

Research Article

## Evolution of the *Pfcr* and *Pfmdr1* Markers and Revert of Chloroquine Sensitive *Plasmodium falciparum* in a Seasonal Malaria Chemoprevention Setting in Cameroon

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## Abstract

*Plasmodium falciparum*, the cause of the most lethal malaria in humans, which still remains a threat to global health, with children and pregnant women being the most affected. This burden is highly pronounced in areas where malaria transmission is seasonal. The emergence of drug-resistance poses a major obstacle to the control of malaria. However predicting decreased or increased sensitivity to anti-malarial and fixation of multidrug resistance genotypes is vital in the fight against malaria. In order to assess how drug policies can impact this burden in a seasonal malaria chemoprevention setting in Cameroon, we investigated molecular changes in *Pfprt* and *Pfmdr1* genes in pre and post seasonal malaria chemoprevention in Cameroon. To assess the evolution of these markers, finger-prick whole blood samples were collected on 3MM Whatman filter paper from over 405 children that were diagnosed positive for malaria. DNA was extracted from and dry blood spot and genotyped. Chi square test was used to test for significance in the distribution of the markers within the two time points or periods. Out of the 405 samples from different patients that were genotyped for *Pfprt* and *Pfmdr1*. The proportion of *pfprt* genotypes stood at 33.3% wild-types, 51.9% mutants and 14.2% mixed infection. That of *Pfmdr1* stood at 25.3% wild-type, 63.5% mutants and 11.2% mixed infections. Pearson's chi square/Fischer's exact test revealed that the frequencies of the SNPs of the *pfprt* and *Pfmdr1* gene were significantly associated with their distribution within the two time points ( $p \leq 0.000001$ ).

**Keywords:** *Plasmodium falciparum*, molecular markers, status, seasonal malaria

## 1. Introduction

Human malaria is caused by an apicomplexan parasite of the genus *Plasmodium*. Among the species responsible for human malaria, *Plasmodium falciparum* is the most life threatening. This species is responsible for the highest proportion of infectious disease in malaria endemic countries [1]. Malaria is the most important parasitic disease in terms of human suffering, resulting in the highest number of cases and deaths in malaria endemic countries. Globally there are estimated 229 million malaria cases, with the African region accounting for 94% of the total number of cases with majority of the case from sub-Saharan Africa [2].

Over the past years, Cameroon has implemented complimentary malaria control strategies based on WHO recommendations. Including the distributions of insecticide treated bed nets and implementation of intermittent preventive treatment in pregnant women [3, 4]. Replacing Chloroquine with Amodiaquine as first line treatment between 2002 and 2004 and the adoption of Artemisinin based combination therapy as first line treatment in 2004 [5, 6]. Despite all these, malaria is still one of the leading causes of morbidity and mortality in Cameroon with children under the age of 5 being the most affected especially in the north and far north regions [7, 8]. In Cameroon, malaria transmission varies with respect to the different ecological zones with a major seasonal peak from July to October in the Sahelian and sub Sahelian ecological regions [8]. In these high risk ecological regions, with very high seasonal peaks accompanied by a spike in mortality and morbidity, WHO recommends an intervention known as seasonal malaria chemoprevention (SMC), which is based solely on preventing infection in children under the ages of five with Sulphadoxine Pyrimethamine and Amodiaquine (SPAQ) [36].

The strategy is to administer monthly SPAQ doses to children under 5 years during the malaria peak transmission season. Non-inclusion of children above the age of five as well as adult and the development of resistance against SPAQ by the parasite, are some of the challenges in the reduction of malaria burden in this zone, through this strategy. The unfortunate spread of resistance to SPAQ and to most, if not all anti-malaria drugs, some of which were once effective and safe, like Chloroquine, is the principal cause of long standing malaria burden especially in this regions [9]. Chloroquine resistance was first reported in the Thai-Cambodian borders in the late 1950s [10]. It then spread to other malaria endemic regions of the globe [11, 12].

In the 1990s almost all of Sub-Saharan African countries had reported Chloroquine resistant *Plasmodium falciparum*. In Cameroon, the first Chloroquine treatment failure was documented in 1985 and was withdrawn as treatment for uncomplicated malaria in 2002 [5, 13]. Although significant progress has been made to understand the resistance mechanisms to administered anti-malarial drugs, the genetic basis of drug resistance is vital to implement strategies for efficient malaria control. Single nucleotide polymorphism in the genes “*Plasmodium falciparum* multidrug resistance 1” (*Pfmdr1*), located in chromosome 5 [14, 15], “*Plasmodium falciparum* chloroquine resistance transporter” (*Pfcr1*), on chromosome 7 modulate the level of chloroquine resistance [16]. Several studies have confirmed a strong association between the mutant alleles *Pfcr1*-T76 and *Pfmdr1*-Y86 and high level of chloroquine resistant [17, 12, 13]. Before and after the replacement of Chloroquine as first line treatment, studies showed low Chloroquine failure rate in the North and Far North regions also known as

seasonal malaria chemoprevention setting in Cameroon as compared to the Southern regions [5, 13].

These regions also account for the highest number of malaria cases and deaths in Cameroon with children under age of 5 being the most affected [7]. Chloroquine, was the most preferred antimalarial in many countries that were prone to malaria endemic *Plasmodium falciparum*. The drug was safe, effective, widely available, remarkably inexpensive with a long half-life and protect from early relapse ([12, 18]. Chloroquine could be administered to pregnant women as well as infants. The advantages of Chloroquine are simply too strong. With this high rate of prevalence of malaria in these regions before and even after the introduction seasonal malaria chemoprevention, we then set out to investigate the Evolution of the *Pfcr1* T76 and *Pfmdr1* Y86 markers in a seasonal malaria chemoprevention setting in Cameroon. Reason being that the years of reliance on antimalarial other than Chloroquine could have led to the revert of Chloroquine sensitive *Plasmodium falciparum* [12, 19, 20, 21], before or even after the introduction of seasonal malaria chemoprevention in Cameroon.

## 2. Materials and Methods

### 2.1 Study Area

The study was conducted in the North and Far North regions of Cameroon with Garoua and Maroua respectively as regional capitals. These regions fall within the major Sahelian geo-ecological region of Cameroon. Garoua is in the north region of Cameroon and lies at coordinates 6°24'N & 10°46'E. Garoua is bounded to the North by the Far North region, to the South by the Adamawa region, to the West by Nigeria, to the East by Tchad and Southeast

by the Central African Republic. Garoua serves as a river port in years when the rainfall is abundant. The temperature here is on average at 31°C for most of the year and the vegetation is Guinea-Savanna. It receives an annual average rainfall of 380 mm with just about 4 months of rainy season [22]. The population is predominantly Muslim with agriculture as the prominent activity of the region.

Maroua is in the Far North region of Cameroon. And lies at the coordinates, 5°0'N & 12°27'E. Mount Maroua dominates the skyline of the city. The climate of Maroua is Sahelian, being hot and dry for most of the year. Maroua lies along the same belt stretching from the West African country of Gambia through Burkina Faso and Niger to Northern Nigeria and across Tchad and towards Sudan. This region experiences low precipitation, and therefore prone to malaria epidemics. The predominant religion is Sufi Islam and most indigenes engage themselves in Agriculture (cotton farms) or raise cattle (Non-Fulani's and Fulani's respectively). They also engage in trade with neighboring Tchad and Nigeria [23].

## 2.2 Study population

Children between the ages of 6-120 months with primary diagnosis of malaria confirmed by a positive CareStart™ Malaria HRP2 pf (CAT NO: G0141, ACCESSBIO) Ag RDT and who fulfill all inclusion and none of the non-inclusion criteria, were enrolled in the study, after obtaining consent from their parents or guardians.

## 2.3 Study samples

Study participants were recruited from randomly selected health districts in the North and the Far North regions of Cameroon in 2016 prior to the implementation of SMC and in 2021 five years after

the implementation of SMC. Febrile self-presenting patients who fulfilled all of the inclusion and none of the non-inclusion criteria's were eligible for the study. An axillary temperature was measured to check a fever. Febrile patients were considered to have an axillary temperature of  $\geq 37^{\circ}\text{C}$ . A malaria rapid diagnostic test (RDT) was used to diagnose the patient for plasmodium falciparum infection. This was further confirmed by microscopy. Finger-prick blood samples were obtained from patients reported as being infected with plasmodium falciparum. No additional clinical assessment was carried out at the time of recruitment. Together, over 405 finger prick blood samples collected on Whatman#3mm filter paper were air-dried, sealed individually in zip lock bags and stored at room temperature for DNA extraction and further molecular analysis.

## 2.4 DNA extraction

Plasmodium falciparum genomic DNA was extracted from dried blood spots on 3mm Whatmann filter papers by chelex (Bio-Rad laboratories SIGMA) method as previously described by Plowe and coworkers [24]. The dry blood spot was cutout and placed in a micro-centrifuge labeled tube. 1 ml 0.5% Saponin was then added to it and incubated overnight at 4°C. The following day the saponin was replaced with 1 ml of phosphor buffer saline (1X PBS). After 20-30 minutes of incubation at 4°C, the PBS was discarded and the blood spot transferred to 20% hot chelex in a heating block. This mixture was then vortexed and centrifuge at 10,000 rpm for 2 minute and the supernatant transferred into a new tube. The tube was again centrifuge and the supernatant transferred to a final tube which was then stored at 20 °C.

## 2.5 DNA amplification

The extracted DNA was used for PCR amplification and the left over returned -20°C for storage. The *Pfcr1* and *Pfmdr1* genes of the parasitic DNA were amplified using pairs of forward and reverse primers obtained from Inquaba Biotec (Pretoria, South Africa) flanking codons 76 and 86 of the respective genes in a two round PCR. Both codons were amplified in a T3 thermo-cycler (Biometra UK) [24]. The products of the first amplifications were used as substrates for the second amplification. For the amplification, reactions volumes of 25µl reaction mixtures were used, each of which contain; nuclease free water, 10 X thermopol buffers, 10mM dNTPs, Taq Polymerase and substrate. In the first round of the nested PCR for the gene *Pfmdr1* the primers MDR1F (5'-GCGCGCGTTGAACAAAAAGAGTACCGCTG-3') and MDR2R (5'GGGCCCTCGTACCAATTCCTGAACTCAC-3') were used. Pre-denaturation was done at 95°C for 5minutes followed by 30 cycles of denaturation at 95°C for 30s annealing at 45°C for 30s and extension at 65°C for 45s with a final extension at 72°C for 5minutes.

The primers MDR3F (5'-TTTACCGTTTAAATGTTTACCTGC-3') and MDR4R (5'-CCATCTTGATAAAAAACACTTCT-3') were respectively used as forward and reverse primers for the second round of the nested PCR. Pre-denaturation was done at 95°C for 3minutes, followed by 25 cycles of denaturation at 95°C for 30s annealing at 45°C for 30s and extension at 65°C for 45s with a final extension at 65°C for 3minutes. For the *Pfcr1* gene, CRTP1 (5'-CCGTTAATAATAAATACACGCAG-3') and CRTP2 (5'-CGGATGTTACAAACTATAGTTACC-3') were respectively used as forward and reverse primers for

the first nested reaction. The PCR reaction mixture was subjected to the following conditions. Pre-denaturation was done at 94°C for 3minutes, followed by 45 cycles of denaturation at 94°C for 30s annealing at 56°C for 30s and extension at 60°C for 1 minute with a final extension at 60°C for 3minutes. For the second nested reaction, CRTD1 (5'-TGTGCTCATGTGTTTAAACTT-3') and CRT (5'-CAAACTATAGTTACCAATTTG-3') were respectively used as forward and reverse primers. The PCR reaction mixture was subjected to the following conditions. Pre-denaturation was done at 95°C for 5minutes, followed by 30 cycles of denaturation at 92°C for 30s annealing at 48°C for 30s and extension at 65°C for 45s with a final extension at 65°C for 5 minutes.

## 2.6 Restriction fragment length polymorphism

The PCR amplicon were then subjected to restriction fragment length polymorphism using site-specific restriction enzymes (New England Biolabs). The *pfcr1* alleles were analyzed by incubating the amplicon at 37°C overnight with the restriction enzyme Apo I. ApoI cleaves *pfcr1*-76K but not *pfcr1*-76T [24]. Analysis of the *pfmdr1* alleles were performed by incubation the amplicon at 37°C overnight with the restriction enzyme Afl III. Afl III cleaves the coding sequence of allele *pfmdr1*-86N but not that of *pfmdr1*-86Y [25].

## 2.7 Agarose gel electrophoresis

The amplified DNA and the restricted products were subjected to electrophoresis in a 2 % agarose gel, stained with 0.5µl Ethidium Bromide and visualized under ultraviolet light. Chloroquine sensitive and resistant haplotypes were analyzed. Haplotypes were considered resistant if they all represented combinations of resistant related mutations and

sensitive if they represented combinations of wild-types allele or mixed if both forms were present.

### 3. Results

Among the participants who were recruited in the study, 405 dried blood spots filter papers had sufficient information to be returned for further analysis. Dried blood spots from unidentifiable participants, or with duplicated patient code as well as those with incomplete patient's code or information were not considered for further analysis. Among the 405 samples, 201/405(49.6%) were collected in 2016 and the remaining 204/405 (50.4%) in 2021. DNA was successfully extracted from all the 405 samples as confirmed by a Nano drop analysis. The *Pfcr1* and the *Pfmdr1* genes were successfully amplified in the 201 samples, giving a genotyping success rate of 100%. For the samples collected in 2021, 144 *pfcr1* and 183 *pfmdr1* genes were successfully amplified, giving a genotyping rate of 70.6% (144/204) and 89.7% (183/204) respectively. The amplified DNA was then assessed for single

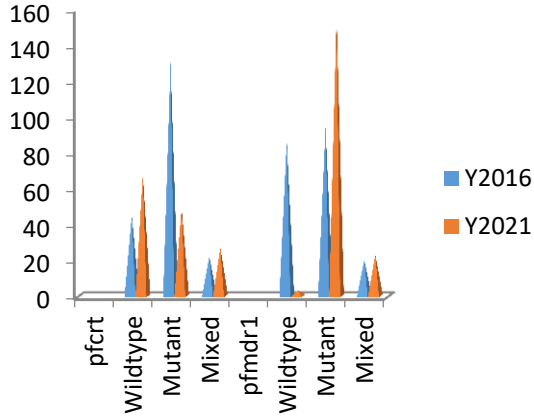
nucleotide polymorphisms (SNPs) at codons 76 and 86 of the *Pfcr1* and *Pfmdr1* gene respectively.

The observed frequencies of the investigated SNPs in *Pfcr1* and *Pfmdr1* loci for both years are summarized in table 1. The *Pfcr1* gene harboring the wild type allele (K76) susceptible AAA nucleotide, coding for Lysine (Lys) was observed in 97/384 (25.3%) of the analyzed samples. Among which 94/201(46.8%) were sampled in 2016 and 3/183(1.6%) in 2021. The *Pfcr1* gene harboring the mutant allele (67T) susceptible ACA nucleotide coding for threonine (Thr) was observed in 244/384(63.5%). There were 87/201 (43.3%) sampled in 2016 and 157/183(85.8%) in 2021. Mixed infection containing both the Wild-type and mutant alleles were observed in 43/384(10.9%) among which 20/201(10%) were sampled in 2016 and 23/183 (10.9%) in 2021. The *Pfmdr1* gene harboring the wild type allele (N86) susceptible TGA nucleotide, coding for Asparagine (Asn) was observed in 115/345 (33.3%) of the analyzed samples. among which 47/201(24.4%) were sampled in 2016 and 68/144(47.2%) in 2021.

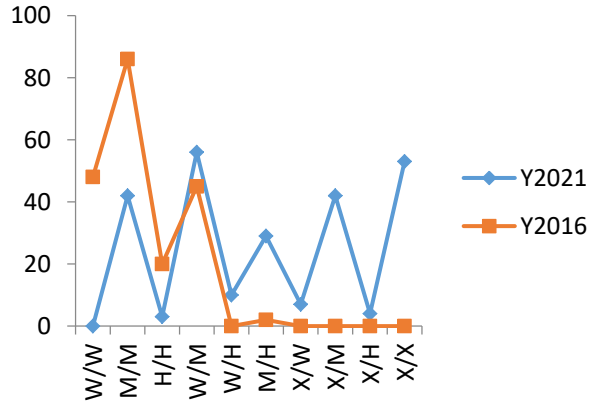
**Table 1:** Proportion of single nucleotide polymorphism on *Pfcr1* and *Pfmdr1* genes

Gene	Locus	Amino acid	Genotype	Alleles	Y2016	Y2021	Total	95% CI
<b>Pfmdr1</b>	TGA	Asn	Wild type	N86	94(46.8%)	3(1.6%)	97(25.3%)	626.6; -529.6
	TGT	Tyr	Mutant	86Y	87(43.3%)	157(85.8%)	244(63.5%)	566.7; -322.7
	TGA/TGT	Asn/Tyr	Mixed	N86Y	20(10%)	23(12.6%)	43(11.2%)	40.6; 2.4
<b>Total</b>					201/201	183/204	384/405	564.28; -363.28
<b>Pfcr1</b>	AAA	Lys	Wild type	K76	47(24.4%)	68(47.2%)	115(33.3%)	190.9; -75.9
	ACA	Thr	Mutant	76T	130(64.7%)	49(34.0%)	179(51.9%)	604.1; -425.1
	AAA/ACA	Lys/Thr	Mixed	K76T	22(10.9%)	27(18.8%)	49(14.2%)	56.3; -7.3
<b>Total</b>					201/201	144/204	345/405	

A= Adenine, C= Cytosine, G= Guanine, T=Thymine, Asn=N =arginine, Tyr=Y= tyrosine, Lys=K= Lysine, Thr=T=Threonine, Pfcrt= Plasmodium falciparum Chloroquine resistant transporter gene, Pfmdr1 = Plasmodium falciparum multidrug resistant gene 1, CI=confidence interval, Y2016= year 2016, Y2021= year 2021



**Figure 1;** Distribution of *Pfcrt* and *pfmdr1* point mutations in the investigated population in accordance with the year when the samples were collected  
Key: Y2016 = the year 2016 and Y2021 = the year 2021



**Figure 2;** Variation of allelic combination of *Pfcrt* and *pfmdr1* SNPs between 2016 and 2021  
Key; Y2016 = the year 2016 and Y2021 = the year 2021, W= wild type, M= mutant, H= heterozygous (mixed) and X= negative PCR

**Table 2:** Various *Pfcrt* and *Pfmdr1* combination obtained from restriction analysis to determine single nucleotides polymorphisms in the respective genes

Category	Y2016	Y2021	Total	CI
No mutation	48(23.9)	0(0.0)	48(14.1)	335.8 -286.8
Single mutant	86(42.8)	66(47.1)	111(32.6)	188.9 -77.9
double mutant	108(53.7)	74(52.9)	182(53.4)	300.2 -119.2
<b>Total</b>	201	140	341	

CI= Confidence interval

Pearson’s chi square/Fischer’s exact test of 31.7 and 105.5 for *pfcrt* and *pfmdr1* genes respectively revealed that the frequencies of their SNPs were significantly associated with their distribution between the two years ( $p_v \leq 0.000001$ ), with an increase in the mutant alleles between 2016 and 2021 figure 1. All mutants and SNPs were evaluated for both 2016 and 2021. A breakdown of the unique combination of mutants is shown in table 2 and the

variation for each unique combination of SNPs for 2016 and 2021 is shown on figure 2.

**4. Discussion**

The aim of the current study was to investigate the evolution of *Pfcrt* and *Pfmdr1* single nucleotide polymorphisms between pre SMC and post SMC in Cameroon. *Pfmdr1* and *Pfcrt* are molecular markers of resistant to one of the drug use for seasonal

malaria chemoprevention. These markers have also been proven to be strongly associated with a once effective and safer anti malaria drug call Chloroquine. Owing to the role Chloroquine has played in the fight against malaria and it comparatively low resistant in the seasonal malaria chemoprevention region before it replacement in 2002 as well as the introduction of seasonal malaria chemoprevention in Cameroon. Single nucleotides polymorphism was used to investigate the prevalence of mutations alleles in *Pfcr1* and *Pfmdr1* genes, all of which are associated with Chloroquine resistance *Plasmodium falciparum*. A large proportion of *Plasmodium falciparum* isolates in this study 179/345(51.9%) carried the Chloroquine resistant *Pfcr1*-76T allele. Previously it has been reported that the Chloroquine resistant (CQR) mutant *Pfcr1*-K76T is a strong predictor of overall Chloroquine resistant [26] and that *Pfmdr1* encodes a trans-membrane transporter and that point mutations in this gene modulate the level of Chloroquine resistance [16, 27].

We observed a high proportion of *Pfcr1*-K76T mutant alleles in 2016. This was not in line with previous study conducted in Cameroon by leonado et al which lead to the ban of Chloroquine importation and used in Cameroon in 2002. In this study they demonstrated a decreasing gradient in Chloroquine resistance *plasmodium falciparum* towards the Sahelian northern Cameroon [5]. This difference may be due to the continual used of quinolone based antimalarial or even the continual usage of Chloroquine after its withdrawal. The continual usage of this molecule may have enhances the prevalence of these resistant markers in the parasitic population. This results also differs from other studies conducted in Ethiopia, Zambia, Malawi , French guinea, new pupa guinea [12, 19, 21, 28] and 2 East African countries [20, 29]

where the frequency of the *Pfcr1*-76T resistance allele was repopulated with the sensitivity ones after many years of Chloroquine withdrawal. Here the complete withdrawal of Chloroquine and quinoline related anti-malaria drugs lead to a reduction in drug pressure and an increase in Chloroquine susceptible *plasmodium falciparum*. It appears that CQ sensitive *Plasmodium falciparum* isolates are yet to be back in a seasonal malaria chemoprevention setting in Cameroon even though we experience a decrease in *pfcr1* mutant alleles between 2016 and 2021.

This decrease was disproportionately followed by a paralleled increased in *pfmdr1* mutant alleles within the same period. *Pfmdr1* is thought to transport and accumulate CQ in the parasites, food vacuole, mutations N86Y interfere with the transportation of the anti -malaria drugs leading to reduced CQ sensitivity [30-32]. Although genetic transformation experiments have found that *pfmdr1* mutations can modulate the level of chloroquine resistance once it has been conferred by mutations in *pfcr1*, mutations in *pfmdr1* are by them insufficient to confer chloroquine resistance [19]. Recent studies have reported that *pfmdr1* mutations, in addition to *pfcr1* mutations, are no more strongly associated with chloroquine treatment failure than *pfcr1* mutations alone [6] and that *pfmdr1* mutations do not add to the predictive value of *pfcr1* mutations for chloroquine treatment failure [20]. Even if *pfmdr1* mutations do contribute to treatment failure, they require *pfcr1* mutations to exert an effect on the response to chloroquine.

Therefore, persistence of *pfmdr1* mutations in a population with a very low prevalence of *pfcr1* mutations would not be expected to reduce chloroquine efficacy in that population [32-34]. The



decrease in *pfcr* mutant allele, might be explained by an advantage of the *pfcr* K76 allele over its mutant form after removal of CQ pressure owing may be to the complete withdrawal of CQ from the non-official market. It can also be due to increase in awareness by the population on the efficacy of artemisinin based antimalarial drugs [30, 31]. A similar trend was observed in south Cameroon where it was concluded that chloroquine could not be reintroduce for the treatment of *Plasmodium falciparum* malaria since the *pfcr* mutant allele was still comparatively high even though the study demonstrated a decrease in this *pfcr* mutant allele towards the seasonal malaria chemoprevention region [13]. A similar decrease in *pfcr* mutant allele in these regions has also been described earlier by Mbacham et al, Moye et al and Leonado et al [5, 6, 35] where they demonstrated a comparative reduction in Chloroquine resistance *Plasmodium falciparum* in these regions.

## 5. Conclusion

Chloroquine sensitive plasmodium falciparum is yet to return to the seasonal malaria chemoprevention setting in Cameroon.

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## Conflicts of interest

We declare that there is no conflict of interest.

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