

Full Length Research Paper

Assessing the polymorphism of DHFR gene from *Plasmodium falciparum* in the south of Côte d'Ivoire

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Since 2005, Côte d'Ivoire has adopted new strategies of malaria management including free provision of Artemisinin-based Combination Therapy (ACT) to children less than five years of age and sulfadoxine-pyrimethamine (SP) as Intermittent Preventive Treatment (IPT) for pregnant women. However, introduction of ATCs raises concerns about the extensive use of cheap SP which could increase *Plasmodium falciparum* resistance level to SP. Therefore, this study aimed to determine the prevalence of the Asn-108 marker in three sites in Southern Côte d'Ivoire. After obtaining consent, blood samples were collected in Anonkoua-kouté, Port-Bouët, and Ayamé sites from 180 patients over 2 years of age and having simple *P. falciparum* malaria. *P. falciparum* genomic DNA extracted from these samples was amplified by nested-PCR with pfdhfr specific primers. The amplification products were revealed by electrophoresis on 1.5% agarose gel and then sequenced according to Sanger method. After sequencing, the prevalence of mutation points associated with *P. falciparum* resistance to pyrimethamine was determined. For the three study sites, 180 DNA fragments, of which 165 (165/180 or 91.66%) were successfully sequenced. Analysis of the 165 sequences indicated a prevalence of 61.29% (76/124) of the Asn-108 mutant allele versus 17.41% (27/155) of the wild type Ser-108 allele. Results also indicated that the prevalence of Ser-108-Asn mutation were 69.07, 69.04 and 82.75% for Anonkoua-Kouté, Port-Bouët and Ayamé, respectively. More than a decade after the adoption of SP as IPT for pregnant women, the prevalence of the marker Asn-108 was relatively high in Anonkoua-kouté, Port-bouët and Ayamé.

Key words: Pfdhfr, Asn-108, Côte d'Ivoire, sulfadoxine-pyrimethamine, resistance, antimalarial drug.

INTRODUCTION

Malaria remains a major cause of morbidity worldwide. According to the World Health Organization (WHO), 219 million cases of malaria were recorded in 2017, of which

345,000 led to death with 93% occurrence in Africa (WHO, 2018). For children under five years of age, the deaths were estimated to 61% in 2017 (WHO, 2018).

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The treatment of this disease involves antimalarial drugs, because effective vaccine is not yet available. However, malaria control is limited by *Plasmodium falciparum* resistance to most antimalarial drugs. Indeed, high levels of chloroquine resistance have forced some countries to abandon chloroquine as first-line treatment in favor of sulfadoxine-pyrimethamine (SP). However, resistance to this drug has emerged regarding treatment failures reported in Africa, Asia, Indonesia and South America (Adnan et al., 2018; Ratcliff et al., 2007; Ravi (2016); Shannon and Miriam (2015); Vladimir et al., 2010).

Pyrimethamine and sulfadoxine act synergistically to inhibit two important enzymes in the pathway of parasite folate biosynthesis namely dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) (Kasturi et al., 2018; Yaro, 2009). Mutations in *pfdhfr* and *pfdhps* genes confer resistance to pyrimethamine and sulfadoxine respectively, with an *in vitro* decrease in *P. falciparum* sensitivity related to the number of mutations in each gene (Ingrid and John, 2010; Vladimir et al., 2010). These mutations are correlated with treatment failure in clinical trials (Ratcliff et al., 2007; Yaro, 2009).

The presence of mutations in *pfdhfr* gene appears to be more important in treatment failure than mutations in *pfdhps* gene (Sankar et al., 2010). Indeed, the triple mutation in codons 108, 51 and 59 of *pfdhfr* gene increases the risk of *in vivo* resistance to SP by 4.3 (OR; 95% CI: 3.0-6.3, meta-analysis of 16 studies) (Picot et al., 2009). Detection of Ser-108-Asn mutation is predictive of the presence of the other two mutations.

In Côte d'Ivoire, since 2005, SP has been used as intermittent preventive treatment (IPT) in pregnant women and children as recommended by the WHO (WHO, 2016). However, introduction of ATCs raises concerns about the extensive use of cheap (SP) which could increase *P. falciparum* resistance level to SP. This study, conducted in three sites in Southern Côte d'Ivoire, aims to determine the prevalence of key mutations associated with *P. falciparum* resistance to pyrimethamine (*dhfr* codons N51, C59, S108) in patients with uncomplicated malaria.

MATERIALS AND METHODS

Study site

This study was carried out in three localities of Côte d'Ivoire (malaria endemic zone) as part of a monitoring study of antimalarial drug resistance. The study included a standard questionnaire to collect socio-demographic data from participants and blood collection for molecular testing. The study was conducted from February to August, 2015 at the Anonkoua Kouté health center and the general hospitals of Port-Bouët and Ayamé. All these sites (Anonkoua Kouté 5°25'55.90" N ; 4 02'45.27" W, Port-Bouët 5°15'20" N et 3°57'52" W and Ayamé 5°36'12,43" N ; 3°09'19,36" W) are located in the Southern region of Côte d'Ivoire where climate is equatorial, with annual rainfall exceeding 1700 mm and temperature varies between 27 and 33°C. Malaria is seasonal, predominating in the rainy season from June to September with

prevalence peaks in October-November. *P. falciparum* is the dominant specie with more than 90% of identified malaria parasites (Adja et al., 2011). Anonkoua-kouté Health Centre, Port-Bouët and Ayamé General Hospitals were selected based on high annual incidences of malaria cases.

Study population and blood sample collection

All suspected cases of malaria at Anonkoua-kouté health center, Port-Bouët and Ayamé general hospitals were randomly selected for the study. After informed consent, patients' socio-demographic data were recorded from the questionnaire; then blood samples were collected from participants over 2 years of age and suffering from uncomplicated *P. falciparum* malaria detected after microscopy test. Approximately 2-5 ml of venous blood was drawn and collected in Ethylene Diamine TetraAcetic (EDTA (BD Vacutainer®-367844)) containing tube and 50 µl of whole blood was placed on Whatman 3 MM (Whatman Inc., Maidstone, United Kingdom) filter paper using a micropipette. Blood spots on filter paper were dried for approximately 60 to 120 min at room temperature. Unused blood in EDTA tube was stored in at - 20°C for further analysis.

Extraction of *P. falciparum* genomic deoxyribonucleic acid (DNA)

Plasmodial DNA was extracted from filter paper blood spots with methanol (Miguel et al., 2013). Indeed, fine cuts of spots were immersed in 1 ml of Wash Buffer (950 µL PBS 1X and 50 µl 10% saponin) and then incubated at 4°C overnight. The wash buffer was removed and 150 µl of methanol were added. After 20 min incubation at 4°C, the methanol was gently removed and the samples were dried at room temperature for 2 h before adding 300 µl of sterilized water. Samples were then heated at 99°C in a thermo-mixer for 30 min to extract the DNA. The DNA extracts were aliquoted into a 1.5 ml Eppendorf tube and stored at - 20°C.

Amplification of the *pfdhfr* gene

The *pfdhfr* gene was amplified by nested PCR using specific pair of primers and commercial DNA polymerase kit (5X FIREPol® Blend Master Mix (Solis Biodyne)) with mM MgCl₂. This kit is a pre-mix (for the reaction mixture) ready to use composed of DNA polymerase (FIREPol® DNA polymerase), buffer (5x Blend Master Mix Buffer), MgCl₂ (7.5 mM MgCl₂) and dNTPs (2 mM dNTPs of each).

For primary PCR, the primer pairs used for amplification of the *pfdhfr* gene were *dhfr_M1* (5'TTTATGATGGAACAAGTCTGC) / *dhfr_M7* (CTAGTATATACATCGCTAACA). The primary PCR (25 µL) reaction contained 0.625 µl of each primer, 3 µl of plasmodial DNA, 5 µl of Taq DNA polymerase and 15.75 µl of milliQ water. The cycling parameters used were as follow: Initial denaturation at 95°C for 15 min followed by 30 denaturation cycles at 95°C for 30 s, annealing at 58°C for 2 min and extension at 72°C for 2 min. Terminal extension step was set at 72°C for 10 min.

The second PCR was carried out on amplification products (amplicon) of the primary PCR in a reaction volume of 50 µL containing: 1.25 µl of each primer, 5 µl of amplification product (amplicon), 5 µl of Taq DNA polymerase and 37.5 µl of milliQ water. The primer pairs used for the secondary Polymerase chain reaction (PCR) were *dhfr_M9* (5'CTGGAAAAA AATACATCAC ATTCATATG) / *dhfr_M3* (5T GATGGAACAAGTCTGC GACGTT). The secondary PCR cycling operation was performed as follow: Initial denaturation at 95°C for 15 min followed by 30 denaturation cycles at 95°C for 30 s, annealing at 60°C for one min and extension at 72°C for 1 min.

Table 1. Study population profile.

Site	Gender	
	Female n (%)	Male n (%)
Anonkoua-Kouté	37 (59,67)	25 (40,33)
Port-Bouët	39 (66,10)	20 (33,9)
Ayaamé	35 (59,32)	24 (40,68)
Total	111 (61,66)	69 (38,34)

Table 2. Blood samples used for molecular analysis of pyrimethamine chemoresistance.

Sites	Sampling period (2015)	Age groups (years)	Average age (years)± SD	Blood samples
Anonkoua-kouté	February - March	2 to 53	16.60 ±14.30	62
Port - Bouët	April - May - July	2 to 62	16.69±13.24	59
Ayamé	June - July - August	2 to 55	15.84±14.87	59
Total				180

Terminal extension step was set at 72°C for 10 min.

Detection and analysis of PCR products

The amplification products were transferred on a 1.5% agarose gel containing ethidium bromide (EtBr). After migration, the gel was visualized under UV lamp using the UV transilluminator (Gel Doc™ EZ Imager (Bio-Rad)).

Sequencing amplification

Amplified DNA fragments (*pfdhfr* gene) of *Plasmodium falciparum* were subjected to sequencing according to the Sanger method by the Company Eurofins MWG opéron (Cochin sequencing platform). Samples were dropped in a microplate (Greiner Bio-one-652270B) that was sent to the platform for sequencing. The DNA sequences received after sequencing reaction were recovered fast. The software BioEdit was used to analyze the sequences in order to search for possible mutations.

Statistical analysis of data

Data were collected based on standard questionnaire that was tested and validated. They were analyzed using the statistical software R; version 3.2.2 (Core Team R, 2013). The χ^2 comparison test of three mean values was used to compare the prevalence of the molecular marker of pyrimethamine resistance (*pfdhfr* S108N). The χ^2 test was used to determine whether the molecular marker prevalence can be considered to be all equal (hypothesis H0) or if two or more prevalence are different (alternative hypothesis Ha). A difference and/or statistical association was considered significant if p-value < 0.05.

RESULTS

Profile of selected patients

A total of 180 persons with uncomplicated malaria were

selected for this study, including 111 (61.66%) females and 69 (38.34%) males (Table 1). Patients' ages ranged from 2 to 62 years, with mean ages in Anonkoua-kouté, Port-bouët and Ayamé of 16.60, 16.69 and 15.84 years respectively. Thus, 180 blood samples were collected in the study sites (Table 2).

DNA sequencing assessment

For all the study sites, 180 DNA fragments were isolated, of which 165 (165/180, or 91.66%) were successfully sequenced. Molecular analysis of these fragments showed that the number of sequenced DNA fragments with success varied according to the presence of interest codons. Thus, 124 (75.15%), 126 (76.63%) and 155 (93.93%) DNA fragments were successfully sequenced for the nucleotides position 153, 177 and 324 corresponding to the amino acids Asn-51-Ile, Cys-59-Arg and Ser-108-Asn where mutations were observed (Table 3). Sequencing of the DNA region leading to the Ser-108-Asn mutation was more successfully performed (155/165; 93.93%).

Polymorphism of the *pfdhfr* gene

Prevalence of individual alleles of the *pfdhfr* gene and molecular analysis of corresponding genotypes

For the three study sites, our results indicated that the prevalence of isolates carrying the Ile-51 (61.29%), Arg-59 (54.76%), Asn-108 (74.19%) mutations were higher than those of wild isolates Asn-51 (15.32%), Cys-59 (15.07%), Ser-108 (17.41%) of *pfdhfr* gene (Table 4). Molecular analysis of the genotypes corresponding to

Table 3. Mutation status of sequenced DNA extracted from patients.

Sequenced fragments	Mutations	Success	Failure
<i>Pfdhfr</i> (n = 165)	Asn-51-Ile	124	11
	Cys-59-Arg	126	39
	Ser-108-Asn	155	10

Table 4. Prevalence of individual alleles of *pfdhfr* gene.

Codons	Alleles	Study sites (N=165)		
		Staff	(%)	
Dhfr_51	Wild Type (N)	Asn-51	19	15.32
		Ile-51	76	61.29
		Phe-51	13	10.40
	Mutants	Lys-51	2	1.61
		Leu-51	6	4.83
		Pro-51	6	4.83
		Ser-51	2	1.61
dhfr_59	Wild type (C)	Cys-59	19	15.07
		Arg-59	69	54.76
		Ala-59	2	1.58
	Mutants	Gly-59	21	16.66
		Leu-59	2	1.58
		Ser-59	9	7.14
		Trp-59	4	3.17
dhfr_108	Wild type (S)	Ser-108	27	17.41
		Asn-108	115	74.19
		Ala-108	4	2.58
	Mutants	Phe-108	2	1.29
		His-108	3	1.93
		Thr-108	2	1.29
		Val-108	2	1.29

"N" represents the total number of isolates successfully sequenced. "n" represents the number of isolates for which the codons of interest (51, 59, 108) or nucleotides (153, 177, 324), of the sequence.

pfdhfr gene showed a predominance of triple mutant (75/165, or 45.45%) and double mutant (50/165, or 30.30%) genotypes. The results also indicated that isolates carrying the IRN (triple mutant), NRN (double mutant) and ICN (double mutant) genotypes were observed with prevalence of 31.51, 9.09 and 7.87%, respectively, compared to 13.93% for isolates carrying the NCS (wild type) genotype (Table 5). Single mutant genotypes were also observed with a prevalence of 10.30%.

Prevalence of the Asn-108 mutation of the *pfdhfr* gene polypeptide in Anonkoua-Kouté, Port-Bouët and Ayamé

Our results showed that the Ser-108-Asn mutation was observed at 69.09%, 69.04% and 82.75% respectively for Anonkoua-Kouté, Port-Bouët and Ayamé (Table 6). For the same mutation (Ser-108-Asn), the highest prevalence was observed in Ayamé (82.75%). Analysis also revealed no significant difference between the prevalence of the

Table 5. Prevalence of genotypes corresponding to *pfdhfr* in the three sites.

Haplotype	N51I	C59R	S108N	Blood (N=165)	
				n	Proportion
Wild types	N	C	S	23	13.93
				17	10.30
Single mutant	N	C	<u>I</u>	2	1.21
	<u>I</u>	C	S	4	2.42
	N	C	<u>F</u>	2	1.21
	N	C	<u>N</u>	4	2.42
	N	C	<u>V</u>	2	1.21
	<u>L</u>	C	S	2	1.21
	<u>P</u>	C	S	1	0.60
				50	30.30
Double mutant	N	<u>G</u>	<u>A</u>	2	1.21
	N	<u>S</u>	<u>N</u>	4	2.42
	<u>L</u>	<u>G</u>	S	4	2.42
	<u>F</u>	C	<u>N</u>	4	2.42
	N	R	N	15	9.09
	I	C	N	13	7.87
	N	G	N	4	2.42
	N	A	A	2	1.21
	P	W	S	2	1.21
				75	45.45
Triple mutant	<u>S</u>	<u>G</u>	<u>N</u>	2	1.21
	<u>F</u>	<u>L</u>	<u>N</u>	2	1.21
	<u>I</u>	<u>G</u>	<u>H</u>	2	1.21
	<u>F</u>	<u>S</u>	<u>N</u>	4	2.42
	I	R	N	52	31.51
	<u>I</u>	<u>R</u>	<u>H</u>	2	1.21
	<u>K</u>	<u>G</u>	<u>N</u>	1	0.60
	<u>I</u>	<u>W</u>	<u>N</u>	2	1.21
	<u>I</u>	<u>S</u>	<u>N</u>	2	1.21
	<u>F</u>	<u>G</u>	<u>N</u>	4	2.42
<u>P</u>	<u>G</u>	<u>N</u>	2	1.21	

An uppercase letter in the "genotypes" column represents the code for an amino acid. Amino acids resulting from the mutation are underlined and in bold. The prevalence correspond to the number of observations on the number of success per gene.

Ser-108-Asn mutation determined from isolates for Anonkoua-kouté, Port-Bouët and Ayamé (p = 0.344).

DISCUSSION

This study indicated that in 2015, the prevalence of the Ser-108-Asn mutation (Asn-108) was observed at the same level of prevalence in Anonkoua-kouté (69.09%), Port-Bouët (69.04%) and Ayamé (82.75%). These data could be explained by the presence of *P. falciparum* potentially pyrimethamino-resistant isolates. The prevalence of this mutation was higher than those obtained in 2008 at Anonkoua-Kouté in Abidjan (49%)

and Ayamé (54%) in blood isolates from individuals with malaria symptoms (Ako et al., 2014). Lower proportions were obtained by other authors in 2001 (50%) and 2006 (46.4%) at Yopougon in Abidjan (Djaman et al., 2002, 2010) and at Adzopé (35.4%) in 2010 (Ouattara et al., 2010).

In addition, a study of marker dynamics indicated that the prevalence of the Ser-108-Asn mutation increased significantly in Anonkoua-kouté between 2002 and 2008, with an average of 43% (Ako et al., 2012, 2014). In view of all these results, the prevalence of Asn-108 mutation has increased significantly in this part of the country.

This finding is also important because the sulfadoxine-pyrimethamine combination is recommended in intermittent

Table 6. Frequencies of the wild Ser-108 and Asn-108 mutant alleles of the *pfdhfr* gene at Anonkoua-Kouté, Port-Bouët and Ayamé.

Codon	Alleles		Anonkoua-Kouté (N=55)		Port-Bouët (N=42)		Ayamé (N=58)		p-value of the test χ^2
			n	%	n	%	n	%	
dhfr_108	Wild	Ser-108	6	10.90	11	26.19	10	17.24	0.682
	Mutants	Asn-108	38	69.09	29	69.04	48	82.75	0.344

"N" represents the total number of successfully sequenced isolates per study site. "n" represents the number of successfully sequenced isolates for codon. dhfr_108. The list of other mutants is given in Table 3.

preventive treatment of pregnant women in Côte d'Ivoire (MSHP, 2013). Despite its prohibition in the curative treatment of malaria attacks, SP could be used by some population in Anonkoua-kouté, Port-Bouët and Ayamé and perhaps in other towns (Granado et al., 2009, 2011). The data obtained could also be explained by the increased use of SP (Tinto et al., 2007) in unofficial markets because of withdrawal of chloroquine. This increased use of SP could be explained by non-recommended therapeutic practices such as self-medication (Gokpeya et al., 2013; Kouadio et al., 2006) encouraged by easy access to the molecule already available in the country before 1996 (Henry et al., 1996, 2002). Indeed, Min (2012) mentioned that poor populations prefer to turn to unofficial markets to obtain SP and CQ, which remain inexpensive antimalarial molecules. According to Granado et al. (2009, 2011) and Orostegui et al. (2011), unofficial markets are found in large cities such as Abidjan or San-Pedro in Côte d'Ivoire (Granado et al., 2009, 2011; Orostegui et al., 2011). About 45 illicit sales outlets for pharmaceutical products, including various antimalarial drugs, were counted in such areas in Abidjan in 2005 (Granado et al., 2011). Populations with low purchasing power may explain the recourse to unofficial retailers (Kizito et al., 2012). This uncontrolled use of SP could promote the development of high drug pressure, which could lead to the selection of

resistant parasites at Anonkoua-kouté, Port-bouët and Ayamé.

In addition to drug pressure, pyrimethamine resistance in these three localities could be explained by the use of poor quality antimalarial drug. Indeed, the use of poor-quality antimalarial drug can have multiple consequences, including an increased risk of developing drug resistance, as sub-therapeutic doses of drugs will be ineffective in destroying all parasites (Newton et al., 2010; Shunmay et al., 2015).

Elsewhere in sub-Saharan Africa, high rates of *P. falciparum* resistance have been found. Indeed, results of monitoring for *P. falciparum* chemo resistance have shown the following results: in Burkina Faso, the reported rate of Asn-108 mutation was 63.8% (Somé et al., 2016); 92% in Gabon (Ghyslain et al., 2011); 93% in Senegal (Daouda et al., 2013). As the Asn-108 mutation, additional mutations (Asn-51-Ile and Cys-59-Arg) have also been identified. All mutations at codons 51 and 59 were associated with that of codon 108. Parasites carrying these additional Asn-51-Ile and Cys-59-Arg mutations associated with the Ser-108-Asn mutation have higher pyrimethamine resistance than those carrying the Ser-108-Asn mutation alone (Mathieu et al., 2007; Gregson and Plowe, 2005). Compared to the prevalence of 17.33 and 27.27% reported by Ako respectively for Dabakala, Anonkoua-Kouté, Ayamé sites (Ako et al., 2012, 2014) and Bonoua and Samo sites

(Ako et al., 2012), the prevalence of the triple mutant IRN (31.51%) increased compared to the sensitive strain (NCS). A high prevalence of triple-mutant *P. falciparum* parasites reduces the efficacy of sulfadoxine-pyrimethamine as an intermittent preventive treatment against malaria in infants and children (Gosling et al., 2009; Nankabirwa et al., 2010), undermines the ability of sulfadoxine-pyrimethamine to clear existing *P. falciparum* infections in asymptomatic pregnant women, and shortens the post-treatment prophylactic period, following Intermittent Preventive Treatment during pregnancy (Desai et al., 2016).

Conclusion

The study indicates that the prevalence of alleles associated with pyrimethamine chemoresistance represented by the *dhfr* Asn-108 mutation has increased in Anonkoua-kouté, Port-Bouët and Ayamé. It also indicates an increase in the prevalence of the genotypes that confer pyrimethamine resistance. The study revealed an increase in potentially pyrimethamino-resistant isolates despite the withdrawal of SP as a first-line antimalarial treatment. These high proportions of known mutations in the *pfdhfr* gene could be in favour of a decrease in the SP efficacy in Côte d'Ivoire.

ETHICAL CLEARANCE AND INFORMED CONSENT

The study was conducted in accordance with the Declaration of Helsinki and approval was received from the National Committee for Ethics and Research (CNER) of the Ministry of Health and AIDS of Côte d'Ivoire. After appropriate information and explanation, the adult participants, parents or legal guardians of all children who wished to participate in the study gave their written consent prior to sampling.

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