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Use of saliva for monitoring *Plasmodium falciparum* chemoresistance to pyrimethamine in three sites in Southern Côte D'ivoire

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Current malaria diagnostic methods require blood sampling, which can be a barrier due to needle-related discomfort or cultural taboos surrounding blood. Saliva, being minimally invasive to collect, offers a promising alternative. This study aims to utilize saliva to monitor *Plasmodium falciparum* resistance to pyrimethamine at three sites in southern Côte d'Ivoire. Blood and saliva samples were collected from 94 patients over 2 years old with microscopically confirmed uncomplicated *P. falciparum* malaria across three localities. The genomic DNA of *P. falciparum* was extracted, amplified using nested PCR with primers specific to the pfdhfr gene, and sequenced using the Sanger method. The sequencing results were then analyzed to determine the prevalence of pfdhfr mutations (N51I, C59R, S108N) associated with pyrimethamine resistance in *P. falciparum*. Data analysis was performed using R statistical software, version 3.2.2. A total of 153 DNA fragments of the pfdhfr gene were sequenced, comprising 86 blood DNA fragments and 67 salivary DNA fragments. Successful sequencing rates for blood DNA fragments were 75.58% (N51I), 76.74% (C59R), and 94.18% (S108N), compared to 85.07% (N51I), 86.56% (C59R), and 94.02% (S108N) for salivary DNA fragments. Sequence analysis revealed mutation prevalences in the pfdhfr gene of 61.53% (N51I), 54.54% (C59R), and 74.07% (S108N) in blood, and 49.12% (N51I), 63.79% (C59R), and 79.36% (S108N) in saliva. Notably, no significant difference was observed between the mutation prevalences in blood and saliva (p = 0.44). Molecular analysis revealed that the sensitive NCS haplotype (51N59C108S) was detected in 17 out of 153 isolates, with a prevalence of 13.96% (12/86) in blood and 7.46% (5/67) in saliva. In contrast, the IRN triple mutant haplotype (51I59R108N) was observed in 48 out of 153 isolates, with a prevalence of 31.4% (27/86) in blood and 31.34% (21/67) in saliva. Notably, the prevalences of the IRN triple mutant haplotype did not differ significantly between blood and saliva (p = 0.685). This study demonstrated that the prevalence of genotypes conferring resistance to pyrimethamine reached comparable levels in both blood and saliva isolates. Over a decade after the adoption of sulfadoxine-pyrimethamine as intermittent preventive treatment for pregnant women, the prevalence of the Asn-108 allele and the triple mutant IRN haplotype remained relatively high in Anonkoua-kouté, Port-Bouët, and Ayamé, Côte d'Ivoire.

Key words: Pfdhfr, saliva, Côte d'Ivoire, sulfadoxine-pyrimethamine, resistance, antimalarial drugs, *Plasmodium falciparum*.

INTRODUCTION

Malaria remains a major public health problem. According to the World Health Organization, nearly 249 million cases of malaria were recorded worldwide in 2022, including 608,000 deaths, 95% of which occurred in Africa (WHO, 2023). The vast majority of deaths were among children under five, accounting for 78% of all malaria deaths (WHO, 2023). Despite efforts to combat malaria, the disease remains a serious public health problem. The emergence of *Plasmodium falciparum* resistance to almost all available antimalarial drugs is making the situation more complicated and difficult.

Southeast Asia (SEA) is considered the epicentre for the evolution and spread of resistance against all major classes of antimalarials (Hassett and Roepe, 2019). Faced with the spread of *P. falciparum* resistance to almost all antimalarial drugs, the WHO has recommended the use of artemisinin-based combination therapies (ACTs) as first-line treatment for uncomplicated *P. falciparum* malaria in all malaria-endemic countries (WHO, 2015). Since 2005, Côte d'Ivoire has adopted the free dispensing of Artemisinin-based Combination Therapies (ACTs) to children under five and Sulfadoxine-Pyrimethamine (SP) as intermittent preventive treatment (IPT) to pregnant women (Touré et al., 2021).

However, this situation gives rise to fears of widespread use of cheap SP, which could increase the level of *P. falciparum* resistance to SP and reduce susceptibility to ACTs (PNLP, 2022). It is therefore necessary to set up a system for monitoring *P. falciparum* resistance to pyrimethamine and artemisinin derivatives in Côte d'Ivoire to ensure early warning and better management of malaria. The chemosensitivity of antimalarial drugs can be determined by four different methods, namely *in vivo* therapeutic efficacy studies, *in vitro* tests, molecular marker studies and the measurement of drug concentrations. Molecular marker studies are important for determining any early signs of antimalarial drug resistance. Several studies have shown a correlation between the development of antimalarial drug resistance and the presence of polymorphisms in the genes of the *P. falciparum* parasite (Niba et al., 2021; Plowe, 2009).

For example, mutations in the *pfcrt* gene spanning codons 72-76 have been associated with CQ resistance in *P. falciparum*, while the K76T mutation is characteristic of CQ resistance (Niba et al., 2021; Eboumbou et al., 2009). In addition, point mutations in the *pfdhfr* and *pfdhps* genes are associated with resistance to pyrimethamine and sulfadoxine respectively (Desai et al., 2016; Gesase et al., 2009; Picot et al., 2009) with an *in vitro* decrease in *P. falciparum* susceptibility linked to the number of mutations in each gene. Polymorphisms at codons 436, 437, 540, 581 and 613 of *pfdhps* and at codons 16, 51, 59, 108 and 164 of the *pfdhfr* gene have been identified as markers of resistance to sulfadoxine and pyrimethamine (Niba et al., 2021). Furthermore, all the chemosensitivity study methods mentioned above use blood sampling by capillary puncture (at the fingertip) or venipuncture (Putaporntip et al., 2011).

However, blood sampling is often an obstacle, as some people are reluctant to have their blood sample taken because of their cultural habits (blood taboo), or because of fear linked to the trauma of the puncture, especially when the sample is taken repeatedly (Achieng et al., 2020; Chatio et al., 2016; Bohaen et al., 2013; Baiden et al., 2012). Previous studies have demonstrated the performance of saliva for the detection of *Plasmodium* DNA and molecular markers of antimalarial drug resistance (Dagnogo et al., 2024; Chai et al., 2022; Aninagyei et al., 2020; Al-Shehri et al., 2019; Dagnogo et al., 2017). This study aimed to use saliva to study the chemosensitivity of *P. falciparum* to pyrimethamine.

MATERIALS AND METHODS

Study site

This prospective study was conducted from February to August 2015 at three healthcare facilities in southern Côte d'Ivoire, characterized by an Atrean climate with high rainfall (over 1,700 mm annually) and temperatures between 27 and 33°C. Malaria transmission in this region is seasonal, peaking during the rainy season (June-September) with highest prevalence and incidence rates in October-November. In this region, *P. falciparum* is the predominant malaria species, accounting for over 90% of parasite infections. The primary malaria vectors are *Anopheles gambiae sl* and *Anopheles funestus* complexes (Akre et al., 2021). The study sites were selected due to their high annual malaria incidence and favorable environmental conditions, including equatorial epidemiological characteristics, hydrography, and vegetation, which support high Anopheles densities (PNLP, 2020; Sadia-Kacou et al., 2021). The Anonkoua-kouté health centre and Ayamé general hospital were selected due to their high annual malaria incidence and their long-standing role as key sites for multicentre clinical trials conducted by the Pasteur Institute of Côte d'Ivoire's Malaria Unit. Additionally, the Port Bouët general hospital was chosen for its high malaria incidence and distinct environmental features, particularly its marshy terrain used for market gardening.

Study population and sample collection

All patients clinically suspected of having malaria at the Anonkoua Kouté health centre, Port- Bouët and Ayamé general hospitals during this study period were eligible. However, following informed consent, blood and saliva samples were collected from patients

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Table 1. Samples used for molecular analysis of pyrimethamine chemoresistance.

Site	Period of collections in 2015	Brackets age	Biological products	Conffetis collected
Anonkoua-kouté	February - March	2 to 53 years	Blood	32
			Saliva	32
Port - Bouët	April - May - July	2 to 62 years	Blood	31
			Saliva	31
Ayamé	June - July - August	2 to 55 years	Blood	31
			Saliva	31
Total				188

over 2 years of age with an axillary or rectal temperature greater than 37.5°C and with uncomplicated *P. falciparum* malaria confirmed microscopically by a thick drop and a blood smear. A total of 188 samples, including 94 blood samples and 94 saliva samples (from the 94 patients included in this study) were used for molecular analysis of pyrimethamine chemoresistance (Table 1).

Blood and saliva sampling

Blood

From each patient with microscopically confirmed malaria, approximately 2 to 5 mL of venous blood was drawn and collected in an EDTA tube. Approximately 50 µL of whole blood was spotted onto Whatman 3 MM filter paper using a micropipette with filter cones. The paper containing the blood spots was dried for approximately 60 to 120 min at room temperature in a dust-free environment.

Saliva

Ten to fifteen minutes after rinsing the mouth with tap water, 5 mL of saliva per patient was collected in a sterile bottle. Using a micropipette and filter cones, 50 µL of saliva was deposited on Whatman 3 MM filter paper and the resulting confetti was dried for approximately 60 to 120 min at room temperature in a dust-free environment.

Extraction of *P. falciparum* genomic DNA

Plasmodium DNA was extracted with methanol from blood confetti (Miguel et al., 2013). Thin cuts of blood confetti were immersed in 1 mL of wash buffer (950 µL of 1X PBS plus 50 µL of 10% saponin) and then incubated overnight at 4°C.

The wash buffer was removed and washed before adding 150 µL of methanol. After incubation for 20 min, the methanol was gently removed and the samples were dried at room temperature for 2 h before adding 300 µL of sterile water. The samples were then heated to 99°C in a thermo-mixer for 30 min to elute the DNA. After removing the confetti debris, the DNA extracts were aliquoted into a 1.5 ml Eppendorf tube and stored at -20°C. The NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to check DNA concentration and purity using a 260/280 ratio. The extracts were then stored at -20°C for possible future use.

Amplification of the *pfdhfr* gene

The *pfdhfr* gene was amplified by nested PCR using a pair of specific primers and a commercial DNA polymerase kit called 5X FIREPol® Blend Master Mix with mM MgCl2. The composition of this kit constituted a pre-mix for the reaction mixture. For the primary PCR, the primer pair used for amplification of the *pfdhfr* gene was dhfr_M1 (5'TTTATGATGGAACAAGTCTGC) / dhfr_M7 (CTAGTATATACATCGCTAACA). The primary PCR of the gene was performed in a 25 µl reaction mixture containing 0.625 µL of each primer, 3 µL of plasmodial DNA, 5 µL of Taq polymerase, and 15.75 µL of milliQ water. The reaction was then run on a PTC-100TM thermal cycler (Eppendorf Mastercycler) using the following program: initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 95°C for 30 s, hybridization at 58°C for 2 min, and extension at 72°C for 2 min, with a final terminal extension at 72°C for 10 min.

The secondary PCR was performed on the primary PCR amplification products in a 50 µl reaction mixture containing 1.25 µL of each primer, 5 µL of primary PCR product, 5 µL of Taq polymerase, and 37.5 µL of milliQ water. The primer pairs used for secondary PCR were dhfr_M9 (5' CTGGAAAAAATACATCACATTCATATG) and dhfr_M3 (5' TGATGGAACAAGTCTGCGACGTT). Secondary PCR was run on the same thermal cycler as the primary PCR, using the following program: initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 95°C for 30 s, hybridization at 60°C for 1 min, and extension at 72°C for 1 min, with a final terminal extension at 72°C for 10 min.

Detection and analysis of PCR products

Amplification products were migrated onto a 1.5% agarose gel containing Ethidium bromide (BET). After migration, the gel was recovered and then observed under a UV lamp using the UV transluminator (Gel DocTM EZ Imager). The presence or absence of bands was used to judge PCR efficiency.

Sequencing amplification

Amplified DNA fragments (*P. falciparum pfdhfr* gene) were sequenced using the Sanger method by Eurofins MWG opéron (Cochin sequencing platform). After the sequencing reaction, the DNA sequences received were recovered in fasta form. In the case of this work, these were the sequences corresponding to the *pfdhfr* gene of the isolates collected. The sequences were analyzed for mutations using BioEdit software. The loci of interest, that is,

Table 2. Profile of patients and samples collected.

Site	Collection period	Age groups	Average age (years)	Number of patients	Types of samples	Number of samples collected
Anonkoua-kouté	February - March, 2015	2 to 53 years	16.60	32	Blood	32
					Saliva	32
Port - Bouët	April - May - June, 2015	2 to 62 years	16.69	31	Blood	31
					Saliva	31
Ayamé	June - July - August, 2015	2 to 55 years	15.84	31	Blood	31
					Saliva	31
Total	-	-	-	94		188

codons at positions 51, 59 and 108 of the PfDHFR polypeptide or nucleotides at positions 153, 177 and 324 of the *pfdhfr* gene, were identified and analysed after parallel alignment of two or more DNA sequences, including the reference sequence of the *pfdhfr* gene, maximizing the number of identical nucleotides or residues, while minimizing the number of mismatches and gaps.

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and received approval from the National Ethics and Research Committee (CNER) of the Côte d'Ivoire Ministry of Health and AIDS. Written informed consent was obtained from adult participants and the parents or legal guardians of participating children after providing them with thorough information and explanations, prior to sample collection.

Statistical analysis of data

Data were collected using a standard questionnaire that had been tested and validated. They were then entered and analyzed on R statistical software; version 3.2.2 (R Core team, 2017). The χ^2 test for comparison of three means was used to compare the prevalences of the molecular marker of pyrimethamine resistance (pfdhfr S108N). The χ^2 test was used to determine whether the prevalences of the molecular marker can be considered to be all equal (null hypothesis H0) or whether at least two prevalences are different (alternative hypothesis Ha). A statistical difference and/or association were considered significant if p of the χ^2 test < 0.05.

RESULTS

Profile of patients and samples selected

A total of 94 people infected with *P. falciparum* were included in the study, 58 (61.7%) of whom were female and 36 (38.3%) male. The patients ranged in age from 2 to 62 years, with an average age in Anonkoua-kouté, Port-bouët and Ayamé of 16.60, 16.69 and 15.84 years respectively. A total of 188 samples (blood and saliva) were collected from all three study sites (Table 2).

Sequencing results

Sequencing results revealed that the number of successfully sequenced DNA fragments from patient isolates varied depending on the biological sample and the presence of codons of interest (Table 3). Out of 188 pfdhfr gene fragments, 153 (81.38%) were successfully sequenced, comprising 86 blood DNA fragments and 67 salivary DNA fragments (Table 3). Successful sequencing rates for specific nucleotide positions were: 75.58% (position 51), 76.74% (position 59), and 94.18% (position 108) in blood samples, and 85.07% (position 51), 86.56% (position 59), and 94.02% (position 108) in saliva samples. Notably, sequencing of the DNA region associated with the Ser-108-Asn mutation was highly successful in both blood (94.18%) and saliva (94.02%) samples.

Polymorphism of the *pfdhfr* gene in biological products

Frequency of individual alleles of the pfdhfr gene and molecular analysis of the corresponding genotypes

The frequencies of Asn-51-Ile, Cys-59-Arg, and Ser-108-Asn mutations in the pfdhfr gene varied across biological samples. The Asn-51-Ile mutation frequency ranged from 49% to 62% (Table 4), with the highest frequency observed in blood (61.53%) compared to saliva (49.12%), although the difference was not significant ($p = 0.33$). The Cys-59-Arg mutation frequency increased from 54.54% in blood to 63.79% in saliva. The Ser-108-Asn mutation showed the highest frequencies, with 74.07% in blood and 79.36% in saliva, and no significant difference

Table 3. Sequencing results for DNA extracted from blood, saliva and urine isolates according to mutations.

Sequenced		Blood (n=86)		Saliva (n=67)	
Fragment	**Change**	**Success**	**Chess**	**Success**	**Chess**
Pfdhfr (N=153)	Asn-51-Ile	65 (75.58)	21 (24.42)	57 (85.07)	10 (14.93)
	Cys-59-Arg	66 (76.74)	20 (23.26)	58 (86.57)	9 (13.43)
	Ser-108-Asn	81 (94.19)	5 (5.81)	63 (94.03)	4 (5.97)

Table 4. Frequency of individual alleles of the *pfdhfr* gene in blood, saliva and urine.

Codons	Strains and mutations observed		Biological products		p of the test-χ^2
			Blood n = 65 (%)	**Saliva n = 57 (%)**	
dhfr_51	Sauvage	Asn-51	10 (15.38)	17 (29.82)	0.159
	Mutants	Ile-51	40 (61.53)	28 (49.12)	0.338
		Phe-51	7 (10.76)	6 (10.52)	0.674
		Other	8 (12.30)	6(10.52)	
			Blood n = 66 (%)	**Saliva n = 58 (%)**	
dhfr_59	Sauvage	Cys-59	10 (15.15)	10 (17.24)	0.7428
	Mutants	Arg-59	36 (54.54)	37 (63.79)	0.115
		Gly-59	11 (16.66)	3 (5.17)	-
		Other	9 (13.63)	8 (13.79)	0.271
dhfr_108	Sauvage	Ser-108	14 (17.28)	8 (12.69)	0.742
	Mutants	Asn-108	60 (74.07)	50 (79.36)	0.443
		Other	7 (8.64)	5 (7.93)	0.259

Amino acids derived from the *pfdhfr* mutation are in bold and underlined. "n" represents the number of isolates successfully sequenced per codon and per biological product. Ile-51, Arg-59, Asn-108 refer to the mutations Asn-51-Ile, Cys-59-Arg and Ser-108-Asn respectively. The list of other mutants can be found in appendix 1 (appendix 1). The χ^2 test could not be performed due to values below 5 in several cells.

was observed between the two biological samples (p = 0.44). The wild-type strain (Ser-108) remained low in both blood (17.28%) and saliva (12.69%). Rare mutations (frequency < 5%) in the pfdhfr gene were also identified (Appendix 1). The study found no significant difference in the frequencies of individual alleles of the *P. falciparum* pfdhfr-ts gene between blood and saliva, suggesting saliva is a reliable alternative to blood for studying allele polymorphism. Molecular analysis revealed variations in susceptible and mutant strain frequencies across biological samples. The NCS allelic form (susceptible haplotype) was observed in 11.11% of isolates, with a prevalence of 13.96% in blood and 7.46% in saliva (Table 5). The triple mutant haplotype with the IRN genotype was identified in 31.37% of sequences, with no significant difference in frequency between blood and saliva (p = 0.685). No significant differences were found in the prevalences of mutant haplotypes (single, double, and triple mutants) between blood and saliva. Other mutant haplotypes were identified, but at very low frequencies (< 5%) (Appendix 2).

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

The Ser-108-Asn mutation was observed in 68.97 and 95.24% of blood and saliva respectively in Anonkoua-kouté and 83.33 and 65.22% respectively in Ayamé, compared with frequencies of 68.18 and 84.21% respectively in blood and saliva in Port-Bouët (Table 6). For the same mutation, the highest frequencies determined in saliva were recorded in Anonkoua-kouté (95.24%) and Port-Bouët (84.21%). The analysis revealed no significant difference between the Ser-108-Asn frequencies determined from isolates from Anonkoua-kouté, Port-Bouët and Ayamé in blood (p =

Table 5. Frequency of different allelic forms of the *pfdhfr* gene in blood, saliva and urine after mutation.

***pfdhfr* haplotypes**	**Genotypes**			**Blood (n=86)**		**Saliva (n=67)**		**p of test χ^2**
	N51I	**C59R**	**S108N**	**n**	**%**	**n**	**%**	
Sensitive haplotypes	N	C	S	12	13.96	5	7.46	0.4185
				9	10.47	9	13.43	0.347
Single mutant haplotypes	N	C	N	2	2.33	5	7.46	-
	Other			7	8.13	4	5.97	-
				26	30.23	25	37.31	0.447
Double mutant haplotypes	N	R	N	8	9.30	12	17.91	-
	I	C	N	7	8.14	3	3.48	-
	Other			11	12.79	10	14.92	-
				39	45.34	28	41.79	0.827
Triple mutant haplotypes	**I**	R	N	27	31.40	21	31.24	0.685
	Other			12	13.95	7	10.44	-

A capital letter in the "genotypes" column represents the single-letter code for the amino acids (appendix 2). The amino acids resulting from the mutation are underlined and in bold. The frequencies determined correspond to the number of observations out of the number of successes per gene. The list of other mutant haplotypes can be found in Appendix 2 (Appendix 2). The χ^2 test could not be performed due to values of less than 5 in several cells.

Table 6. Prevalence of Asn-108 mutations in the *pfdhfr* gene polypeptide in Anonkoua-Kouté, Port-Bouët and Ayamé.

Asn-108-Asn	***pfdhfr* gene**			
	Anonkoua-Kouté	**Port-Bouët**	**Ayamé**	**p of the test-χ^2**
Blood	20/29 (68.97%)	15/22 (68.18%)	25/30 (83.33%)	0.344
Saliva	20/21 (95.24%)	16/19 (84.21%)	15/23 (65.22%)	0.036

The frequencies of the wild-type Ser-108 strain and other mutations are described in the appendix (Appendix 3). The frequencies of the wild-type Cys-580 strain and other mutant alleles are described in the appendix (Appendix 4). The χ^2 test could not be performed due to values of less than 5 in some cells.

0.344). However, there was a significant difference between the frequencies of the Ser-108-Asn mutation measured in saliva (p = 0.036) (Table 6).

DISCUSSION

Systematic and effective monitoring of parasite genes is a necessary strategy for controlling *P. falciparum* resistance to antimalarial drugs. To achieve this, it is essential to use molecular biomarkers to identify and understand the genetic mutations that cause *P. falciparum* resistance to antimalarial drugs. To date, the methods used to study this chemoresistance are all invasive because they require blood sampling. In this study, saliva (a non-invasive method) was used to monitor *P. falciparum* resistance to pyrimethamine at three sites in southern Côte d'Ivoire. The study shows that in 2015, the frequency of the Ser-108-Asn (Asn-108) mutation was high in both saliva (79.36%) and blood (74.07%) in patients with uncomplicated malaria. No significant difference was observed between the frequencies of these two biological products (p = 0.443). These results consolidate those of the diagnostic performance of saliva previously presented by Dagnogo et al. (2024) in Anonkoua-kouté, Port-Bouët and Ayamé in Côte d'Ivoire (Dagnogo et al, 2024).

These data could be explained by the presence of potentially pyrimethamine-resistant *P. falciparum* isolates. The frequency of this mutation in blood and saliva isolates is higher than those obtained in 2008 in Anonkoua-Kouté in Abidjan (49%) and Ayamé (54%) in blood isolates from individuals with malaria symptoms (Ako et al., 2014a). Lower proportions were obtained by other authors in 2022 (50.43%) in India (Rana et al., 2022), in addition, a marker dynamics study indicated that the prevalence of the Ser-108-Asn mutation had increased significantly in Anonkoua-kouté between 2002 and 2008, with an average of 43% (Ako, 2014b). The increase in the frequency of this mutation could be explained by the fact that the sulfadoxine-pyrimethamine combination is still in circulation

because it is recommended as intermittent preventive treatment for pregnant women in Côte d'Ivoire (PNLP, 2022). The data obtained could also be explained by the increased use of self-medication for MS on parallel markets as a result of the effective withdrawal of chloroquine. In addition, the increase in pyrimethamine resistance in these three localities could be explained by the use of poor-quality antimalarials, whose sub-therapeutic doses will be ineffective in destroying all the parasites (Shunmay et al., 2015).

This drug pressure could be at the origin of the circulation of pyrimethamine-resistant strains in the three study sites, with an increase in the prevalence of mutations conferring resistance to pyrimethamine in *P. falciparum* (Ile-51, arg-59 and Asn-108) in the *pfdhfr-ts* gene in the three study sites. Indeed, these mutations are fixed in the parasite and confer resistance to pyrimethamine, implying that the efficacy of the Sulfadoxine-Pyrimethamine (SP) combination as Intermittent Preventive Treatment would be limited in this region (Berzosa et al., 2017).

Other studies in sub-Saharan Africa have reported high frequencies of these mutations. Researchers have reported frequencies of 63.8% in Burkina Faso (Somé et al., 2016), 97.39% in Niger (Issa et al., 2022) and 92.5% in Nigeria (Zhao et al., 2021). As with the Asn-108 mutation, parasites carrying the additional Asn-51-Ile and Cys-59-Arg mutations associated with the Ser-108-Asn mutation increase the level of *in vitro* resistance to antifolate drugs and sulphadoxine-pyrimethamine (Tessema et al., 2015). In addition, for the two biological products (blood and saliva), there was no significant difference between the frequencies of IRN triple mutant genotypes (31.4% in blood isolates and 31.34% in saliva isolates) and NCS susceptible genotypes (13.96% in blood isolates and 7.46% in saliva isolates). Compared with the frequencies of 17.33 and 27.27% reported by Ako for the Dabakala, Anonkoua-Kouté and Ayamé sites respectively (Ako, 2014) and the Bonoua and Samo sites (Ako et al., 2012), the frequency of the IRN triple mutant increased compared with the susceptible strain (NCS). Elsewhere in the West African region, this increase in the frequency of IRN triple mutant genotypes was observed by Zhao et al. (2021) (64%) in Nigeria and l'Episcopia et al. (2023) (81.1%) in Benin (Zhao et al., 2021; l'Episcopia et al., 2023).

This study has an essential limitation related to saliva. Indeed, compared to blood, whole saliva collection presents limitations in terms of sample quality, such as the risk of extrasalivary contaminants, which must be minimized during collection, and the excessive presence of proteins in the sample, which may be indicative of an underlying infection.

Large quantities of protein or foreign contaminants can remain in the DNA extract, making quantification inaccurate and possibly influencing the quantity/quality ratio (Caizhi et al., 2023).

Conclusion

The main objective of this study was to use saliva to analyse the polymorphism of the *pfdhfr* gene, *a* molecular marker of *P. falciparum* chemoresistance to pyrimethamine. The study shows 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, in both saliva and blood. It also showed an increase in the prevalence of genotypes conferring pyrimethamine resistance in these three sites. The study showed an increase in potentially pyrimethamine-resistant isolates, confirming saliva as a biological product that can be used to monitor chemoresistance to antimalarial drugs.

In addition, the study uses a non-invasive sampling method that can replace the collection of blood samples for antimalarial resistance surveillance studies. This is particularly useful for longitudinal cohort monitoring in clinical trials of antimalarial drugs, where it is necessary to repeat sampling in healthy individuals. The method also reduces the need for blood sampling in epidemiological studies where large-scale malaria screening is required. This non-invasive molecular analysis tool also helps to clarify or complement the results of therapeutic efficacy tests to better guide antimalarial drug use policies.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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APPENDIX

Appendix 1. Frequencies of other mutant alleles of the *pfdhfr* gene in blood and saliva.

Codons	Strains and mutations observed		Blood (N=86)	Saliva (N=67)
			Effectifs (%)	Effectifs (%)
			Blood (n=65)	Saliva (n=57)
dhfr_51	Wild type (N)	Asn-51	10 (15.38)	17 (29.82)
		Ile-51	40 (61.50)	28 (49.12)
		Phe-51	7 (10.76)	6 (10.52)
	Mutants	Lys-51	1 (1.53)	0 (0)
		Leu-51	3 (4.61)	0 (0)
		Pro-51	3 (4.61)	5 (8.77)
		Ser-51	1 (1.53)	0 (0)
		Met-51	0 (0)	1 (1.75)
		Thr-51	0 (0)	0 (0)
		Val-51	0 (0)	0 (0)
			Blood (n=66)	Saliva (n=58)
dhfr_59	Wild type (C)	Cys-59	10 (15.15)	10 (17.24)
		Arg-59	36 (54.54)	37 (63.79)
		Ala-59	1 (1.51)	1 (1.72)
		Gly-59	11 (16.66)	3 (5.17)
	Mutants	Leu-59	1 (1.51)	1 (1.72)
		Ser-59	5 (7.57)	0 (0)
		Trp-59	2 (3.03)	2 (3.44)
		Pro-59	0 (0)	2 (3.44)
		Asn-59	0 (0)	0 (0)
		Val-59	0 (0)	0 (0)
		Phe-59	0 (0)	2 (3.44)
		Tyr-59	0 (0)	0 (0)
			Blood (n=81)	Saliva (n=63)
dhfr_108	Wild type (S)	Ser-108	14 (17.28)	8 (12.69)
		Asn-108	60 (74.07)	50 (79.36)
		Ala-108	2 (2.46)	0 (0)
	Mutants	Phe-108	1 (1.23)	1 (1.58)
		His-108	2 (2.46)	0 (0)
		Thr-108	1 (1.23)	0 (0)
		Val-108	1 (1.23)	0 (0)
		Asp-108	0 (0)	1 (1.58)
		Lys-108	0 (0)	0 (0)
		Gly-108	0 (0)	1 (1.58)
		Pro-108	0 (0)	0 (0)
		Ile-108	0 (0)	1 (1.58)
		Arg-108	0 (0)	1 (1.58)

Appendix 2. Frequencies of other mutant genotypes corresponding to the *pfdhfr* gene in blood and saliva.

Haplotype	N51I	C59R	S108N	Blood		Saliva	
				n	Proportion	n	Proportion
WT	N	C	S	12	13 .96	5	7.46
				9	10.47	9	13.43
	N	C	T	1	1.16	0	0.00
	I	C	S	2	2.33	1	1.49
	N	C	F	1	1.16	1	1.49
	N	C	N	2	2.33	5	7.46
	F	C	S	0	0.00	1	1.49
SM	N	C	V	1	1.16	0	0.00
	L	C	S	1	1.16	0	0.00
	P	C	S	1	1.16	0	0.00
	N	C	D	0	0.00	1	1.49
	N	C	R	0	0.00	0	0.00
	N	C	P	0	0.00	0	0.00
	N	W	S	0	0.00	0	0.00
				26	30.23	25	37.31
	N	G	A	1	1.16	0	0.00
	N	S	N	2	2.33	0	0.00
	L	G	S	2	2.33	0	0.00
	F	C	N	2	2.33	1	1.49
	N	R	N	8	9.30	12	17.91
	I	C	N	7	8.14	3	4.48
	N	G	N	2	2.33	1	1.49
	N	A	A	1	1.16	0	0.00
	P	F	S	0	0.00	1	1.49
	M	R	S	0	0.00	1	1.49
	P	G	S	0	0.00	1	1.49
	F	C	G	0	0.00	1	1.49
	P	A	S	0	0.00	1	1.49
	F	C	S	0	0.00	1	1.49
	I	W	S	0	0.00	1	1.49
	I	R	S	0	0.00	1	1.49
DM	I	F	S	0	0.00	0	0.00
	N	P	N	0	0.00	0	0.00
	I	C	D	0	0.00	0	0.00
	T	N	S	0	0.00	0	0.00
	N	W	N	0	0.00	0	0.00
	N	Y	N	0	0.00	0	0.00
	P	W	S	1	1.16	0	0.00
				39		28	41.79
	S	G	N	1	1.16	0	0.00
	F	L	N	1	1.16	0	0.00
	I	G	H	1	1.16	0	0.00
	F	S	N	2	2.33	0	0.00
	I	R	N	27	31.40	21	31.34
	I	R	H	1	1.16	0	0.00
	K	G	N	1	1.16	0	0.00
	I	W	N	1	1.16	0	0.00
	I	S	N	1	1.16	0	0.00
	F	G	N	2	2.33	0	0.00

Appendix 2 Contd. Frequencies of other mutant genotypes corresponding to the *pfdhfr* gene in blood and saliva.

TM	P	G	N	1	1.16	1	1.49
	P	R	R	0	0.00	1	1.49
	F	R	N	0	0.00	1	1.49
	I	L	N	0	0.00	1	1.49
	F	P	I	0	0.00	1	1.49
	F	P	N	0	0.00	1	1.49
	F	W	N	0	0.00	1	1.49
	V	R	N	0	0.00	0	0.00
	I	V	K	0	0.00	0	0.00
	V	R	K	0	0.00	0	0.00
	T	L	R	0	0.00	0	0.00
	T	F	K	0	0.00	0	0.00

WT : Wild Type, SM : Simple Mutant, DM : Double Mutant, TM : Triple Mutant.

Appendix 3. Frequencies of wild-type strain Ser-108 and other mutations in codon 108 of the *pfdhfr* gene polypeptide

Blood	**Anonkoua-Kouté (n=29)**		**Port-Bouët (n=22)**		**Ayamé (n=30)**	
	Numbers	**%**	**Numbers**	**%**	**Numbers**	**%**
Ser-108	3	10.34	6	27.27	5	16.67
Asn-108	20	68.97	15	68.18	25	83.33
Ala-108	1	3.44	1	4.54	0	0
Phe-108	1	3.44	0	0	0	0
His-108	2	6.89	0	0	0	0
Thr-108	1	3.44	0	0	0	0
Val-108	1	3.44	0	0	0	0

Saliva	**Anonkoua-Kouté (n=21)**		**Port-Bouët (n=19)**		**Ayamé (n=23)**	
	Numbers	**%**	**Numbers**	**%**	**Numbers**	**%**
Ser-108	1	4.76	2	10.53	4	17.39
Asn-108	20	95.24	16	84.21	15	65.21
Asp-108	0	0	0	0	1	4.34
Phe-108	0	0	0	0	1	4.34
Gly-108	0	0	0	0	1	4.34
Ile-108	0	0	0	0	1	4.34
Arg-108	0	0	1	5.26	0	0

Appendix 4. Frequencies of wild-type strain Cys-580 and mutations in codon 580 of the *pfK13 propeller* gene polypeptide.

Blood	**Anonkoua-Kouté (n=30)**		**Port-Bouët (n=32)**		**Ayamé (n=31)**	
	Numbers	**%**	**Numbers**	**%**	**Numbers**	**%**
Cys-580	28	93.33	**29**	90.63	**28**	90.32
Tyr-580	**0**	0.00	0	0.00	0	0.00
Ser-580	1	3.33	1	3.13	1	3.23
Pro-580	1	3.33	2	6.25	0	0.00
Gly-580	0	0.00	0	0.00	1	3.23
Arg-580	0	0.00	0	0.00	1	3.23

Saliva	**Anonkoua-Kouté (n=27)**		**Port-Bouët (n=28)**		**Ayamé (n=19)**	
	Numbers	**%**	**Numbers**	**%**	**Numbers**	**%**
Cys-580	14	51.85	**5**	17.86	**16**	84.21
Tyr-580	0	0.00	0	0.00	1	5.26
Trp- 580	3	11.11	0	0.00	0	0.00
Arg-580	5	18.52	6	21.43	0	0.00
Gly-580	2	7.41	6	21.43	0	0.00
Pro-580	1	3.70	1	3.57	0	0.00
Gln-580	1	3.70	1	3.57	0	0.00
Ser-580	**1**	3.70	**3**	10.71	**0**	0.00
His-580	0	0.00	1	3.57	0	0.00
Thr-580	0	0.00	2	7.14	0	0.00
Asn-580	0	0.00	1	3.57	0	0.00
Val-580	0	0.00	1	3.57	0	0.00
Ala-580	0	0.00	1	3.57	0	0.00
Asp-580	0	0.00	0	0.00	1	5.26
Phe-580	0	0.00	0	0.00	1	5.26