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Full Length Research Paper

Genetic diversity and multiplicity of *Plasmodium falciparum* infections in an urban setting of perennial and intense malaria transmission in southern Côte d'Ivoire

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Abstract

Malaria is the primary cause of clinical diagnostic examination and the major human pathology in tropical areas worldwide. In Côte d'Ivoire, it represents the principal cause of population death and studies focusing on better understanding of the structure and extent of genetic diversity among malaria pathogen strains, using molecular-based DNA markers are limited. This study was conducted to assess the pattern of genetic variability of malaria parasites and multiplicity of infection in a panel of 64 *P. falciparum* isolates, using *msp-1* and *msp-2* markers. Nested PCR methods used for the isolates genotyping showed high variability among pathogen strains with a total of 25 shared alleles. As *msp-1* markers, K1 and MAD20 were highly polymorphic with five alleles in each other. FC27 and 3D7 respectively detected 7 and 6 different allele-sizes for *msp-2* markers. Furthermore, polyclonal infections were more frequent (92.20%) in infected patients and the mean value of *He* was 0.50 (*msp-1*) and 0.70 (*msp-2*). Relationships between MOI and patients' age were poor but, parasitaemia only was correlated with IC50-QN (r= -0.236). Cluster analysis categorized the isolates into four main groups. The cluster 4 was only consisted of a single isolate (W634) genetically distinct from isolates within the other clusters. This study provides useful information that could be used as a guide for the currently implemented programs of malaria control strategies.

Keywords: Chemosensitivity, genotyping, markers, molecular, *msp*, MOI, Wassakara, Yopougon.

Background

Malaria, caused by *Plasmodium falciparum*, is still one of the major public health concerns and the most damaging and devastating parasitic human disease in many tropical and subtropical areas around the world (Touray et al., 2020; Yang et al., 2021). The parasite, transmitted to people by an insect vector referred to as anopheles mosquitoes, can annually affect multiple vertebrate hosts and lead to the death of millions of people (Pumpaibool et al., 2009; Kouna, 2022). According to the recent report by World Health Organization (WHO) in 2020, more than 241 million infected persons are recorded each year. In 2020, the estimated number of people dying from malaria was about 627,000, which was increased

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up to 69,000 compared to 2019, making it the world's heading endemic and parasitic disease. In thirty-two Sub-Sahara African countries, malaria represents the main human mortality cause with about 93% of malaria-related deaths (OMS, 2021).

The improvement of current control strategies and intervention programs implemented through awareness-raising campaigns in Côte d'Ivoire, the use of long-lasting insecticidal nets (LLINs) freely distributed to the general population, free administration of intermittent preventive treatment during pregnancy with at least three doses of sulfadoxine-pyrimethamine (SP), and the control of subsequent spread of mosquitoes (destruction of mosquito breeding sites, and sanitation of the living environment) have contributed to slightly reduce the mortality rate from 3,340 to 3,104 in 2016 and 2020 (PNLP CI, 2020), respectively. However, these results remain fragile and not enough sufficient due to the low-rate use of LLINs, which accounted for only 63% of the general population instead of 80%, being the expected rate to get up a significant impact (PNLP CI, 2020), and the long-term use of artemisinin-based combination therapies (ACTs) since 2009 as first-line treatment drugs.

However, several authors have reported that malaria transmission to humans is caused by more than 70 different anopheles mosquito species (Molina-Cruz et al., 2016).

As a consequence, sexual reproduction involving the recombination events of genetically distinct gametes during the diploid phase of *P. falciparum* parasite's life circle in *Anopheles* could provide a high degree of haploid differentiation and broaden the genetic diversity of parasite populations (Aly et al., 2009; Bennink et al., 2016). This can occur when a mosquito feeds on the blood of an individual infected bearing multiple parasite strains or when the same mosquito feeds on the blood of more than one individual hosting distinct parasite strains (Soe et al., 2017). Factors that significantly affect the genetic variability and the patterns of population structure of the malaria parasite are strongly inherent to intense malaria transmission and epidemiological settings (Mara et al., 2013; Usman-Yamman et al., 2021). In human population living in highly endemic conditions, multiple forms of parasites, including novel genotypes evolving from parental parasite strains may be transmitted to a single or several individual hosts (Molina-Cruz et al., 2016). It has been reported that the genotypic and phenotypic diversity of malaria parasites enhances their ability to survive and thrive to control measures such as therapeutic drugs and to adapt and avoid the barriers to human immune system (Nsanzabana, 2019; Ullah et al., 2022).

In addition, the developed and highly complex genetic variability of the parasite also confers the ability to develop multiple drug resistance (Patgiri et al., 2019). Likewise, the non-compliance with effective doses of antimalarial drugs in many poor and developing countries and their frequent applications could be the causes of key mutations in the genome of *P. falciparum* and the occurrence of parasite's resistant strains (Trebissou et al., 2014; Zhao et al., 2021). Thus, the emergence and spreading of resistant strains counteract efforts to control malaria in many parts of the world, especially in African countries (Bogreau et al., 2006; Tuedom et al., 2021).

Analyses of *P. falciparum* genetic diversity are usually focused on the applications of putative neutral molecular markers such as microsatellites (Mohd Abd Razak et al., 2016), single nucleotide polymorphism (SNP) based on minor genome variations within the parasite population (Neafsey et al., 2012; Papa Mze et al., 2020), PCR-restriction fragment length polymorphism (PCR-RFLP) (Mara et al., 2013; Santamaría et al., 2020) and molecular typing of the polymorphic regions of merozoite surface protein (Yavo et al., 2016; Ullah et al., 2022). Among these, the asexual blood antigens also known as merozoite surface protein 1 (*msp-1*) and merozoite surface protein 2 (*msp-2*) are the most widely and attractive candidates markers used for assessing the genetic diversity and multiplicity of infection of *P. falciparum* population (Yavo et al., 2016; Ullah et al., 2022). These genetic markers or single copy genes with extensive polymorphism, both in sequences and sizes, which is mostly generated by intragenic repeats that are variable in copy number and length of the repeat unit and located on different chromosomes (Ghanchi et al., 2010). They have known to be useful tools both in molecular epidemiology investigations and the ability to detect treatment failures from new infections in anti-malarial drug clinical trials (Blackman et al., 1990; Cowman & Crabb, 2006; Boyle et al., 2014; Usman-Yamman et al., 2021). Furthermore, *msp-1* and *msp-2* are the highly expressed parasitic genes on the merozoite surface (Mustafa et al., 2017), which is an important advantage for the genetic characterization purposes of clones using these protein-types, and the development of a fully effective malaria vaccine (Tanabé et al., 1998; Ullah et al., 2022). Updating the centralized archiving of the collected data could help to efficiently understand better the biological mechanisms of the occurrence of different *msp-1* and *msp-2* haplotypes (Oboh et al., 2020) and the dynamics of gene flow mobility.

The urbanization rate in Sub-Sahara African countries remains high and impedes malaria transmission. Hence, increasing the number of non-immune individuals with most being at risk of contracting potentially severe forms of the disease (Bogreau et al., 2006). Unfortunately, Yopougon, one of the most populated and the largest district of Abidjan, harbours several slum areas with poor urban sanitation facilities. These precarious urban contexts are suitable for malaria transmission as revealed by previous studies carried out in the district of Yopougon (Wang et al., 2006; Koné et al., 2015). Moreover, its strategic position in the borders of "Ebrié" lagoon makes it the main focus of sub-regional immigration where a large workforce from neighbouring countries such as Mali and Burkina Faso continuously settle down in the tertiary sector or to develop economic activities. Pumpaibool et al. (2009) and Ullah et al. (2022) have reported that *P. falciparum* transmission rate and the migration of infected human inhabitants, which differ in each endemic area, could affect the

genetic variation and the structure of the parasite population. Many investigations have indicated that intensified control efforts could result in the reduction of genetic diversity of the parasite populations and in some cases lead to a clonal structure of the parasite. Furthermore, certain antimalarial drugs extensively used in the disease management strategies can exhibit selective controls and alter the genetic diversity of *P. falciparum* species (Soe et al., 2017). As earlier stated, the assessment of the pattern of genetic variability within the malaria parasite population using *msp-1* and *msp-2* markers is essential to deepen the understanding of the populations structure (Usman-Yamman et al., 2021) and to identify new target of interest that can be used to develop new drugs or vaccines and efficiently manage the spreading of parasite (Yang et al., 2021). It is, therefore, necessary to carry out a thorough characterization of the parasite isolates collected in Côte d'Ivoire for disease control purposes.

This present study was carried out to investigate the genetic diversity and multiplicity of *P. falciparum* infections among infected patients. The study should provide useful data for monitoring parasite transmission dynamics and effective evaluation of the impact of the current implemented malaria control interventions in the country.

MATERIALS AND METHODS

Study site

The study was carried out in Yopougon, a district of Abidjan, the economic capital of Côte d'Ivoire, located between 2° and 9° West longitude and 4° and 11° North latitude (Koné et al., 2015). Globally, Abidjan is located in a malaria endemic region, the southern forests and coast areas of Côte d'Ivoire with a warm and humid tropical climate. Malaria occurs all the year with high transmission during the rainy seasons. More than 95% of infections are caused *P. falciparum* (MSHP et PNLP, 2019). Yopougon is one of the largest and populated district of Abidjan with a surface of 153 km^2. It is limited by the "Banco" Forest in the northwest and the "Ebrié" Lagoon in the south and south-eastern part. The population is estimated to 1,071,543 (7004 inhabitants per km^2) (INS, 2014). The urban community-based health facility (FSU-COM) of "Wassakara" in Yopougon was selected for patient malaria diagnostic test (Figure 1). According to previous studies, malaria is the primary cause for clinical examination in patients coming for consultation at the "Wassakara" FSU-COM. High infections rate in febrile patients are malaria-related (Wang et al., 1997; Koné et al., 2015; Yavo et al., 2016). The peak of patient attendance for consultation was positively correlated with malaria cases during rainfall period in June (N. Cécilia, Université Lille 2, France, Rapport de stage de fin de 5ème année de Pharmacie).

MATERIALS

Biological material is made of 64 *P. falciparum* clinical isolates collected from infected patient blood who came for a medical diagnostic test during the period of the *ex-vivo* monitoring of malaria susceptibility from February 2014 to August 2015 in FSU-COM Wassakara. The patients retained also were tested positive for asexual *P. falciparum* monoinfection and at least 18-year-old.

METHODS

Blood sample collection

Blood samples were collected from febrile patients with axillary temperature ≥ 37.5°C. All patients were residents of the Yopougon district and freely consented to the study conditions by signing the informed consent form. The blood sample collections were carried out using the finger prick method and a rapid diagnostic test kit (CareStart Malaria HRP2/PLDH (Pf/VOM) COMBO) was used for the detection of *P. falciparum*, the causal agent of malaria disease. The whole venous blood (4 ml) was collected from each tested positive patient to an asexual *P. falciparum* monoinfection and transferred into a 15 ml Vacutainer® tubes (BD-367844 LH) previously containing Ethylene Diamine TetraAcetic (EDTA). Afterwards, patients were taken into account for malaria disease and supported free from any charge treatments. The collected samples were stored on dry ice at 4 °C and taken to the molecular biological laboratory of CSRS (Centre Suisse de Recherches Scientifiques) for DNA extraction and PCR-amplification using nested methods.

Parasitaemia analysis

The Parasitaemia was determined using a microscopic observation test of each patient's blood samples. Thick and thin blood smears were prepared and analysed according to the WHO protocol for blood film preparation with 10% Giemsa staining (WHO, 2016). The parasite density estimate was performed using the number of asexual forms of the *P. falciparum* as described by Silué *et al*., (Silué et al., 2008). The blood samples were then spotted onto filter paper (Whatman® FTA™ Mini Card whatman™) and dried at the conditions of room temperature for *msp-1* and *msp-2* gene analysis.

Extraction of *Plasmodium falciparum* DNA

Plasmodium Genomic-DNA was extracted and purified from filter paper dried blood spots using Zymo "Quick-gDNA™ Blood Mini prep extraction kit" (Zymo Research D3024, Inqaba Biotec West Africa Ltd, Ghana Branch) according to the manufacturer's instructions. At the end of the extraction process, 100 µl volume of all parasite's DNA extracts were stored at -20°C.

Genotyping of *P. falciparum* isolates

The genotyping was performed using each parasite DNA extracts and merozoite surface protein (*msp-1* and *2*) markers. The conserved polymorphic regions, block 2 of *msp-1* and block 3 of *msp-2* genes were amplified using nested PCR methods (Gnagne et al., 2019). The sequences of each primer used in the study are shown in Table 1. The nested PCR methods were carried out in two distinct enzymatic reactions or amplifications.

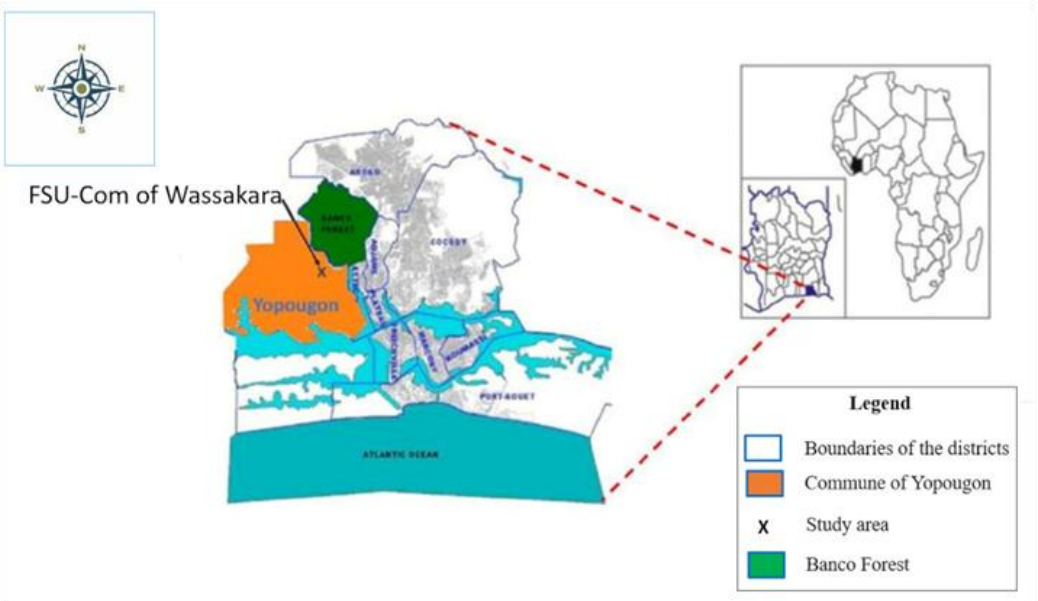

Figure 1. Location of the community-based urban health facility (FSU-COM) of Wassakara.

Table 1. Sequences of primers used for typing of polymorphic regions of *P. Falciparum* isolates using *Pfmsp-1* and *Pfmsp-2* gene loci

MSP	Amplification PCR	Primer	Allele sequence	Allele size (pb)
msp-1				
	PCR1			
		Ml-OF	CTAGAAGCTTTAGAAGATGCAGTATTG	27
		Ml-OR	CTTTGTTACCATCGGTAATTCTT	23
	Nested			
	K1	M1-KF	AAATGAAGAAGAAATTACTACAAAAGGTGC	30
		M1-KR	GCTTGCATCAGCTGGAGGGCTTGCACCA	28
	MAD20	M1-MF	AAATGAAGGAACAAGTGGAACAGCTGTTAC	30
		M1-MR	ATCTGAAGGATTTGTACGTCTTGAATTACC	30
	RO33	M1-RF	TAAAGGATGGAGCAAATACTCAAGTTGTTG	30
		M1-RR	CATCTGAAGGATTTGCAGCACCTGGAGATC	30
msp-2				
	PCR1			
		M2-OF	ATGAAGGTAATTAAAACATTGTCTATTATA	30
		M2-OR	CTTTGTTACCATCGGTACATTCTT	24
	Nested			
	FC27	M2-FCF	AATACTAAGAGTGTAGGTGCARATGCTCCA	29
		M2-FCR	TTTTATTTGGTGCATTGCCAGAACTTGAAC	30
	3D7	M2-ICF	AGAAGTATGGCAGAAAGTAAKCCTYCTACT	28
		M2-ICR	GATTGTAATTCGGGGGATTCAGTTTGTTCG	30

In the first-round reaction, the mixed template consisted of 2.5 µl (each pair of primer *msp-1* and *-2*), five µl Taq DNA Polymerase (5x HotFirepol MasterMix) and five µl genomic DNA in a reaction final volume of 25 µl. In reaction 2, found to

be more specific to the targeted genomic DNA regions, two µl of the first PCR product was used as a template. The volume of *msp-1* and *msp-2* specific primers were used as previously described. DNA amplifications were performed in a *Mastercycler Gradient 5331* (Eppendorf, Hamburg, Germany) using the following program: an initial denaturation for 5 min at 95 °C; 31 cycles of denaturation for 15 s at 95 °C, 62 °C annealing for 90 s and extension at 72 °C for 2 min; and a final extension for 10 min. The nested PCR products were separated in 1.5% agarose gels and stained with 0.5 μg/mL of ethidium bromide. The PCR product spots were visualized under UV transillumination (BioDoc-It^{TM} Image System) and the allele sizes were estimated using a reference 100 bp molecular marker size ladder.

Drug susceptibility assay

The infected erythrocytes were washed three times in RPMI 1640 medium (Invitrogen, 51800-043). Samples with parasitemia rates ranging from 0.1 to 0.3% were used for drug susceptibility tests (Trager & Jensen, 1976). If the parasitemia rate exceed 0.3%, a dilution was needed using a proper volume of uninfected blood (O^+). Parasites were incubated at 37°C in candle jars for 72h with drug serial dilutions and SYBR Green was used to measure the parasite growth inhibition. Parasite sensitivity to eight drugs from *Medicines for Malaria Venture* (MMV), including amodiaquine (AQ), artesunate (AS), chloroquine (CQ), piperaquine (PIP), lumefantrine (LUM), pyrimethamine (PYR), pyronaridine (PYN) and quinine (QN) was assessed in 96-deep well PCR plates. The inhibitory concentration at 50% or IC50 was used to determine the effectiveness of e*x-vivo* test of each antimalarial drug (Table 2). The IC50 was calculated using a non-linear regression model with *In-Vitro Analysis and Reporting Tool* (IVART) software (Le Nagard et al., 2011).

Data analysis

The data were analyzed to determine the distribution of *msp-1* and *msp-2* allelic families in an infected human host using the PCR products. The allele distribution was graphically visualized with GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA, www.graphpad.com). The electrophoretic profiles of each allele on 1.5% agarose gel were used to assess the monoclonal and polyclonal infection rates in the analysed isolates. Samples were considered monoclonal infections when only one PCR-amplified product was detected from each of the loci. In contrast, multiple infections were defined when the isolate samples have more than one PCR amplified allele-fragments. The multiplicity of infection (MOI) was computed by dividing the total number of detected *P. falciparum msp-1* and *msp-2* genotypes by the total number of infected patients (Yavo et al., 2016). The Spearman's rank correlation coefficients (rho) were estimated to assess the linear relationship between parasitaemia, age, IC50 and MOI (*msp-1* and *msp-2*) using *corrplot* packages implemented in R version 4.1.2. A correlation was considered significant at the threshold of 0.05 (*P value ≤ 0.05*). The genetic variability of the parasite population was determined through the allelic richness or expected heterozygosity (*He*) defined as the probability that two randomly selected clones from a population will carry distinct alleles at each marker loci. *He* was calculated according to the following formula:
He = [n/ (n - 1)] [(1 - Σpi2)] (Nei, 1978), where n is the number of *P. falciparum* isolates analysed (sample size) and pi represents the frequency of each different allele at a locus in the total population.
The values of expected heterozygosity (from 0 to 1) were used to identify the pattern of genetic diversity in the parasite populations.
Thus, the *P. falciparum* population was considered not diversified when the value of *He* is 0 and highly diversified for the value of 1. Data from the allele profiles were also used to classify the *P. falciparum* population into different homogenous clusters. The presence or absence of each allele was coded as 1 or 0 and scored in a binary data matrix. A genetic distance matrix was generated using the Euclidean method with a *dist* function in R. The cluster analysis was performed based on the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) methods using the *dendextend* packages of R (Galili, 2015). Genetic diversity among the identified clusters was assessed through the number of different alleles (nA), the number of alleles per marker (nA/marker) and allele sizes.

RESULTS

Study patients' characteristics

A total of 64 *P. falciparum* isolates were collected from infected patients who visited the health centre of FSU-COM Wassakara for medical diagnostic during the *ex-vivo* monitoring of malaria susceptibility from February 2014 to August 2015. Patients essentially were female sex with 57.80% and 42.20% for male. All patients were positive or strict adherence to the Rapid Diagnostic Test (RDT) and microscopy test. The sex ratio was 0.73 and patients' age ranged from 18 to 69-year-old with a mean of 28.45. The parasite density varied from 112 to 401320 P/µl with a geometric mean of 15652 P/µl in the total population (Table 3).

Genetic polymorphism of *msp-1* and *msp-2* genes

Nested PCR consisted of specific amplification of the three allelic families K1, RO33, and MAD20 of *msp-1* gene and two (IC/3D7 and FC27) of *msp-2* gene in this study. A total of three hundred and thirty-seven fragments or genotypes were identified for the two genes *msp-1* (177 different genotypes) and *msp-2* (160 genotypes) within the 64 *P. falciparum* isolates. These genotypes only had 25 shared alleles with an average of five alleles per locus. The *msp-1*

Table 2. Inhibitory concentration thresholds of antimalarial drugs at 50% or IC50.

Compounds		AQ	AS	CQ	PIP	LUM	PYR	PYN	QN
thresholds (nM)	CI_{50}	40	40	100	80	100	100	100	400

AQ : ammodiaquine ; AS : artesunate ; CQ : chloroquine ; PIP : piperaquine ; LUM : lumefantrine ; PYR : pyrimethamine ; PYN : pyronaridine et QN : quinine.

Table 3. Characteristics of the study population.

Patients' sex		Patients' ages			Parasitaemia		
Women	Men	mini	maxi	mean	Pd (P/µl)	Gm	IC 95%
57.8%	42.2%	18	69	28,45	112-401320	15652	[9715; 25216]

Mini: Minimum; Maxi: Maximum; Pd: Parasite density; Gm: Geometric mean; IC: Interval of confidence.

gene showed 53 K1-genotypes with five different allele sizes (100, 150, 200, 500 and 700 bp). Two alleles (K1 500 and K1 700 bp) were identified as minor with frequencies of 0.04 and 0.02 (minor allele frequencies or MAF <0.05) and the most frequent allele was K1 200 bp (Figure 2a). With RO33, 58 genotypes were identified. Only two alleles (160 and 1000 bp) were common to these genotypes (Figure 2b). For MAD20, a total of 66 genotypes were detected with 150, 250, 500, 800 and 1000 bp as allele sizes. Only MAD20 250 pb was a minor allele with a frequency of 0.03 (Figure 2c). The *msp-2* gene was able to identify 68 3D7-genotypes with allele sizes ranging from 300 to 800 bp and 92 FC27-genotypes with allele sizes varying from 100 to 700 bp. The minor alleles were 400 pb and 100 pb for 3D7 and FC27, respectively (Figure 2d and 2e).

Monoclonal and of multiple infections detected in the 64 *P. falciparum* isolates

The *msp-1* and *msp-2* gene amplification products were successful with clearly detectable allele-profiles in 89.06 and 96.88% of the 64 *P. falciparum* isolates, respectively.
Monoclonal infection rate for *msp-1* and *msp-2* respectively cumulated a total of 15.80 (3.50% K1, 3.50% RO33 and 8.80% MAD20) and 16.10% (12.90% IC/3D7 and 3.20% FC27). The multiple infection rates of *msp-1* cumulatively accounted for 84.20% (7.00% K1+Ro33, 12.30% K1+MAD20, 14.00% Ro33+MAD20 and 50.90% K1+Ro33+MAD20) and the multiplicity of infection (MOI) was 3.11. With respect to *msp-2*, 83.90% of infections were polyclonal and the MOI value was 2.58. Overall, the mean value of MOI (*msp-1+msp-2*) was 2.85 and 92.20% of samples were polyclonal infections (Table 4; Figure 3). The allelic richness or *He* was high for the *msp-1* and *msp-2* genes with respective values of 0.5 and 0.7 and an average of 0.6.

Relationship between patients' age, MOI (*msp-1* and *msp-2*), parasitaemia and IC50

Spearman's r correlation coefficient was no significant between patient's ages and parasitaemia (r = -0.087; *P value* = 0.493). Furthermore, there was any significant correlation between patients' parasitaemia and IC50s, with the exception of the IC50 of quinine (QN), for which the correlation was negative with the value of -0.236. The correlation between the multiplicity of infection (*msp-1* and *msp-2*) and parasitaemia also was not statistically significant. The drug concentrations needed for the inhibition of 50% parasites or IC50 of piperaquine (PIP) were significant with IC50_AQ (r=0.534, P=0.007), IC50_QN (r= 0.493; p= 0.018) and IC50_PYN (r= 0.731; p<0.05). The relationship between IC50_PYN and IC50_AQ was positive and significant (r=0.465, p=0.021) (Table 5 and S1).

Hierarchical Clustering analysis of *P. falciparum* isolates using molecular data from *msp-1* and *msp-2* markers

Cluster analysis of the 64 *P. falciparum* isolates, based on similarities of their genomic regions and the UPGMA method, revealed four main clusters at about 10.5 Euclidean distance units. The isolates within cluster 1, 2 and 3 were more genetically similar, but distinct from W634, the only member of the cluster 4 (Figure 4).
Cluster 1 was the largest group with 55 isolates (85.93%) and cluster 4 only accounted for 1 isolate (1.56%). Clusters 2 and 3 consisted of 2 and 6 isolates, respectively. A total of 25 alleles were detected from the 64 *P. falciparum* isolates using

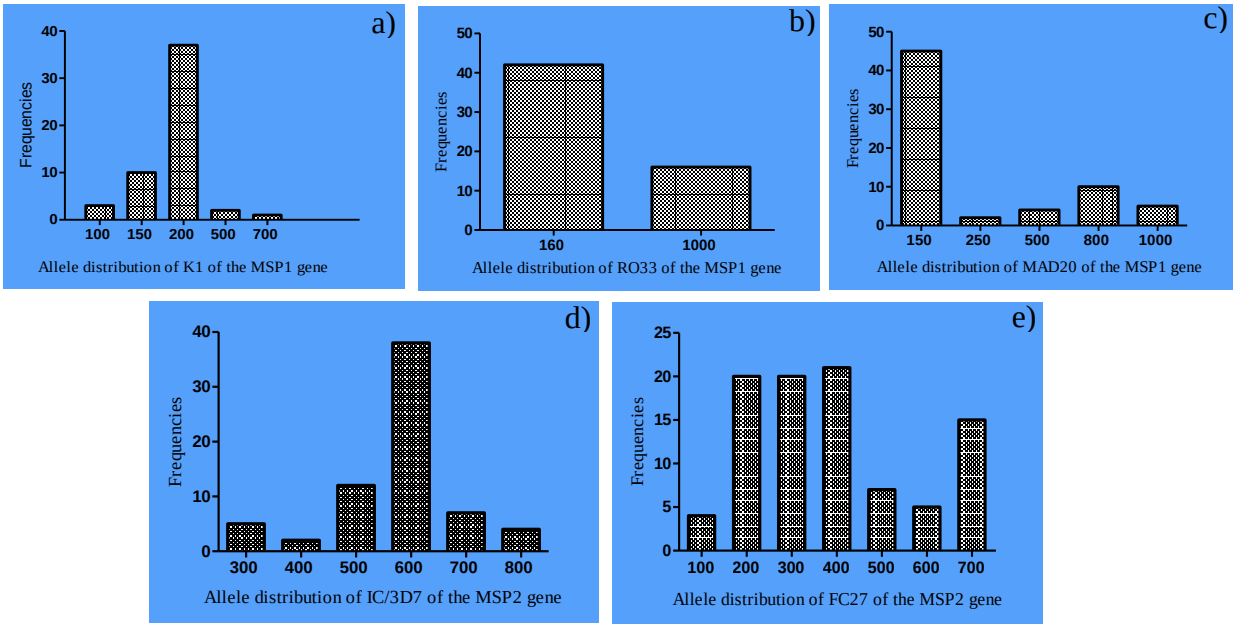

Figure 2. Distribution of msp-1 (a, b and c) and msp-2 (d and e) allele in the total population.

Table 4. Proportion of multiple and monoclonal infections within the 64 *P. falciparum* isolates.

Polyclonal infection				**Monoclonal infection**					
Haplotypes	N	Total	Proportion (%)	Alleles	N	Total	Proportion (%)	*He*	MOI
K1/RO33	4	57	7.00	K1	2	57	3.50	-	-
K1/MAD20	7	57	12.30	RO33	2	57	3.50	-	-
RO33/MAD20	8	57	14.00	MAD20	5	57	8.80	-	-
K1/RO33/MAD20	29	57	50.90	*msp-1*	8	57	15.80	-	-
msp-1	48	57	84.20	IC1/3D7	8	62	12.90	0.50	3.11
msp-2	52	62	83.90	FC27	2	62	3.20	0.70	2.58
msp-1+msp-2	59	64	92.20	*msp-2*	10	62	16.10	-	2.84

N: Number of isolates; He: Expected heterozygosity; MOI: Multiplicity of infection.

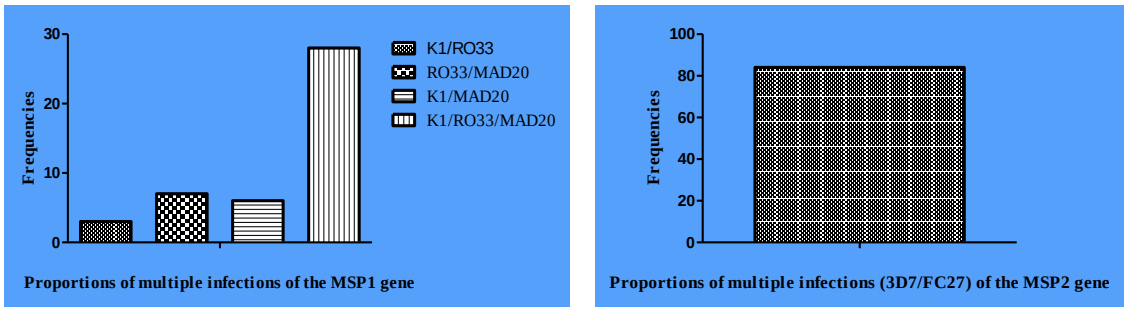

Figure 3. Proportion (%) of multiple infections of *msp-1* and *msp-2* genes.

msp-1 and *msp-2* markers.

In cluster 1, 22 of the 25 alleles (88%) were identified, including 10 alleles for *msp-1* marker and 12 with *msp-2*. With the *msp-1* markers, four different alleles were found using each gene locus KI and MAD20, and only two with RO33 marker. The allele sizes ranged from 100 to 500 bp (K1), 160

Table 5. Spearman's rank correlation coefficients between age, parasitaemia, MOI and IC50.

Parameter	Pd	Age	msp1	msp2	CI50_ AQ	CI50_ AS	CI50_ CQ	CI50_ PIP	CI50_ LUM	CI50_ PYR	CI50_ PYN	CI50_ QN
Pd	1											
Age	-0.087	1										
msp1	0.015	0.339	1									
msp2	-0.035	-0.119	0.295	1								
CI50_AQ	-0.114	0.088	0.073	0.140	1							
CI50_AS	0.028	-0.093	0.107	0.102	0.288	1						
CI50_CQ	0.104	-0.002	-0.047	0.068	0.220	0.254	1					
CI50_PIP	-0.034	0.042	0.028	0.130	**0.534**	0.260	0.266	1				
CI50_LUM	-0.105	0.039	0.070	-0.099	0.122	0.007	-0.032	0.182	1			
CI50_PYR	-0.062	0.191	0.214	0.255	0.331	-0.077	0.026	0.172	0.155	1		
CI50_PYN	-0.034	0.066	0.152	0.250	**0.465**	0.150	0.198	**0.731**	0.179	0.302	1	
CI50_QN	**-0.236**	-0.065	0.097	0.220	**0.432**	0.383	0.124	**0.493**	0.127	0.347	**0.437**	1

Pd: parasite density or parasitaemia.

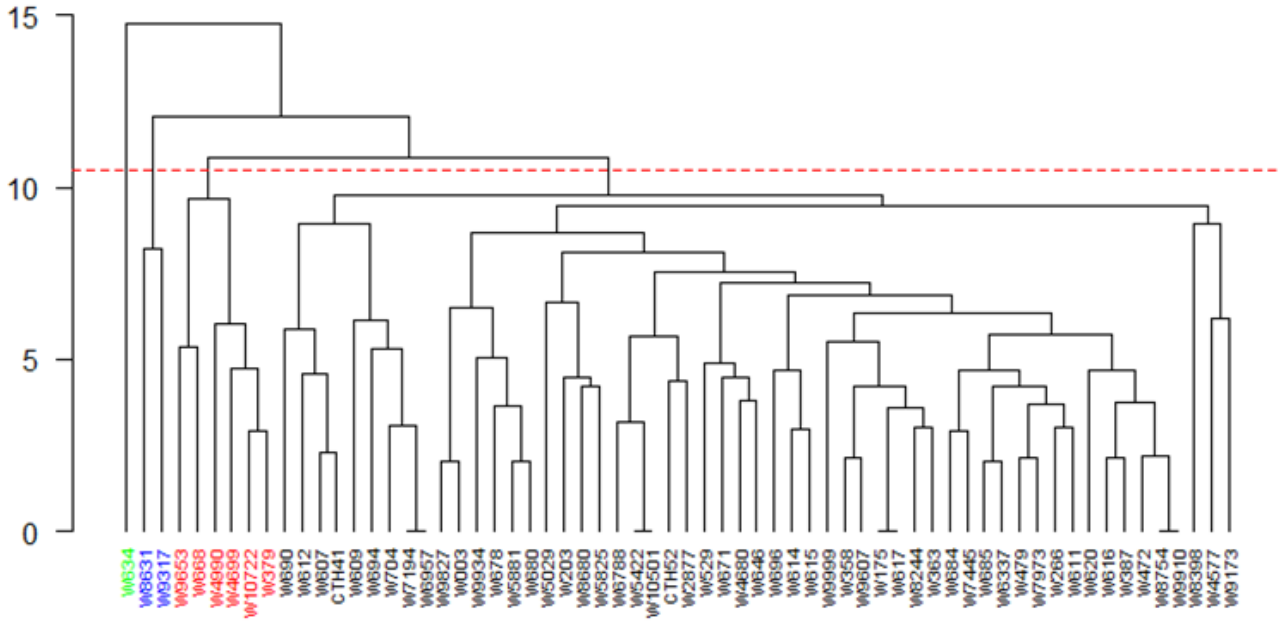

Figure 4. Genetic relationships among 64 *P. falciparum* isolates based on molecular data of *msp-1* and *msp-2* markers and UPGMA methods. Cluster 1 (black); cluster 2 (blue); cluster 3 (red); and cluster 4 (green).

to 1000 bp (RO33) and 150 to 800 bp (MAD20). 3D7 and FC27 of the *msp-2* marker revealed five and seven different alleles, respectively. The sizes of these alleles ranged from 300 to 800 bp (3D7) and 100 to 700 bp (FC27). The minimum and the maximum number of alleles per locus respectively were 0.2 and 2. All isolates in cluster 1 revealed an extensive fragment length polymorphism of the *msp-1* and *msp-2* genes (Table 6).

Cluster 2 recorded nine alleles (36%) in the total population. K1 of *msp-1* gene loci accounted for two different alleles, while RO33 and MAD20 revealed only a single allele each. The K1 allele fragment sizes were 100 and 150 bp and 160 bp for RO33 and 250 bp for MAD20. 3D7 and FC27 markers identified two (500 and 600 pb) and three alleles (100, 400, and 700 pb), respectively. MAD20-250 pb was the private allele only detected in this cluster (Table 6).

In cluster 3, 13 alleles that represent 52% of the total detected alleles were identified with an average of 2.6 alleles per locus. Different alleles were found in this cluster with *msp-1* markers. K1 and MAD20 each revealed two alleles, which measured

Table 6. Cluster characteristics based on the number and allele size using *msp-1* and *msp-2* markers.

Group	Ni	Number of alleles					nA	nA/ marker	Allele size (bp)				
		msp-1			*msp-2*				*msp-1*			*msp-2*	
		K1	RO33	MAD20	3D7	FC27			K1	RO33	MAD20	3D7	FC27
Groupe 1	55	4	2	4	5	7	22	4.4	100-700	160-1000	150-800	300-800	100-700
Groupe 2	2	2	1	1	2	3	9	1.8	100-150	160	250	500-600	100-700
Groupe 3	6	2	1	2	4	4	13	2.6	150-200	160	150-800	400-700	200-600
Groupe 4	1	3	2	1	2	1	9	1.8	200-500	160-1000	150	300-600	400
Total population		5	2	5	6	7	25	5.0	100-700	160-1000	150-1000	300-800	100-700

Ni: Number of isolates; nA: Number of different alleles; and nA/marker: Number of different alleles per locus.

150 and 200 bp, and 150 and 800 bp, respectively. RO33 only detected one allele (160 bp). The *msp-2* markers including 3D7 and FC27 respectively detected four alleles with sizes ranging from 400 to700 bp and from 200 to 600 bp. The number of alleles per locus for each isolate ranged from to 0.4 and 1.8 (Table 6).

Finally, cluster 4 only represents one isolate (W634) and recorded a total of nine alleles, of which six were *msp-1* alleles and 3 *msp-2* alleles with an average number of 1.8 alleles per locus (Table 6 and S2).

Mono and polyclonal infection rates in each cluster defined

Monoclonal infection using the *msp-1* gene in cluster 1 was 27.27% and multiple infections rate represented 72.73% including 25.45% of dimorphic infections with K1/RO33 (5.45%), K1/MAD20 (7.27%) and RO33/MAD20 (12.73%), and 41.82% trimorphic infections (Table 7). The MOI was 2.61 and the *msp-2* gene was detected in 83.02% of samples, which were jointly infected by more than one parasite strain using 3D7 and FC27 allele profiles. The MOI with *msp-2* gene loci was 2.41. In cluster 2, all infections were polyclonal (Table 7). The K1/MAD20 and K1/RO33/MAD20 genotypes each accounted for 50% of malaria infections. The average multiplicity of *P. falciparum* isolates per infected patient (MOI) was 3 and 2.5 for the *msp-1* and *msp-2* genes respectively (Table 7).

Monoclonal infection rates were 33.33% in cluster 3 and 66.67% of polyclonal infections for *msp-1* gene. The multiple infections were made of 16.67% dimorphic genotype (K1/MAD20) and 50% of isolate samples were infected by three different malaria parasite strains or trimorphic infections. The *msp-2* gene detected 16.67% and 83.33% of mono and polyclonal infections, respectively. The *msp-1* and *msp-2* gene loci respectively recorded 3.33 and 3.16 as the multiplicity of infection rates.

All infections in cluster 4 were polyclonal for both genes and multiple infections in the total population represented 84.20 and 83.90 % using *msp-1* and *msp-2* genes, respectively (Table 7).

DISCUSSION

P. falciparum isolates, the causal agent of the most severe form of malaria, were collected from infected patients at the earlier disease growth stage in Yopougon, the commune of Abidjan with the highest population density. The structure and pattern of genetic polymorphism in 64 genotyped isolates, and the multiplicity of infection was assessed using molecular data of *msp-1* and *msp-2* genes. This study revealed high genetic variability among malaria tested isolates with an important allele richness. Similar results have been previously reported in Côte d'Ivoire and elsewhere in Africa (Yavo et al., 2016; Mohammed et al., 2018; Somé et al., 2018; Gnagne et al., 2019). The high allelic variability among collected isolates could be due to the sexual reproduction mode of malaria pathogens during the plasmodium life cycle in the Anopheles midgut, the disease vector. Indeed, the parasite life cycle is initiated when *Anopheles* female blood meal contains both male and female *Plasmodium* gametocytes with diverse genetic backgrounds, creating genetically different multiple zygotes after recombination events. In mosquito midgut basal lamina, the zygotes are transformed into ookinetes and then into oocysts, and release thousands of different sporozoites into mosquito haemolymphs, which will be later transmitted to mosquito during a blood meal (Molina-Cruz et al., 2016). In some cases, a mosquito can feed on the blood of more than one individual hosting distinct parasite strains (Soe et al., 2017) or multiple mosquitoes can transmit to hosts different strains of the parasite. As result, high frequencies of several new isoforms of allelic subfamilies of *msp-1* and *msp-2* could be introduced by intragenic recombination events during crossing over in meiotic cell division and provide a rich haploid state differentiation, thereby broadening the genetic diversity

Table 7. Proportion (%) of multiple infections of *P. falciparum* isolates per cluster.

Cluster	Infection rate (%)					
	msp-1			*msp-2*		
	Monoclonal	Polyclonal	MOI	Monoclonal	Polyclonal	MOI
Cluster 1	27,27	72.73	2.61	16.96	83.02	2.41
Cluster 2	00.00	100.00	3.00	00.00	100.00	2.50
Cluster 3	33.33	66.67	3.33	16.67	83.33	3.16
Cluster 4	00.00	100.00	5.00	00.00	100.00	3.00
Total population	15.80	84.20	3.11	16.10	83.90	2.58

MOI: multiplicity of infection.

of parasite populations (Jongwutiwes et al., 1992; Aly et al., 2009; Bennink et al., 2016; Touray et al., 2020). The stratification of the clinical isolate population into four main clusters using hierarchical clustering analysis also confirmed the important variability of the pathogen isolates in Yopougon. Each cluster would be made of parasite isolates that were genetically similar in their genome. However, the isolates from clusters 1, 2 and 3 could be genetically closer to each other. The isolate W634 collected from a single individual host was the only member of the cluster 4. It was more divergent from the parasite strains within the other clusters at the molecular level. This isolate could be derived from particular gametocytes (male and female) with alleles that may be different in sizes and sequences from those of the gametocytes of the other isolates. These findings suggest that isolates among clusters were genetically divergent in the genome and multiple parasite strains could be transmitted to human hosts by different anopheles mosquito species or by mosquitoes harbouring different genetic strains of the malaria parasite (Molina-Cruz et al., 2016; Soe et al., 2017). Thus, several mosquito species should be potential vectors and responsible for malaria transmission in the municipality of Yopougon and the current set of malaria strains could be considered highly valuable for the disease management and effective use of drugs and vaccines.

In addition, the higher parasitaemia rate in Yopougon suggested that this commune of Abidjan could be considered an intense endemic malaria area, where several infected mosquitoes can transmit the disease to a single healthy person host (Mara et al., 2013; Yavo et al., 2016; Gnagne et al., 2019). It has been previously observed that the malaria disease epidemiology in the Yopougon area was moderate (mesoendemic area) (Wang et al., 2006; Koné et al., 2015). During the last two decades, it has evolved into a hyperendemic malaria area (Dagnogo et al., 2020). This new endemic condition in this southern area of Côte d'Ivoire could demonstrate the implication of a favourable environmental mutation for the parasite spread and growth, making it possible for the appearance of new isolate strains. Therefore, it increases the parasite genetic polymorphisms associated with gene flow dynamics through the community density in Yopougon. This was in line with parasite's prevalence and different patterns recorded in two localities (Obom and Asutsuare) of Ghana (Abukari et al., 2019). Furthermore, Yopougon has an increasing dynamics population density and is located on the borders of "Ebrié" lagoon. This geographic and strategic location allowed a daily movement of populations, which are permanently in interaction with other communities that came from neighbouring foreign countries. Yakubu et al. (2019) and Ullah et al. (2022) also reported that areas with a higher human migration could record high allelic frequencies and an important prevalence of allele combinations. On the other hand, a low and stable malaria transmission with a low genetic diversity has been observed in the eastern forest areas of Malaysia, (Mohd Abd Razak et al., 2016).

Another factor to the substantial genetic diversity of parasite strains or isolates is the occurrence of some mutations due to the abuse use of drugs to treat malaria in the disease endemic settings (Mairet, 2020). In Southeast Asia, resistance to artemisinin-based combination therapies CTAs was previously reported (Ariey et al., 2014; Thuy-Nhien et al., 2017). An informal trade of antimalarial drugs in the market referred to as the "black market" has been also identified in Yopougon, as well as in its neighbouring communes such as Abobo and Adjamé (Trebissou et al., 2014). The poor quality of antimalarial drugs eventually can alter the conformation of the target proteins' structure and select alleles in the parasite population involved in reducing the disease susceptibility because of the emergence of resistance genes to CTAs (Ashley et al., 2014; Abukari et al., 2019). This study also showed a high multiplicity of infection or MOI as well as multiclonal infection rates in clinical isolates of *P. falciparum* collected from a single infected person host. These findings aligns with previous studies conducted in Côte d'Ivoire, Gabon, and Benin (Ogouyèmi-Hounto et al., 2013; Yavo et al., 2016; Lagnika et al., 2022) and Kenya (Touray et al., 2020). However, the values of polyclonal infection rates were higher

than those recorded in other different epidemiological contexts in Nigeria and Senegal (Oboh et al., 2020), Congo (Singana et al., 2019), and Ethiopia (Mohammed et al., 2018). The MOI is greater than or equal to three different *P. falciparum* genotypic parasites should predict malaria treatment failures and the emergence of multiple resistant alleles to the first-line of antimalarial drugs (Yakubu et al., 2019). The high MOI in this endemic area may suggest intense malaria transmission and people continuously exposed to the disease, and eventually favours the development of specific immunity in the population when a single host is co-infected by several *P. falciparum* variants. In South East Asia areas, residual malaria transmission has been observed, with a low MOI and predominant monoclonal infection rates, reflecting reduced exposure, low immunity, and disease eradication efforts (Mohd Abd Razak et al., 2016). Regular monitoring of *P. falciparum* strains according to WHO standards should be urgently considered for the implementation of effective control strategies to ensure the effectiveness of antimalarial treatments by reducing the genetic diversity of *P. falciparum* strains. Thus, the effective control of malaria should be based on the use of new broad-spectrum medicines or vaccines.

The high genetic diversity in this study also was associated with higher trimorphic and dimorphic infection rates, resulting in a high predictive expected heterozygosity (*He*) of the parasite strains in Yopougon. The average *He* value recorded in this commune was consistent with those reported in recent studies in Obom, Ghana (Abukari et al., 2019), Mbita, Kenya (Touray et al., 2020), Pointe-Noire, Congo (Singana et al., 2019), Kosofe, Nigeria and Kédougou, Senegal with different endemicity profiles.

Different epidemiological settings, patients' age and level of subject exposure to the disease in the study sites, intrinsically induce modulation of immune responses through host gene expression variability, of which the clinical manifestations are associated with genotypic charges of *msp-1* and *msp-2* genes (Kimenyi et al., 2019). Thus, this study revealed in Yopougon, joint trends of pre-eminence of the K1 alleles with a maximum of two alleles per patient and MAD20 (three alleles per patient) for *msp1* gene, and the FC27 allele type with a maximum of five alleles per patient for *msp2* gene. The extent of allelic variability at each *msp1* and 2 locus indicates a pattern of epidemiological areas with extremely high allelic diversity of parasitic clones. The main contributors essentially are the genetic recombination process and the importance of persistent malaria infections, which are favourable to the production of polymorphic gametocytes. The dominance of K1 allele family in collected isolate samples from infected patients has been already demonstrated in Côte d'Ivoire (Yavo et al., 2016; Gnagne et al., 2019). Furthermore, similar results have been shown in sub-Saharan Africa areas such as Burkina Faso (Somé et al., 2018; Sondo et al., 2021), Benin (Lagnika et al., 2022), Senegal (Oboh et al., 2020), Nigeria (Yakubu et al., 2019; Oboh et al., 2020), Republic Democratic of Congo (Singana et al., 2019), Malawi, Sao Tome, Tanzania and Uganda (Mwingira et al., 2011). In contrast, the number of MAD20 alleles was similar to that of K1 in this study. These results could be explained by the dynamics of the host-parasite interaction because K1-type alleles were majority found in malaria isolates (Yavo et al., 2016; Mohammed et al., 2018). The importance of MAD20-type allele frequency would be, therefore, induced by environmental conditions favourable to the development and growth of these parasitic genotypes. These results could have major clinical consequences. In *ex-vivo* studies (not yet published) on the same *P. falciparum* isolates, the sensitivity of chloroquine to disease isolates was increased in contrast to lumefantrine and pyrimethamine, the two major antimalarial drugs that are usually associated with CTAs to globally eradicate malaria. Mwingira et al. (**2011**) revealed a similar allelic trend in eight countries investigated in sub-Saharan Africa. The RO33-type allele family showed a dimorphic parasite gene profile. The number of RO33 alleles was low compared to those reported in other part of Côte d'Ivoire (Yavo et al., 2016; Gnagne et al., 2019). These differences in the number of RO33-type alleles could be explained by the different sample sizes used in each study. In populations with diverse genetic backgrounds, the larger the population size, the higher the number of detected different alleles. The multiplicity of infection and parasitaemia were not statistically correlated in this study. The lack of relationships between these two malaria infection parameters should indicate that they were naturally independent parameters and it could be unable to predict or estimate the parasitaemia rate using the observed multiplicity of infection rates in intense endemic areas. Ogouyèmi-Hounto et al. (2013) have also observed similar results using common inclusion criteria in southern Benin, a country sharing the same environmental conditions as Côte d'Ivoire. Both countries are located in West Africa in sub-equatorial climate regions with perennial malaria transmission. However, some investigations have shown a linear relationship between parasitaemia and MOI at the disease transmission level, host acquired immunity and epidemiological endemicity (Mayengue et al., 2009; Aubouy et al., 2015). Specific allelic forms of the *Pfmsp-1* gene were involved in the quick invasion of the parasite into red blood cells, favouring a high parasitaemia and the occurrence of intense transmission of malaria infection (Patgiri et al., 2019). The parasite density in infected and symptomatic subjects was not related to the patient's age and MOI in this study. Furthermore, patients' parasitaemia only was negatively and significantly correlated with quinine IC50. This finding suggests that a higher concentration of quinine is needed to reduce *P. falciparum* parasites strains in infected patients and lower the parasitaemia rate, indicating the effectiveness of quinine in malaria control strategies. The relationships between the tested eight molecules using the Spearman rank showed a positive and significant correlation between piperaquine (PIP) and amodiaquine (AQ), quinine (QN) and pyronaridine (PYN). The correlation between PYN and AQ was positive and significant. AQ, PIP and PYN are usually associated drugs with artemisinin-based combination therapies (ACTs) in the first-line treatment of uncomplicated

malaria. Their integrity has therefore ensured the effectiveness of ACTs. Quinine (QN) usually is a molecule needed for the treatment of severe malaria. In Côte d'Ivoire, the based-quinine drugs are still effective and are part of the therapeutic arsenal for malaria control (PNLP, Elimination du paludisme en Côte d'Ivoire d'ici 2030, Avancées et défis. 5ème colloque scientifique sur le paludisme en Côte d'Ivoire, Faculté de Medecine, Cocody, Abidjan, du 17 au 18 Avril 2019). Thus, the monitoring of *P. falciparum* populations and the suitable management of antimalarial drugs, through the decision making on health care policy, could guarantee the effectiveness of the molecules against parasite resistance or cross-resistance. This could contribute to the development of new and cheaper antimalarial drugs and efficiently reduce the burden of the complex polyclonal infections. The use of *Pfmsp-1* and *Pfmsp-2* markers in the investigation of the genetic variability of the parasite population within or between countries should be taken into account for the development of an effective, broad-spectrum vaccine capable of boosting host-specific immunity and defeating malaria.

CONCLUSION

The 64 genotyped *P. falciparum* isolates, collected from infected human hosts and at least 18 years old in Yopougon, using *msp-1* and *msp-2* genes revealed high genetic diversity among malaria parasite strains. The polyclonal infection rate was more frequently observed in infected populations than monoclonal infections and the commune of Yopougon recorded a high parasitaemia and multiplicity of infection (MOI). Cluster analysis allocated all the parasite strains into four mains groups. The cluster 4 only made of the isolate W634, collected from an infected individual host and identified as a single particular isolate, was genetically divergent from the isolates within the other clusters. It also revealed how the parasite strains are highly variable in sizes and sequences. The relationships between parasitaemia and tested eight molecules have shown that only the quinine-based drugs could efficiently reduce the malaria parasite strains in infected patients. However, new broad-spectrum medicines are needed to effectively control the parasite's transmission because of predominate multiclonal infections. The results of this study provide valuable and useful information using molecular and *ex vivo* data for malaria transmission to humans in intense endemic areas to guide the implemented intervention programs and the effective disease control strategies in Côte d'Ivoire. Furthermore, the study should be extended to several epidemiological and endemic areas in Côte d'Ivoire to understand better the structure of the parasite population, assess the effectiveness of the current malaria control programs and improve the malaria control strategies.

Authors' contributions

KDS and XD whose initiatives through the CSRS-MMV project led to the establishment of a chemosensitivity platform at CSRS. They were principal investigators, trainers. KDS and BB coordinated the protocol ethics approval process, designed and supervised the data collection. KT. EMAB, and IY provided transportation of isolates from FSU-COM in Wassakara to the chemosensitivity platform at CSRS, determined parasite densities of *P. falciparum* isolates, and made DBS sample. AS-PN and AJD were the coordinators of the data analysis. IY randomized sample selection. AJD. AOT and ABA enabled the PCRs to be performed at the IPCI Molecular Biology Laboratory. IY and KTS performed the PCRs tests. IY analysed the data and wrote the initial version of the manuscript. All authors have read and approved the final version of the manuscript.

Ethics Approval

The study has received ethical clearance from the National Research Ethics Committee of Côte d'Ivoire (CNER) under number: 035/MSLS/CNER-dknof May 05th, 2014. It also has been carried out with the agreement from the directorate of the health care facility of FSU-COM Wassakara. Before taking part to the study, patients' consent also was considered by signing a written consent form and any risk associated to their participation was covered by a health insurance policy under the number N°30101211510014 from the company "Génération Nouvelle d'Assurance de Côte d'Ivoire (GNA)". All patients' anonymous and confidentiality were preserved and protected in accordance with the test of the Helsinki Declaration adopted by the 18th World Medical Assembly in 1964 and its amendments, the ICH (International Conference on Harmonization) recommendations. It has been consistent with Good Clinical Practices and all applicable regulatory requirements for clinical studies as well as Côte d'Ivoire's national laws and regulations.

Conflicts of interest

The authors declare no conflicts of interest.

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Data Availability Statement

All data are contained within the article and supplementary files. Additional data are available on request from the corresponding author.

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