Allelic frequencies of mutants of the *Plasmodium falciparum*, quinoline and folate metabolizing genes in the west region of Cameroon

Innocent Mbulli Ali b,1,*, Valery Pacome Kom Tchuenkam b,1, Sandra Sob Tagomo b,1, Hornela Mawamba b,1, Marcel Nyuylam Moyeh c, Emmanuel Nfor Nfor d, Akindeh Mbuh Njia a, Calvino Tah Fomboha a, William Dorian Nanaa, Jean-Paul Chedjou Kenge a, Peter Thelma Ngwa Nibaa e, Germaine Ekobo Ekoyole, Dorothy Fosah Achue, Jude Daiga Bigoga a, Wilfred Fon Mbachama a,**

a MARCAD Program, The Biotechnology Centre, University of Yaounde 1, BP 8094, Yaounde, Centre Region, Cameroon
b Department of Biochemistry, Faculty of Science, BP 67, University of Dschang, Dschang, West Region, Cameroon
c Department of Biochemistry and Molecular Biology, Faculty of Science, University of Buea, BP 63, Buea, South West Region, Cameroon
d Malaria Program, Cameroon Baptist Convention Health Services, BP 01, Nkwen, Bamenda, North West Region, Cameroon
e National Malaria Control Program, Ministry of Public Health, Yaounde, BP 14386, Centre Region, Cameroon

ARTICLE INFO

Keywords: Malaria, Drug resistance, Pfcr, Pfmdr1, Pfdhps, Ngounso, West region, Cameroon

ABSTRACT

The emergence and spread of *Plasmodium falciparum* (*P.f*) drug resistance is still a major concern in Sub-Saharan Africa and warrants that its evolution be monitored continuously. The present study aimed at determining the distribution of key *P.f* drug resistance-mediating alleles in circulating malaria parasites in the West region of Cameroon. A cross sectional hospital-based study was conducted in Dschang and Ngounso in the West region of Cameroon. The *Pfcrt*, *Pfmdr1*, and the *Pfdhps* genes were amplified through nested PCR in 208 malaria-infected samples of the 301 febrile outpatients enrolled. The presence or absence of mutations in the K76T, N86Y, A437G and A581G codons of these *P.f.* genes respectively were determined through restriction digestion analysis. The proportion of different alleles were estimated as percentages and compared between two study sites using the Chi square test. A p value < 0.05 was considered significant. A high prevalence (75.6%) of the 437G allele was observed. It was significantly different between Dschang and Ngounso (62% vs. 89.2%, X2 = 19.6, P = 0.00005).

Equally observed was a 19.2% (95%CI: 13.3 – 25.6) of the *dhps*-581G mutant allele. Furthermore, we observed the *Pfcrt*-76T and *Pfmdr1*-N86 mutations in 73.0% (67.5 – 79.7) and 87.2% (83.2 – 91.9), and 3.0% (0.0 – 9.6) and 12.8% was observed for the *Pfcrt*-K76T and *Pfmdr1*-N86Y respectively. When biallelic haplotypes were constructed from alleles of the three genes, same pattern was seen. Overall, 73% and 87% of circulating *P. falciparum* isolates carried wild type alleles at *Pfmdr1*-N86Y and *Pfcrt*-K76T. On the other hand, we found more parasites with mutant alleles at *dhps* (437G and 581G) loci which may reflect possible drug-related selection of this mutant in the parasite population. Continuous monitoring of these mutations is recommended to preempt a loss in sulphadoxine-pyrimethamine efficacy in malaria chemoprevention programs.

1. Introduction

Malaria remains an important public health concern in tropical countries where it is endemic [1]. In 2019, there was an estimated 229 million malaria cases with approximately 409,000 deaths from 87 malaria-endemic countries. Approximately 94% of estimated cases were detected in Africa [1]. Strategies to fight include accurate and prompt diagnosis, vector control and chemotherapy [2]. Before 2001, mono-therapy with chloroquine and amodiaquine were the first-line drugs for the treatment of uncomplicated malaria. However, extensive review reports showed that the clinical efficacy of mono-therapy significantly declined [3, 4, 5, 6, 7]. The poor clinical efficacy of the...
monotherapies due to drug resistance led the WHO in 2004 to recommend the introduction of combination therapy, notably, artemisinin-based combination therapies (ACTs) [8]. Most countries in sub-Saharan Africa where malaria is endemic adopted ACTs such as artemether-lumefantrine (AL) or artesunate-amodiaquine (AS-AQ) as first-line ACTs. Cameroonian health authorities recommended AS-AQ as the first-line treatment of uncomplicated malaria since 2004 and AL as an alternative therapy since 2006 [9]. The emergence and spread of resistance to artemisinin in Western Cambodia of Southeast Asia [10, 11, 12, 13] prompted global concern given that chloroquine and sulfadoxine-pyrimethamine resistance [14, 15, 16] arose in the same region and then spread to Sub-Saharan Africa. Recent studies also suggest that resistant mutations may emerge independently in these areas and then spread as has been shown in previous studies [17, 18, 19, 20]. Also, the emergence of resistance and tolerance against the long-acting ACT partner drugs, such as amodiaquine and lumefantrine, may consequently affect the efficacy of the ACT partners and increase the emergence of resistance to the short-acting artemisinin-derivatives [21].

Although ACT partner drugs remains effective in Africa, prolonged and abusive use would lead to anti-malarial drug resistance. Antimalarial drug resistance (ADR) would be disastrous for global malaria control. Therefore, in the absence of more treatment options, it is important to monitor the ADR status of P. falciparum parasites. Mutations detected in P. falciparum essential genes including chloroquine resistance transporter gene (Pfcrt), multidrug resistance gene (Pfmdr1), dihydrofolate reductase (Pfdhfr), dihydropteroate synthetase (Pfdhps), Kelch-13 (pfk13), and Plasmodolin (pfpm2) have been used as molecular markers of drug resistance [22, 23]. Single nucleotide polymorphisms (SNPs) have been fundamental in monitoring existing or predicting emerging drug resistance patterns. SNPs in the Pfmdr1 and Pfcrt genes have been associated with resistance to chloroquine [22] amodiaquine [24], lumefantrine, and quinine but also linked to ACTs introduction. In addition, SNPs in pfdhfr and Pfdhps have been associated with pyrimethamine and sulfadoxine resistance respectively [15, 23, 25, 26]. On the other hand, a high proportion of Pfmdr1 N86 and Pfcrt K76 alleles have been recorded in AL treated patients with recurrent parasites [27, 28].

In the Western region of Cameroon, characterized by varied malaria parasite transmission patterns, there is paucity of data on the prevalence of these resistance markers among parasite populations from clinical samples. The present study aimed at evaluating the key SNPs in Pfcrt, Pfmdr1, and Pfdhps genes in circulating P. falciparum infections in Dschang (low transmission) on the western plateau with a transmission intensity of 62.8ib/human/year and Ngounou (high transmission) in the western lowland with a transmission intensity of 90.5ib/human/year.

2. Methods

2.1. Study setting and location

Two localities of the West region of Cameroon; Dschang (5.45, 10.06E and Ngounou 5.96N, 11.23E) were chosen. Dschang is the capital of the Menoua division, one of the six in the West Region. It has an annual rainfall of 2000mm and is situated at 1400m above sea level. It has an equatorial climate with a mountainous relief, characterized by two distinct seasons, the main rainy (mid-March-October) punctuated by a short dry season in July, and the main dry (November-Early March). Ngounou is a village located in Magha Sub-division, in Noun Division of the Western Region of Cameroon. It is situated 60km north of Foumban town, the Head Quarter of the division along the national road number 6 to Banyo. It is situated in the Noun lowlands with an altitude of 720m above sea level and an annual rainfall of 1500mm. As Dschang, it has and equatorial climate characterized by two distinct seasons, the rainy season (April to October) and the dry season (November to March).

2.2. Study design

We carried out a cross-sectional study from May to August 2020 in the district hospital in Dschang and in the Cameroon Baptist Convention Integrated Health centre in Ngouono. An ethical clearance was obtained from the Cameroon Baptist Convention Health Services Institutional Review Board (IRB2019-38 of 05/02/2020). All febrile patients for whom a malaria test was prescribed and who consented to participate in the study were included. Approximately 0.5ml of peripheral blood was collected and distributed as follows: 0.1ml for malaria microscopy, 0.1ml for malaria rapid diagnostic test (CareStart Malaria Ag Pf/Pan) and the rest (0.3ml) was spotted on a Whatman 3MM filter paper (3 spots), air-dried, labelled and stored at minus 20 °C in polyethylene bags containing a small sachet of silica gel.

2.3. Parasite DNA extraction

The malaria parasite DNA was extracted using the Chelex 100 technique as described elsewhere [23]. Basically, the filter paper spotted blood samples were incubated overnight with 1ml 0.5% saponine at +4 °C. Then the spot was washed with 1ml PBS 1x for 30 min at +4 °C. The spot was then introduced in a microtube containing 50ul pre-heated Chelex with 150ul Molecular grade water. The mixture went through three rounds of heating at 100 °C for 10min each time preceded by thorough vortexing for 15sec. After this, the product was centrifuged for 2 min at 14000 rpm. The supernatant was introduced into a new microtube and centrifuged again as in the previous step. The supernatant was collected and stored at -20 °C.

2.4. Amplification of parasite genes by the polymerase chain reaction

The amplification of the parasite DNA was done as previously described by Plowe et al., [29]. The sequence of primers of gene fragments targeted are shown in Table 1, based on a previous study [22, 30, 31, 32]. A total volume consisting of 7 μl of nuclease free water (NFW), 10 μl of OneTaq® Hot Start 2X Master Mix with standard buffer (New England Biolabs, MA, USA), 0.5 μl of each primer 0.4 μM (R2, R3) and 2 μl of DNA extract was composed. The amplification reaction conditions were for dhps; pre-denaturation at 94 °C for 3 min; denaturation at 94 °C for 1 min, primer annealing at 50 °C for 2 min, extension at 72 °C for 2 min x 40 cycles and a termination at 72 °C for 10 min. Regarding the nested PCR, each tube consisted of 8μl of DNAse-free water, 10 μl of OneTaq® Hot Start 2X Master Mix with standard buffer (New England Biolabs, MA, USA), 0.5μl of each nested primers 0.4 μM (K + K/and L + L)/ and 1μl of ampiclon of the primary PCR reaction for a total volume of 20μl. These tubes were subjected to the following amplification conditions: denaturation at 94 °C for 1 min, primer annealing at 45 °C for 1 min, extension at 72 °C for 2 min x 35 cycles and termination at 72 °C for 10 min.

To amplify the Pfmdr1 gene fragment of interest, a total reaction volume of 20 μl was prepared containing 8 μl of DNAse-free water, 0.5 μl of each of the outer primers 0.4 μM (MDR1 and MDR2), 9 μl of OneTaq® Hot Start 2X Master Mix with standard buffer (New England Biolabs, MA, USA) and 2 μl of extract of dDNA. The tubes were then placed in T3 Thermocycler (Biometra, Göttingen, Germany) for amplification under the following reaction conditions: Pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 30s, hybridization at 60 °C for 60s, elongation at 72 °C for 10 min through 30 cycles; and finally termination at 72 °C for 3 min. For the nested reaction, the 20 μl reaction volume consisted of 9 μl of NFW, 0.5 μl of each 0.4 μM MDR3 and0.4 μM MDR4 primers, a 9 μl OneTaq® Hot Start 2X Master Mix with standard buffer (New England Biolabs, MA, USA) and 1 μl of primary PCR ampiclon. The tubes were then placed in a thermocycler (T3 Thermocycler, Göttingen, Germany) for amplification according to the program: pre-denaturation at 94 °C for 5 min, 30 cycles; denaturation at 94 °C for 30s, hybridization at 60 °C for
60s, elongation at 72 °C for 1–10 min, followed by termination at 72 °C for 3 min.

We then amplified the Pfcr cognate fragment of interest under the following reaction conditions. For each tube, a 20μl total reaction volume was prepared consisting of 8μl of DNAase-free water, 0.5μl of each of 0.4 μM CRT-P1 and0.4 μM CRT-P2 primers, 9 μl of OneTaq® Hot Start 2X Master Mix with standard buffer (New England Biolabs, MA, USA.) and 2 μl of DNA extract. Pre-denaturation at 94 °C for 5 min; denaturation at 94 °C for 30s, hybridization at 60 °C for 60s, elongation at 72 °C for 1–10 min for a total of 30 cycles, followed by termination at 72 °C for 3 min. The products of the primary PCR reaction were used as substrate for the nested PCR reaction. For this, a new 20 μl reaction volume was prepared for each tube composed of 9 μl of DNAase-free water, 0.5 μl of each of 0.4 μM CRT-D3 and 0.4 μM CRT-D4 primers, 9 μl of OneTaq® Hot Start 2X Master Mix with standard buffer (New England Biolabs, MA, USA.) and 1 μl of the primary PCR product. The tubes were then placed in the same thermocycler for amplification according to the program: pre-denaturation at 94 °C for 5 min, 30 cycles; denaturation at 94 °C for 30s, hybridization at 60 °C for 60s, elongation at 72 °C for 1–10 min, followed by termination at 72 °C for 3 min.

The amplified Pfmdr-1, Pfhdps, Pfcr cognate gene were revealed using 2% agarose gel electrophoresis and visualized using a UV transilluminator. Samples that amplified the malaria parasite specific fragment targeted by the primers in the semi nested PCR were selected to screen for the mutations related to drug resistance in P. falciparum. The Genomic DNA of HB3, 3D7 and DD2 strains of P. falciparum (from the Sutherland laboratory at the London School of Hygiene and Tropical Medicine, London, UK) were used as positive controls for the studied polymorphisms.

### 2.5. Restriction digestion

Single nucleotide polymorphisms in selected genes were detected using restriction fragment length polymorphism (RFLP-PCR). For the Pfmdr1, the region containing codon 86 was digested using the restriction enzyme AvaI. The master mix (20μl) for each sample of the Pfmdr1 gene was prepared in a 0.2ml microtube: 8.μl nuclease free water, 3.0μl buffer (10XmM), 0.4μl, 1U AvaI and 8μl amplicon respectively. The tubes were incubated overnight at 37 °C and digestion was revealed using 2% agarose gel. The region around codon 76 of the Pfcr cognate gene was digested using the restriction enzyme APO I which cuts or digests the DNA if Lys is present (when there is no mutation). The master mix (20μl) for digestion experiment for each sample of the Pfcr cognate gene was prepared in a 0.2ml microtube: 7.8μl nuclease free water, 3.0μl buffer (10mM), 0.2μl BSA, 1μl 1U APO I and 8μl amplicon. The tubes were incubated overnight at 50 °C and digestion was revealed using a 2% agarose gel electrophoresis as described above. For the dhps cognate gene, restriction digestion of amplicons was performed using codon specific restriction enzymes AvaII for A437G and BstUI for A581G. Each tube consisted of 8μl of DNAase-free water, 3μl of 10X buffer, 1μl of restriction enzyme at concentration 1U and 8μl of amplicon for a total volume of 20μl. The mixtures were then incubated at 37 °C for the AvaII and 60 °C for the BstUI enzyme in an incubator for 1.5 h. The products of digestion were revealed using a 2% agarose gel electrophoresis as described above and interpretation done as per standard operating procedures.

### 2.6. Data analysis

Data from this study were transcribed from paper records unto an Excel sheet (Microsoft Excel; version 2016). Data entry was double-checked to prevent transcription errors. We then used this as the base to build the database of single nucleotide polymorphisms (SNPs) per entry. This de-identified dataset was used to calculate the proportion of each SNP genotyped, alongside their 95% confidence intervals. The Chi square test ($\chi^2$ test) was used to measure differences in genotype proportions between study sites. Where the number of expected observations was less than 5, the Fisher’s test was used. The R software (R Foundation for Statistical Computing, Vienna, Austria; version 4.0.4) was used to analyze the dataset. A $p < 0.05$ was considered significant in all comparisons.

#### 2.6.1. Ethical considerations

Before performing this study, an ethical clearance was obtained from the Cameroon Baptist Convention Health Services Institutional Review Board (IRB2019-38 of 05/02/ , 2020). All study staff were trained on human subject research and good clinical laboratory practice prior to enrolling study participants. All participants provided written informed consent/assent after the study background, objectives, and procedures, measures to minimize risk; the benefits; measures to maintain confidentiality and privacy were explained. Participants who did not provide a written consent were not enrolled in the study, but were treated with the same standard of care as anyone else. The consent included agreement to publication of de-identified data through peer-reviewed manuscripts.

---

**Table 1. Primer sequences and codons targeted for PCR amplification.**

| Gene & PCR reaction | Mutation | Primer identity | Sequence (5’→3’) |
|---------------------|----------|-----------------|-----------------|
| Pfcr primary PCR reaction | K76T | CRT-P1 (1/5) | CGTATAAATATAAATACAGCAG |
| Pfcr primary PCR reaction |  | CRT-P2 (1/5) | CGGATGGTAAAAACATATGTTAC |
| Pfcr nested PCR reaction | K76T | CRT-D3 (1/5) | TGTGCTCAGTGTGTTAAAATT |
| Pfcr nested PCR reaction |  | CRT-D4 (1/5) | AAAACATGATTACAAATTTGG |
| Pfmdr1 primary PCR reaction | N86Y | MDR1 | GCCGCCGTAGACAAAAAGAGTCGCTG |
| Pfmdr1 nested PCR reaction |  | MDR2 | GGGGCGTACCCCATTCGAGAC |
| Pfmdr1 nested PCR reaction |  | MDR3 | TTACCTGGTATTGCTTTGCTG |
| Pfmdr1 nested PCR reaction |  | MDR4 | CCATCCGTGATAAAAAACATCTT |
| Dhps Primary PCR reaction | A437G | R2 | AACCTAAAGTCGTCCTCAA |
| Dhps nested PCR reaction | K | R/ | AATTGTGATTGTCGACAA |
| Dhps nested PCR reaction | K/ | K/ | GTCTGACCTGCTCCTAC |
| Dhps Primary PCR reaction | A581G | R2 | AACCTAAAGTCGTCCTCAA |
| Dhps nested PCR reaction | L | R/ | ATAGATACCTATTTGTAATAGGACCCAGGATTAG |
| Dhps nested PCR reaction | L/ | L/ | ATTACACATTGTCATTGCCAGCCG |

---

I.M. Ali et al. Heliyon 8 (2022) e11861
3. Results

3.1. Demographic distribution of the study population

A total of 301 patients were recruited for this study. Out of this, 208 patients whose isolates provided at least one genotype result were included in the analysis. 105 from Dschang and 103 from Ngounso. 110 (52.9%) were females and 98 (47.1 %) males. The age group 0–29 was the most represented 138 (66.4%). This is summarized in Table 3 below.

3.1.1. Prevalence of the Pfcrtr K76T and Pfmdr1 N86Y mutations

The prevalence of K76T and N86Y mutations was assessed for all the samples positive by PCR. An overall amplification efficiency of 94.7% (95% CI: 92.3–97.7) for the Pfcrtr K76T and 94.2% (91.8–97.5; 95% CI) for the Pfmdr1 N86Y mutations was obtained. The mutations detected are presented in Table 3 below.

3.1.2. Pfcrtr-K76T and Pfmdr1-N86Y alleles in the study sample

The prevalence of K76T and N86Y mutations was determined for all the samples positive by PCR. An overall amplification efficiency of 94.7% (95% CI: 67.5–79.7) had the wild-type allele, 47 (23.9% (95% CI: 18.3–30.4)) the mutant allele and 6 (3.0% (95% CI: 0.0–9.6)) were both mutant and Wild-type for the K76T loci in the study samples. Regarding the Pfmdr1 N86Y gene, we found a prevalence of 87.2% (95% CI: 83.2–91.9) for the wild-type Pfmdr1-N86, and 12.8% (25/196 (83.2–91.9; 95% CI)) harbored both the mutant and the wild-type at this locus (Table 2). No mutant Pfmdr1-N86Y allele was found. Although we found differences in the prevalence of K76T mutation between the sites, these differences were not significant (P = 0.6) as shown in Table S1. With regards to Pfmdr1-N86Y, the prevalence of samples carrying both the mutant and the Wild-type genotype was significantly different between the two sites (P = 0.03; S1). Overall, there was a high proportion of circulating parasites with the wild-type alleles at the analyzed loci in both genes.

3.1.3. Pfdhps A437G and A581G alleles in the study samples

We had an amplification efficiency of 96.6% (94.7–98.9; 95% CI) and 97.6% [94.7–98.9; 95% CI] for the Pfdhps fragments containing the mutations A437G and A581G respectively (Table 3). For the A437G mutation, we had a predominance of the mutant haplotype 152 (75.6%), and 6 (3.0%) having both the mutant and the wild-type. There was a significance difference (P < 0.05, X² = 19.6) between the prevalence of A437G mutations of the two studied sites (Figure 1, lower panel. Table S1). For the A581G mutation, we found a predominance of the wild-type allele 143 (70.4% [64.5–76.8; 95% CI]) with no significant difference between the prevalence in Dschang (72.5% [64.7–81.4]) compared to Ngounso [69.3% (95% CI 60.4–77.9)] at both of these loci (Figure 1. Table S1). We also found the mutant allele in 39 isolates [19.2 % (95% CI 13.3–25.6)] and 21 isolates [10.3% (95% CI: 4.4–16.7)] had both the mutant and the Wild-type alleles. Out of this, 14 were from patients in Ngounso.

Biallelic haplotypes of resistance markers studied.

We constructed biallelic haplotypes from the mutations observed in different genes in monomodal infections. The combinations of multiple mutations found in single genes and in both genes, are shown in Figure 2.

These combination of haplotypes in an isolate, was defined as wild type haplotype when they contained the wild type allele at all loci. Similarly, mutant haplotypes were defined as having the mutant alleles at both loci. The mutant haplotype TY made of the 76T and the 86Y mutation, was obtained. Regarding the Pfcrtr-K76T and the Pfmdr1-N86Y mutations, we found a high proportion of 94.7% (171/196) and 97.5% (192/203) of the total for the double mutation TY in the entire studied population. With 04 from each site. We found a 19.3% [95% CI: 13.1–26.1] (43/222) for the double mutant haplotype GG of the Pfdhps gene. No significant difference was found when the proportions of this double mutant was compared between the two studied sites (S1).

4. Discussion

A cross-sectional study in two sites to assess the prevalence of mutations in the Pfcrtr-76, Pfmdr1-86 and the Pfdhps codon 437 and 538 genes responsible for Plasmodium falciparum resistance in malaria patients was conducted. The Pfcrtr K76T mutation is involved in the resistance of Plasmodium falciparum to amodiaquine and chloroquine (7,25–27), the Pfmdr1 N86Y mutation which is a multi-drug resistance mutation to Plasmodium falciparum is associated to the usual antimalarial drugs namely amodiaquine, quinine, chloroquine, arteether [24, 27] and the Pfdhps which is associated to sulphadoxine and pyrimethamine [25, 33].

Studies assessing the occurrence of resistance-conferring alleles in natural populations of Plasmodium falciparum are important for epidemiological purposes but also for drug policy making. Molecular markers of drug resistance have been shown to provide early warning signs about potential loss in drug efficacy [7, 19, 22, 33]. The primary goal of this study was to assess key single nucleotide polymorphisms of the Pfcrtr, Pfmdr1 and Pfdhps associated with P. falciparum drug resistance in malaria-infected samples in two health facilities in the west region of Cameroon. Of the 301 samples amplified, 208 were positive amplification for Plasmodium falciparum, with 105 from Dschang and 103 from Ngounso giving an overall 69.1% positivity. This percentage positivity could be due to the presence of other species of Plasmodium present in the population which we did not explore in the present study.

The characterization of the Pfmdr1 gene showed a distribution of the dominant Pfmdr1 wild-type allele N86 with an overall prevalence of 87.2% (171/196), 12.8% (25/196) of both mutations and none of the mutant allele 86Y was found in both sites. These findings are consistent with the work of Sulayman et al. [34], who also did not find the 86Y allele.
mutant allele in both study sites. The proportions of mutants obtained in the two sites were different and statistically significant ($P < 0.05$). Equally, the 76T mutation of the $Pfcrt$ gene, which codes for a transporter protein located in the digestive vacuole of $P. falciparum$, was found in 24.7% (24/97) in Dschang and in 23.0% (23/100) in Ngounso. The overall prevalence of the $Pfcrt$ 76T allele found was 23.9%. This finding corroborates with that obtained in Equatorial Guinea (30%) by Berzosa et al. [35], who assessed the mutations in locations of similar urbanity and malaria transmission intensity like our study. Previous studies by Ali et al., (2014, unpublished) in Cameroon showed a higher prevalence (83%) compared to ours. The decrease in the prevalence of the $Pfcrt$ 76T mutation may primarily be due to the withdrawal of chloroquine from the Cameroonian market as a first-line treatment, which would have contributed to the decrease in drug pressure and consequently to the re-emergence of chloroquine sensitive parasites [36, 37, 38, 39].

The results indicate that parasites keep evolving in the presence of selection forces such as drug pressure. While the findings are good for the sensitivity of chloroquine and amodiaquine, the current use of artemether-lumefantrine in malaria case management might be threatened, largely because the CQ and AQ resistance related wild type alleles are selected for by pressure from lumefantrine exposure while parasites with mutant alleles are selected for by CQ and AQ. There is a growing

Figure 1. Frequency of aminoquinoline Upper panel (A: Pfcr7 76 and B: Pfmdr1 86) and antifolate Lower Panel (C: pfdhps 437 and D: pfdhps 581) mutations within the study population of Dschang and Ngounso.

Figure 2. Biallelic haplotypes of $Pfcrt$-K76T and $Pfmdr1$-N86Y markers of resistance in the study sites.
body of evidence from in vitro and in vivo studies that lumefantrine selects for parasites with wild type Pfmdr1-N86 alleles including in efficacy trials [21, 40]. The re-emergence of wild type Pfmdr1 and Pfcr parasites may also be a result of widespread use of artemether-lumefantrine in both prescription settings and over-the-counter. Although we did not quantify the use of Artemether-Lumefantrine in the study areas, in a similar high transmission area in Cameroon, artemether-lumefantrine was noted as the antimalarial of choice purchased by the population both from hospital pharmacies and ambulant or street vendors, followed by quinine tablets. Lumefantrine blood concentrations from widespread exposure during high malaria transmission may thus be playing a role as a directional driver in the emergence of Pfmdr1-N86 alleles in Cameroon [21]. In addition, the population wide use of multiple first line drugs in malaria control programs seems to be advantageous in this context, in which one artemisinin combination drives the emergence of parasites that are susceptible to the alternative combination [41]. In this case, the policy option to use ASAQ and AL as an alternative seems justified. However, there is a strong need to continue monitoring genetic changes associated with resistance to these drugs.

The monitoring of the Pfdfs gene is important to guide policies in malaria chemoprevention in pregnancy and in intermittent preventive treatment in infants and children. In Cameroon, SP is used as a prophylactic treatment for pregnant women while it forms one of the base in the combination used in seasonal malaria chemoprevention [42] in the two northern regions of Cameroon. In Cameroon, most of the studies done to evaluate this resistance is on the pregnant women and children. The present study focused on adults. The prevalence of the codon A437G of the Pfdfs genotype was 75.6%, for the mutant genotype, 3.0% for both genotype and 21.4% for the wild type in the total population. This prevalence is higher than what we found sampling in 2018 (61%, unpublished), much higher than what was found along the slopes of mount Cameroon with samples collected in 2013–2014 [43]. The timing of sample collection might have had some impact on the difference. A recently published study in Dschang found that 59% of parasites carried the dfsps-437G mutation while our study with much recent sample collection (2020) found significantly much more. This may not only reflect the differences in methods used to assess the mutations but may be growing evidence of an expansion of dfsps-A437G mutant parasites in the region. Our results are also similar to those obtained by Chauvin et al. [44] who reported a 76% prevalence of this mutation among pregnant women who had been on SP prophylaxis in Yaoundé. While the sampling was done between 2010 and 2011, the results highlight the possibility that there has been an increase in the prevalence of this mutation in the general population, especially in high transmission areas. Recent studies have reported that the pdfsps 437G has been increasing. Studies across West and central Africa, in neighboring Nigeria and in Zambia have reported high prevalence of this mutation among clinical P. falciparum samples [26, 45, 46]. Similar findings were reported by Ndong-Ngomo et al. [25], who obtained a prevalence of 67% for the dfsps-437G mutation 3 years after policy change to ACTs in Gabon. In some areas of seasonal malaria chemoprevention, more than 80% of the mutation has also reported [47]. There appears not to be a significant difference between the prevalence of the mutation in SMC eligible (3 months-5 years) children and the general population given the rates found in an area of seasonal malaria chemoprevention in Senegal [48]. This high prevalence of the mutation 437G in Pfdfs may indicate that the P. falciparum parasite populations remain subject to high drug pressure and have the potential to rapidly develop more resistant pfdhfr/Pfdfs mutant in the near future, particularly in chemoprophylaxis programs that employ sulphadoxine-pyrimethamine. Drug pressure, in our context, might be resulting from easy access to these drugs by a population with high self-medication rates. The semi-urban setting of N’goungo lies along a trajectory that links Cameroon to Nigeria through informal trade routes. The prevalence of mutations in N’goungo village was much higher than in Dschang; likely explained by increased access and exposure to SP owing to its proximity to trade routes with Nigeria where drugs of questionable quality may be easily procured. A quantification of the tonnage of sulphadoxine-pyrimethamine and amodiaquine/chloroquine in the two localities might throw more light into this assertion. It is possible that easy access from neighbouring Nigeria might be a determinant of the higher proportion of dfsps-437G bearing parasites in this study. In addition, differences in malaria transmission intensity [49] between the two study sites resulting from differences in altitude could account for the increased proportion of circulating parasites bearing resistance alleles in N’goungo. Although studies have shown that even in the presence of the quintuple mutation, SP maintains its efficacy in pregnancy, the search for a viable alternative is increasingly important.

The 581G mutation in Pfdhps plays an important modulatory role in Sulphadoxine resistance. It has been found to reduce efficacy in preventing malaria in pregnant women, as the efficacy of SP in IPT is understood by parasites carrying the A581G mutations [50]. This is consistent with the work of Kateera et al. (2013) in Rwanda who demonstrated that parasites carrying the A581G mutation were associated with low birth weight infants and increased morbidity in patients receiving SP. This mutation, though, did not appear to affect parasitaemia. The 581G resistance-conferring allele was found in 19.3% of all circulating parasites in our study, being higher in N’goungo than in Dschang. It has been shown to vary widely in areas of seasonal malaria chemoprevention [47] settings. It occurs alongside the 540E mutation in East Africa but not in West Africa [15]. Although its effects have been demonstrated authoritatively in pregnancy [50, 51], it is very important, given the 19.3% prevalence we found in our study, to monitor the evolution of this mutation in the study areas and in the wider transmission settings. When we constructed biallelic (Pfdhps A437G-A581G) haplotypes at these dfsps loci we found that the double mutant GG was in 19.3% (43/222) indicating all dfsps-437G parasites also contained the 581G mutation. These mutations that result in the replacement of Alanine (A) by Glycine (G) in the codons 437 and 581 indicates more widespread occurrence of mutations in other gene loci which warrants more studies. The National Malaria Control Program envisages the implementation of IPTi/IPTc as a strategy to curb the high prevalence of malaria in this age group. It is therefore an additional reason why surveillance of these mutations should be part of the deployment and future monitoring and evaluation strategy.

This study has a limitation. We did not genotype for loci associated with drug resistance. Several studies have shown that, especially in high transmission areas, relevant pfdhfr mutations have reached fixation. A body of evidence shows this. A recent assessment of the dhfr mutations in one of the study sites highlights this [52] as well as unpublished data from the authors in several regions in Cameroon. Although the Leu-164 mutation of the dhfr gene could be of interest, it is very rare in west/central Africa. Evaluating status of mutations in more loci from these genes or in other identified and validated genes would add evidence into the occurrence and the role of new and known markers in conferring resistance to antimalarial drugs.

5. Conclusion

This study shows a population of parasites predominantly wild type for Pfcr and Pfmdr1-N86 but predominantly mutants for dfspsA437G and dfspsA581G consistent with findings from other studies. The proportion of the double mutant GG at dfsps437 and dfsps581 highlights the need to further characterize the whole panel of known and new markers of SP resistance to support the routine monitoring of chemoprevention programs such as seasonal malaria chemoprevention and perennial malaria chemoprevention of malaria in Cameroon.

Consent for publication

Information on potential publication of anonymised data was included in the consenting process in the information sheet. All participants were aware and consented to publication of the data reported in this manuscript.

I.M. Ali et al. Heliyon 8 (2022) e11861
Availability of data

All supporting data are within the manuscript and its supplementary tables.

Competing interests

The authors declare no competing interests.

Funding

This research was funded in whole, or in part, by the Wellcome Trust (107741/A/15/Z) and the UK Foreign, Commonwealth & Development Office, with support from the Developing Excellence in Leadership, Training and Science in Africa (DELTAS Africa) programme. For the purpose of open access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission.

Declarations

Author contribution statement

Innocent Mbulli Ali: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Pacom Valery Kom Tchuenkam: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sandra Sob Tagomo, Masamba Hornela: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Marcel Nyuylam Moyeh, Emmanuel Nfor Nfor, Akindeh Mbuh Nji, Germaine Ekobo Eko yol: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Calvino Tah Fomboh, William Dorian Nana: Performed the experiments; Analyzed and interpreted the data.

Jean-Paul Chedjou Kenge, Peter Thelma Ngwa Niha: Analyzed and interpreted the data.

Dorothy Fosah Achu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Jude Daiga Bigoga: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Wilfred Fon Mbacham: Conceived and designed the experiments; Analyzed and interpreted the data.

Funding statement

Dr Innocent Mbulli Ali was supported by Malaria Research Capacity Development in West and Central Africa (MARCAD) Consortium through the Developing Excellence in Leadership, Training and Science (DELTAS) Africa Initiative [grant # DEL-15-010].

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e11861.

Acknowledgements

We wish to thank the hospital authorities of the Dschung District Hospital and the Ngonufo Integrated health Centre of the Cameroon Baptist Convention Health Services. We also wish to thank all participants for their time and willingness to be part of the study.

References

[1] WHO, World Malaria Report 2020: 20 Years of Global Progress and Challenges 299, World Health Organisation, Geneva, 2020, p. 299.

[2] P. Winstanley, S. Ward, R. Snow, A. Breckenridge, Therapy of falciparum malaria in sub-Saharan Africa: from molecule to policy, Clinic. Microbiol. Rev. 17 (3) (Jul. 2004) 612–637.

[3] C.V. Flows, Combination therapy for malaria: mission accomplished? Clin. Infect. Dis. 44 (8) (Apr. 2007) 1075–1077.

[4] N.J. White, Antimalarial drug resistance, J. Clin. Invest. 113 (8) (Apr. 2004) 1084-1092.

[5] E. Ashley, The threat of antimalarial drug resistance, Int. J. Infect. Dis. 45 (Apr. 2016) 15.

[6] J. Lusingu, L. Seidlein, Challenges in malaria control in Sub-Saharan Africa: the vaccine perspective, Tanzania, J. Health Res. 10 (4) (Aug. 2009).

[7] J.H. Sibley, et al., Pyrimerthine-sulfadoxine resistance in Plasmodium falciparum: what next? Trends Parasitol. 17 (12) (Dec. 2001) 582-588.

[8] P.T.N. Niba, et al., Drug resistance markers within an evolving efficacy of antimalarial drugs in Cameroon: a systematic review and meta-analysis (1998-2020), Malar. J. 20 (1) (Dec. 2021) 32.

[9] NMCP, Situation of Malaria Control Progress, National Malaria Control Program, MINSANTE, 2006.

[10] D. Menard, A. Dondorp, Antimalarial drug resistance: a threat to malaria elimination, Cold Spring Harb Perspect Med 7 (7) (Jul. 2017) a025619.

[11] A. Uwimana, et al., Emergence and clonal expansion of in vitro artemisinin-resistant Plasmodium falciparum kelch13 R561H mutant parasites in Rwanda, Nat. Med. 26 (10) (Oct. 2020) 1662–1668.

[12] A.M. Dondorp, et al., Artemisinin resistance in Plasmodium falciparum malaria, N. Engl. J. Med. 361 (5) (Jul. 2009) 455–467.

[13] E.A. Ashley, et al., Spread of artemisinin resistance in Plasmodium falciparum malaria, N. Engl. J. Med. 371 (5) (Jul. 2014) 411–423.

[14] R.M. Packard, The origins of antimalarial-drug resistance, N. Engl. J. Med. 371 (5) (Jul. 2014) 397–399.

[15] L.C. Okell, J.T. Griffin, C. Roper, Mapping sulfadoxine-pyrimethamine-resistant Plasmodium falciparum malaria in infected humans and in parasite populations in Africa, Sci. Rep. 7 (1) (Dec. 2017) 7389.

[16] T.J.C. Anderson, C. Roper, The origins and spread of antimalarial drug resistance: lessons for policy makers, Acta Trop. 94 (3) (Jun. 2005) 269-280.

[17] T. Mitu, Origins and spread of pfδfr mut mutant alleles in Plasmodium falciparum, Acta Trop. 114 (3) (Jun. 2010) 166–170.

[18] S. Vinayak, et al., Origin and evolution of sulfadoxine resistant Plasmodium falciparum, PLoS Pathog. 6 (3) (Mar. 2010) e1000830.

[19] M. Alifrangis, et al., Independent origin of Plasmodium falciparum antifolate Super-resistance, Uganda, Tanzania, and Ethiopia, Emerg. Infect. Dis. 20 (8) (Aug. 2014) 1280–1286.

[20] R.J. Pearce, et al., Multiple origins and regional dispersal of resistant dhps in African Plasmodium falciparum malaria, PLoS Med. 6 (4) (Apr. 2009) e1000555.

[21] S.D. Otienoburu, et al., Selection of Plasmodium falciparum pfcr1 and pfmdr1 polymorphisms after treatment with artemesate-amodiaquine fixed dose combination or artether-lumefantrine in Liberia, Malar. J. 15 (1) (Dec. 2016) 452.

[22] A. Djimde, et al., A molecular marker for chloroquine-resistant Plasmodium falciparum malaria, N. Engl. J. Med. 344 (4) (Jan. 2001) 257–263.

[23] W.H. Wermesdorfer, H. Noedl, Molecular markers for drug resistance in malaria: use in treatment, diagnosis and epidemiology, Curr. Opin. Infect. Dis. 16 (6) (Dec. 2003) 553–558.

[24] F.L. Eyase, et al., The role of Pfmdr1 and pfcr in changing chloroquine, amodiaquine, mefloquine and lumenfantine susceptibility in western-Kenya P. falciparum samples during 2008–2011, PLoS One 8 (5) (May 2013), e64299.

[25] J.-M. Ndung Ngomo, D.P. Mawili-Mbomba, N.P. M’Bondoukou, R. Nikimana Ndung Ella, M.K. Bouyou Akotet, Increased Prevalence of Mutant Allele Pfdhps 437G and Pfdhfr Triple Mutation in Plasmodium falciparum isolates from a Rural Area of Gabon, Three Years after the Change of Malaria Treatment Policy 2016, Malaria Research and Treatment, Apr. 2016, pp. 1-6.

[26] L. Zhao, et al., Widespread resistance mutations to sulfadoxine-pyrimethamine in malaria parasites imported to China from Central and Western Africa, Int. J. Parasitology: Drugs Drug Resist. 12 (Apr. 2020) 1-6.

[27] M.I. Veiga, et al., Globally prevalent Pfmdr1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies, Nat. Commun. 7 (1) (Sep. 2016) 11553.

[28] S.T. Windle, et al., Evidence for linkage of pfmdr1, pfcr and pfk13 polymorphisms to lumenfantine and mefloquine susceptibilities in a Plasmodium falciparum cross, Int. J. Parasitology: Drugs Drug Resist. 14 (Dec. 2020) 208-217.

[29] G.V. Plowe, A. Djimde, M. Bouare, O. Doumbo, T.E. Wellems, Pyrimerthine and prophylactic resistance-conferring mutations in Plasmodium falciparum dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa, Am. J. Trop. Med. Hyg. 52 (6) (Jun. 1995) 565-568.
