoire (Figure 1).

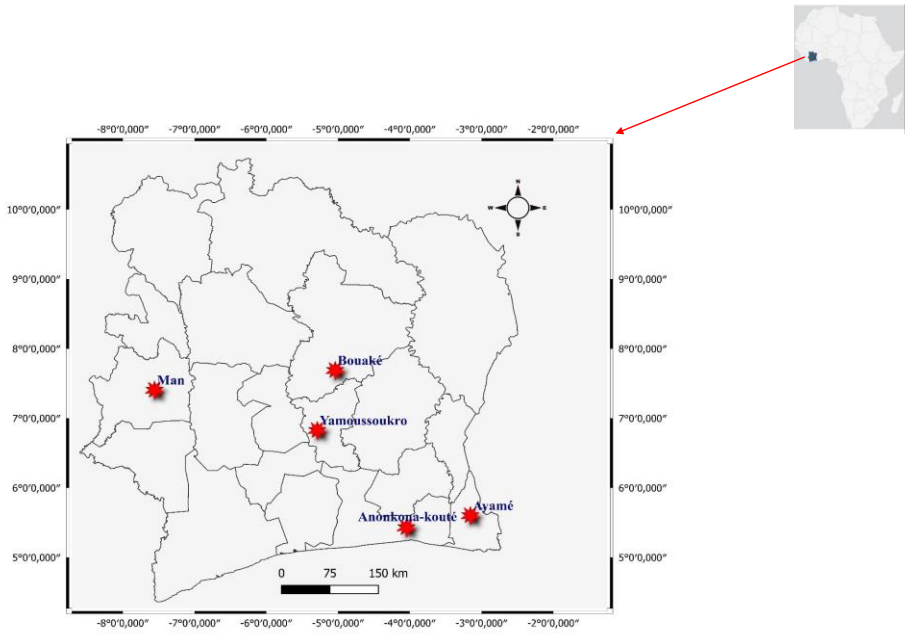

Figure 1. Study sites corresponding to some sentinel sites of the National Malaria Control Program (NMCP).

Purification

The purification was carried out using Promega magnetic beads (Madison, WI, USA) and the automated KingFisher™ Flex Purification System (Thermo Scientific, Vantaa, Finland). The DNA extracts served as templates for purification, following the protocol of Zainabadi et al. (Zainabadi et al., 2017). In summary, the Deep Well 1 storage plate, containing 50 µL of magnetic microbeads and 200 µL of DNA extract, was homogenized. The magnetic field generated by the reaction medium allowed the specific binding of magnetic beads to the DNA matrix present.

Subsequently, the DNA-bead complex was immersed in 500 µL of Wash Buffer 1 (3M Guanidine thiocyanate; 16.7% Isopropanol; 2% Triton X-100; 10 mM EDTA; 5 mM Trizma HCl, pH 7.4, and 0.1% HCl 6N) contained in Deep Well 2. The complex underwent a second wash in 500 µL of Wash Buffer 2 (25% absolute ethanol; 25% isopropanol; 100 mM sodium chloride; and 10 mM Trizma HCl, pH 7.4) contained in Deep Well 3. Finally, the DNA was eluted in 70 µL of elution buffer. During this last step, the magnetic field was neutralized using AE buffer properties, ensuring the isolation of DNA free from any chemical and biological impurities. An aliquot of the purified DNA was stored at -80°C for use as a template in quantitative PCR molecular tests.

Quantitative PCR amplification

The amplification was performed in a 20 µL reaction mix containing 0.25 mM of each primer (Table 1), milliQ water, Hot FIREPOL 5X Evagreen qPCR MixPlus-No ROX (Solis Biodyne), and 5 µL of purified DNA template. The cultured pf3D7 clone was used as a positive control. The qPCR amplification program for the *pfpm2* gene was executed using the CFX96 real-time PCR instrument (Bio-Rad) with the following protocol: 12 min at 95°C; 15 sec at 95°C; 20 sec at 58°C; and then 20 sec at 72°C. For the pfβtubuline gene, the amplification consisted of 40 cycles: 12 min at 95°C; 15 sec at 95°C; 20 sec at 60°C; and then 20 sec at 72°C.

Amplification was carried out in 20 µL of reaction mix containing 0.25 mM of each primer (Table 1), milliQ water, Hot FIREPOL 5X Evagreen qPCR MixPlus-No ROX (SolisBiodyne) and 5 µL of purified DNA template. The cultured pf3D7 clone was used as a positive control. The qPCR amplification program of the *pfpm2* gene inserted in the CFX96 real-time PCR instrument (Bio-Rad) was as follows: 12 min at 95°C; 15 sec at 95°C; 20 sec at 58°C then 20 sec at 72°C. That of the *pf-ß-tubulin* gene included 40 amplification cycles: 12 min at 95°C; 15 sec at 95°C; 20 sec at 60°C then 20 sec at 72°C.

Name	Sequence (5'-3')	Forward
Plasmepsin 2	GGAGATAACCAACAACCATTTAC	Forward
	GTTGTACATTTAACACTTGGG	Reverse
ß-tubuline	TGATGTGCGCAAGTGATCC	Forward
	TCCTTTGTGGACATTCTTCCTC	Reverse

Table 1. List of primers.

Determination of *pfpm2* gene copy number

The number of copies of the *pfpm2* gene (PF3D7_1408000) was determined using a modified EvaGreen-based quantitative PCR protocol (Solis Biodyne, Latvia) as described by Witkowski (Ariey et al., 2014; Witkowski et al., 2017). The *P. falciparum β-tubulin* gene (PF3D7_1408000) served as an unduplicated internal standard, and the 3D7 clone (PF3D7_1408000) was used as a parallel copy control. During each amplification cycle, two replicates using the DNA from the reference 3D7 parasite clone and two replicates of negative controls were included. The number of copies was calculated using the following formula:

$$N = 2^{-\Delta\Delta Ct}$$

Where,
N representing the copy number and Ct the cycle threshold.
$\Delta\Delta C_t=(C_{t\ pfpm2}-C_{t\ pf\beta\text{-}tubuline})_{\ sample}-(C_{t\ pfpm2}-Ct_{\ pf\beta\text{-}tubuline})_{\ PF3D7}$
N>1.6, isolate tested contains multiple copies of the *pfpm2* gene
N ≤ 1.6: the test isolate contains only one copy of the *pfpm2* gene

Statistical analysis of data

For the geographical distribution of isolates carrying multiple copies of the pfpm2 gene, version 3.26.2 of the QGIS geographic information system software was used. Data normality was assessed using the Shapiro-Wilk test (α=0.05) before performing multiple comparisons. Subsequently, either a one-way ANOVA or the Kruskal-Wallis test was conducted.

To analyze trends over time in the five sentinel sites, the Desc Tools package in RStudio version 4.1.3 (R Core Team, 2020) was utilized to perform the Cochrane-Armitage trend test. This test enables the determination of whether a series of proportions (k) varies linearly based on an ordinal variable. In this study, the proportions correspond to the genotypic prevalence of the *pfpm2* gene, while the ordinal variable represents the time period expressed in years. For a one-tailed test (left or right), the following hypotheses were formulated: H_0: z ≠ 0 and Ha_1: z<0 or Ha_2: z>0. If Ha_1 is accepted, the proportions decrease with an increase in the time period. Conversely, if Ha_2 is accepted, the proportions increase with an increase in the time period. Additionally, the test for comparison of two proportions was used to compare patients treated with Antimalarial AL and ASAQ. A difference was considered statistically significant when the p-value was less than the threshold α=0.05.

RESULTS

Isolation and DNA quality control

A total of 1,042 participants were included in this study. Table 2 presents the distribution of participants by site and year, with a maximum of 252 participants in Ayamé and a minimum ranging from 24 to 27 depending on the study region, across all years. In 2016, the highest number of samples was collected, totaling 426 from participants.

From this set, DBS samples were randomly selected to optimize DNA isolation using the Tween 20/Chelex 100 method as described previously. The average amount of DNA obtained was 75.4 ng/mL, 95% CI (45.2-105.6). The mean quality of the DNA extract, expressed as the ratio of optical densities at 280 and 260, was 1.9; 95% CI (1.7-2.1). DNA isolation was subsequently performed on all collected samples to determine the number of copies of the *pfpm2* gene.

Distribution of the exact number of *pfpm2* copies per year and study site

The analysis of the exact number of copies reveals the presence of varying copies of the *pfpm2* gene across all study sites. Between 2013 and 2019, the evolution of the exact number of copies of the Plasmepsin 2 gene can be divided into two distinct phases. Additionally, there is an inverse relationship between the proportion of parasites carrying at most one copy of the Plasmepsin 2 gene and those with more than six copies of the same gene. In 2013, isolates carrying a single copy of the gene were predominant in Man (65.4%, *i.e.*, 17 out of 26 detected isolates) and in Anonkoua-Kouté (13.9%, *i.e.*, 6 out of 43 detected isolates). In Ayamé, this proportion was comparable to that of isolates with more than six copies, representing 36.2% (34 out of 94 collected isolates) and 37.2% (37 out of 94 collected isolates), respectively. In contrast, by 2019, the configuration had completely reversed, with a clear predominance of isolates carrying more than six copies, particularly in Bouaké, where they accounted for over 95% (*i.e.*, 23 out of 24) of isolates among the two identified alleles. The same year in Man, isolates with more than six copies of the gene were still less numerous than those possessing only a single copy (Table 2 and Figure 2).

Regions	Sites: n (%)	Mean log_{10} Paras (95% CI)	2013		2016		2019	
			n (%)	Log_{10} Paras (95% CI)	n (%)	Log_{10} Paras (95% CI)	n (%)	Log_{10} Paras (95% CI)
South (n=385)	ANK: 133 (15.6)	4.39 (4.29-4.50)	43 (5.0)	4.52 (4.34-4.70)	27 (3.2)	4.11 (3.88-4.34)	63 (7.4)	4.43 (4.28-4.58)
	AYM: 252 (29.6)	4.35 (4.29-4.42)	94 (11.0)	4.27 (4.18-4.36)	96 (11.3)	4.33 (4.22-4.45)	62 (7.3)	4.50 (4.36-4.65)
West (n=167)	MAN: 167 (19.6)	4.07 (3.99-4.15)	26 (3.1)	4.32 (4.10-4.54)	87 (10.2)	3.97 (3.87-4.07)	54 (6.3)	4.11 (3.94-4.28)
	BKE: 112 (13.1)	3.44 (3.32-3.57)	-	-	88 (10.3)	3.27 (3.13-3.41)	24 (2.8)	4.08 (3.93-4.23)

Center (n=300)	YMK: 188 (22.1)	4.46 (4.38-4.55)	-	-	128 (15.0)	4.48 (4.38-4.59)	60 (7.0)	4.42 (4.25-4.60)
Total	N=852 (100)	-	163 (19.1)	-	426 (50)	-	263 (30.9)	-

Table 2. Distribution of Dried Blood Spot (DBS) samples over years and study sites.

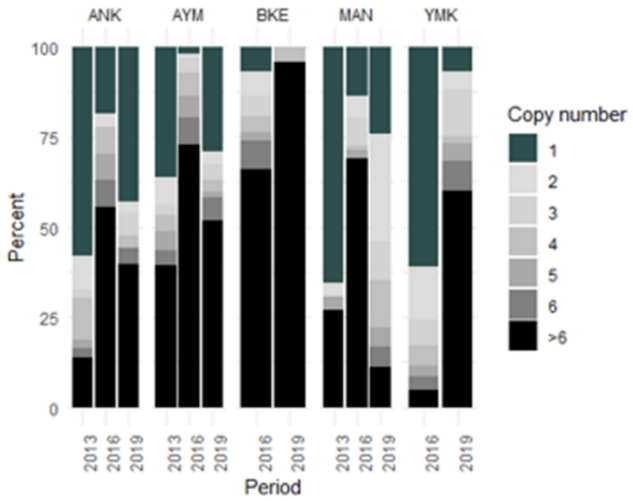

Figure 2. Distribution of exact number of copies per years per study sites.

Geographical distributions of isolates carrying multiple copies of *pfpm2* gene

The term "number of multiple copies" refers to isolates containing at least two copies of the *pfpm2* gene (Table 3). Figure 3 illustrates that these isolates are predominant across all the study sites, with the lowest and highest average proportions observed in Yamoussoukro (56.4% (95% CI: 49.0-64.0)) and Bouake (94.6% (95% CI: 88.2-97.8)), respectively. When the study sites are grouped by regions, as indicated in Table 2, a predominance of isolates carrying multiple copies is evident, with regional average proportions ranging from 66.3% (95% CI: 60.6-71.6) in the Central region to 74.8% (95% CI: 67.4-81.1) in the Western region.

Despite these regional disparities, no significant difference was observed in the average proportions of isolates carrying multiple copies of the *pfpm2* gene (*Chi-square*=4.0421; df=2; p-value=0.1325). The results of the geographical distribution analysis, both at the site and regional levels, highlight a pronounced predominance of alleles with multiple copies, with a scarcity of the single-copy allele in Bouake [3].

Copy number	ANK	AYM	MAN	BKE	YMK	*chi-square*	p-value
≤ 1	57.14 (n=76)	34.13 (n=86)	49.70 (n=83)	19.64 (n=22)	68.61 (n=129)	91.69	2.20E-16
2	9.02 (n=12)	14.46 (n=37)	8.38 (n=14)	9.82 (n=11)	9.57 (n=18)	5.79	0.21
3	5.26 (n=7)	7.14 (n=18)	8.38 (n=14)	15.17 (n=17)	5.31 (n=10)	11.57	0.02
4	5.26 (n=7)	2.38 (n=6)	4.79 (n=8)	6.25 (n=7)	2.65 (n=5)	5.01	0.28
5	2.25 (n=3)	3.57 (n=9)	4.19 (n=7)	8.92 (n=10)	2.65 (n=5)	9.17	0.06
6	2.25 (n=3)	2.38 (n=6)	2.99 (n=5)	7.14 (n=8)	2.12 (n=4)	7.54	0.1
>6	18.79 (n=25)	35.71 (n=90)	21.55 (n=36)	33.03 (n=37)	9.04 (n=17)	49.46	4.67E-10
Total	133	252	167	112	188	-	-

Table 3. Frequency of the exact number of copies of the Plasmepsin 2 gene per study site.

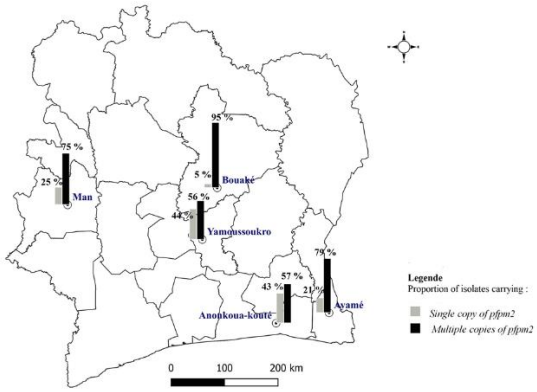

Figure 3. Geographical distribution of the exact number of copies of *pfpm2* gene.

Distribution by year of isolates carrying several copies of *pfpm2*

Distribution over the years of isolates carrying multiple copies of *pfpm-2* gene was analyzed following the geographical distribution of alleles. Figure 4 illustrates the average proportions of the studied alleles per year. In 2013, no statistically significant difference was observed between the proportions of isolates carrying multiple alleles and those carrying a single allele (*Chi-square*=0.3715; p-value=0.4747). The lowest average proportion (53%, 95% CI (45.42-61.11)) was observed in 2013, while it remained around 76% in both 2016 (95% CI (71.41-79.75)) and 2019 (95% CI (79.73-81.32)). A significant difference was found between the study years (Z-test: *Chi-square*=33.449; p-value=5.454e-8).

The trend test further confirms a significant increase over the years (Cochran-Armitage test: $p=3.97e^{-6}$; z=4.6129). Overall, the proportion of isolates carrying each allele was equal in 2013 before increasing and stabilizing at 76% in both 2016 and 2019.

However, the overall configuration of these data conceals more diverse local trends. For example, in Yamoussoukro, the proportions significantly increase over the years (Cochran-Armitage test; $p=2.65e^{-12}$; z=6.99), while in Man, the trend is rather decreasing (Cochran-Armitage test; p=0.0033; z=-2.93). At the Anonkoua-Kouté, Ayamé, and Bouaké sites, the Cochran-Armitage test is not significant (p=0.1907; z=1.30 for Anonkoua-Kouté, p=0.0706; z=-1.80 for Ayamé, and p=0.1885; z=-1.31 for Bouaké)

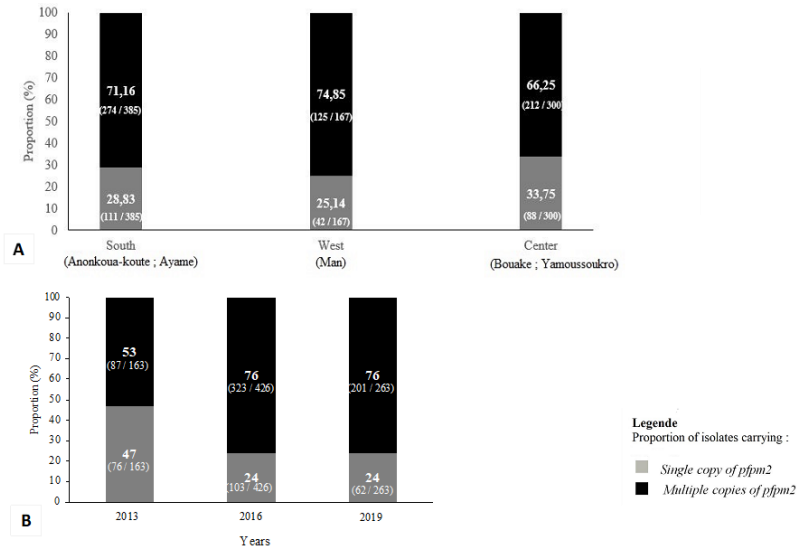

Figure 4. Variation in proportions of isolates carrying multiple copies of *pfpm2* gene. A. Geographical distribution of proportions of isolates carrying multiple copies of *pfpm2* gene. B. Distribution of proportions of isolates carrying multiple copies of *pfpm2* gene over the years. A significant upward trend in the prevalence of alleles with multiple copies was identified by comparing the proportions of multiple copies at the three time points (Cochran-Armitage test: $p=3.97\ e^{-6}$; z=4.6129)

DISCUSSION

Antimicrobial resistance is a major concern in global health due to the significant number of deaths resulting from compromised management of common infections (Murray et al., 2022). It is defined as "occurring when bacteria, viruses, fungi, and parasites evolve over time and no longer respond to drugs, making it more difficult to treat infections and increasing the risk of disease spread, severe forms of illnesses, and death" (WHO, 1967). Artemisinin and its derivatives, used in therapeutic combinations, have been the drugs of choice for malaria management recommended by the WHO since 2005 and are under surveillance (Bosman and Mendis, 2007; Nosten

and White, 2007). In these therapeutic combinations, the role of the first molecule, namely artemisinin or one of its derivatives, is to rapidly reduce the parasite biomass, while the associated drug aims to eliminate the remaining parasites after artemisinin has been cleared from the bloodstream. The emergence of Plasmodium falciparum isolates resistant to artemisinin (Dondorp et al., 2009) and then to artemisinin-based

combination therapies has been reported in Southeast Asia (Amato et al., 2017). In Côte d'Ivoire, the therapeutic arsenal has demonstrated clinical efficacy and includes the use of Artemether-Lumefantrine (AL) as the first-line treatment since 2005, with very good tolerance despite recent observations of delayed parasite clearance.

Furthermore, Dihydroartemisinin-Piperaquine (DHA-PPQ) was introduced as part of the Multiple First-Line Therapy (MFT) following a decision made by PNLP in 2018 (Arrêté N°190 MSHP/CAB of 27 Nov 2018). Given the current context of this strategy, it is essential to monitor the effectiveness of DHA-PPQ as resistance to this combination has been reported several times in sub-Saharan Africa. Indeed, this region has recorded its first strains partially resistant to artemisinin-based Combination Therapies (CTA) (Uwimana et al., 2020; Balikagala et al., 2021), specifically in Mozambique, Mali, Gabon, Burkina Faso, and Uganda (Ebong et al., 2021; Gansané et al., 2021; Moriarty et al., 2021).

The MFT strategy induces greater pharmacodynamic heterogeneity, which contributes to delaying the emergence and slowing down the fixation of already present resistant strains. This approach allows for treating a larger proportion of the population without compromising the future treatment of potentially incurable cases due to high levels of resistance. Consequently, clinical outcomes achieved through MFT are superior to those obtained with a single therapy or a therapy rotation strategy (Boni et al., 2008; Kaboré et al., 2023). This significantly improves the management of uncomplicated malaria in the country, treating 93% of cases in the public sector, 89% in the private sector, and 97% at the community level (PNLP Report, 2019). However, it is not guaranteed that MFT is always optimal when the therapies used exhibit low efficacy due to high drug resistance levels or a risk of decreasing sensitivity, as seen with DHA-PPQ (Leroy et al., 2019). Resistance to Piperaquine could pose a threat to the effectiveness of this approach (Kaboré et al., 2023).

In this study, we have analyzed the number of copies of the pfpm2 gene using the SYBR Green approach and quantitative PCR (Gupta et al., 2018; Inoue et al., 2018; Shrestha et al., 2021).

Since 2013, it has been observed that approximately 53% of P. falciparum isolates carried at least two copies of the *pfpm2* gene. This percentage significantly increased ($p=3.97\ e^{-6}$; $z=4.6129$) to reach a plateau of 76% in 2016 and 2019, corresponding to the period of adopting the combination in the MFT strategy and the deployment of DHA-PPQ in the country. Cases of isolates with duplications of *pfpm2* had previously been reported in Mali between 2011 and 2016, as well as in the West African sub-region (Inoue et al., 2018). In the three targeted regions (West, Center, and South of Côte d'Ivoire), the proportion of isolates with at least 2 copies of *pfpm2* ranged from approximately 66% to 75%. Some specific sites showed particular trends. For instance, in Man in the West region, a significant decrease in the prevalence of isolates with multiple copies was observed ($p=0.0033$ and $z=-2.93$), while in Yamoussoukro in the Center region, the trend was increasing ($p=2.65$ e-12; $z=6.99$) between 2016 and 2019. In Bouaké in 2019, all present isolates carried either 4 copies (approximately 5%) or more than 6 copies (95%) of the pfpm2 gene, representing 100% of isolates with multiple copies.

The high prevalence of isolates carrying multiple copies of pfpm2 across all study sites could be explained by the selection pressure from the use of piperaquine. Furthermore, the observed variations in prevalence could be influenced by internal population movements, which can alter local molecular epidemiology. It is worth noting that the molecule, in the form of bi or triple therapy combinations, was already known since 2006 in the West African sub-region, including Côte d'Ivoire (Menan et al., 2011; Yavo et al., 2011). This previous use of Piperaquine could have contributed to the emergence of isolates carrying multiple copies of *pfpm2*, raising concerns about potential resistance to this therapeutic combination in the country. Indeed, plasmepsin 2 plays a crucial role in *P. falciparum's* resistance mechanism to PPQ. The *pfpm2* gene (PF3D7_1408000), located on chromosome 14, encodes for a protease that participates in hemoglobin degradation (Inoue et al., 2018). This protease is responsible for eliminating the heme accumulated in the parasite following hemoglobin digestion. Heme is toxic to the parasite, but plasmepsin 2 transforms it into a non-toxic polymer called hemozoin. When there are multiple copies of the *pfpm2* gene, it leads to the synthesis of multiple plasmepsin II molecules, a mechanism that counteracts the effect of piperaquine, which aims to prevent heme detoxification (Small-Saunders et al., 2022).

In this study, blood samples from patients were collected using Dried Blood Spots (DBS) as a matrix. DBS have proven to be effective matrices for monitoring antimalarial resistance in endemic countries, as they do not require strict cold chain management and are relatively inexpensive, with a cost of approximately $0.15 USD per sample, compared to around $3.50 USD per sample using commercial kits (QIAamp DNA Blood Mini Kit). By utilizing DBS, we were able to archive a total of 852 samples (Hsiang et al., 2010; Holzschuh and Koepfli, 2022). The method of plasmodial DNA isolation using Tween 20/Chelex 100 was chosen for its cost-effectiveness in the surveillance of antimalarial resistance in our context. Unlike whole blood isolation or commercial kits, this method does not require a cold chain. Moreover, it is known for its high sensitivity, with a DNA recovery rate reaching 15 to 21%, even more sensitive than extractions from whole blood samples (Holzschuh and Koepfli, 2022). During this study, an average DNA concentration of 75.42 ng/mL (95% CI: 45.22-105.63)

with an average purity of 1.9 (95% CI: 1.73-2.07) was obtained.

CONCLUSION

This study revealed the presence of isolates carrying multiple copies of the *pfpm2* gene in five sentinel sites of the national malaria control program, prior to the introduction of DHA-PPQ as the first-line treatment and its deployment in the country. Over the period from 2013 to 2019, proportions of isolates with multiple copies of the *pfpm2* gene exceeding 50% were observed, with a significant downward trend in Man and an upward trend in Yamoussoukro. This situation raises concerns about potential resistance to this therapeutic combination in the country. Therefore, a prior analysis of molecular markers involved in PPQ resistance would have been opportune before the deployment of the DHA-PPQ combination, as its medium or long-term efficacy may be compromised, at least at these study sites.

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