Effects of drug policy changes on the evolution of *Pfmdr1* and *Pfcrt* resistance molecular markers of *Plasmodium falciparum* to chloroquine in Bangui, Central African Republic

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Abstract- **Malaria remains one of the main threats to public health. The emergence of drug resistance is a major obstacle to the fight against malaria. Due to the spread of parasites resistant to antimalarial drugs, treatment guidelines for malaria in the Central African Republic have evolved from monotherapy to artemisinin-based combination therapy. Prediction of decrease or increase in antimalarial susceptibility and fixation of multidrug resistance genotypes is essential in the fight against malaria. To assess the impact of drug policy, we examined molecular changes in the chloroquine-associated *Pfcrt* and *Pfmdr1* resistance marker to monitor the evolution of mutant alleles since the withdrawal of chloroquine from the market following the adoption of Artemisinin based Combined Therapies (ACT) in the Central African Republic. To assess the evolution of these markers, dried blood spots were prepared from 138 children diagnosed positive for plasmodium falciparum malaria by rapid diagnostic test. DNA was then extracted from the blood and genotyped. The chi-square test was used to check for the association between the period of withdrawal and the time of sample analysis. The alleles conferring resistance to chloroquine in the Pfmdr1-86Y genotype showed a significant increase from 1.72% in 2010 to 99.1% in 2021, on the other hand, they was a reduction in the mutant alleles *Pfcrt-76T* and an increase in mixed infection from 0% in 2010 to 3.48% in 2021 (P<0.05). The results demonstrated a clear increase in the *pfmdr1* resistant marker.**

Key words; **Plasmodium, Chloroquine, molecular markers, policy**

Introduction

Despite reports of a decline in global malaria incidence, the WHO African region still recorded 95% of the world's 228 million early malaria cases, with an estimated 602,000 out of 627,000 global deaths in 2021 [1]. Venerable groups like children under the age of 5 years and pregnant women are the most affected. This persistence malaria burden is link to many factors including poverty, ignorance but above all the insufficiency of medical infrastructures [2][3]. Over the years, the Central African Republic (CAR), has implemented complementary malaria control strategies based on WHO recommendations. In particular, the fight against malaria vectors by deploying long-lasting insecticide-treated mosquito nets (LLINs), the use of intermittent preventive treatment among infants and pregnant women [4][5]. Despite The replacement of chloroquine following the adoption of combination therapies based on artemisinin derivatives (ACT) as first-line treatment in 2005 [4], malaria remains one of the main causes of morbidity and mortality in CAR. The replacement of chloroquine was driven by the development and spread of plasmodium falciparum chloroquine resistant strains [6]. Although the mechanism of chloroquine resistance is not yet fully understood, it has been shown to be positively correlated with mutations in the chloroquine resistance transporter gene (*Pfcrt*) that encodes a vacuolar transmembrane protein.The *pfcrtK76T* (substitution of lysine for threonine) genetic mutation, has been reported in all clinical isolates resistant to chloroquine. It is therefore used as molecular markers to monitor chloroquine resistance in field isolates [9]. Mutations in the *Plasmodium falciparum* (*Pfmdr1)* gene especially that at codon N86Y (substitution asparagine with tyrosine) has been shown to play a modulating role in decreasing sensitivity to chloroquine as well as other aminoquinolines such as amodiaquine and mefloquine [10][11].Recent progress in global malaria control is threatened by the emergence of artemisinin resistance in Southeast Asia [1] and its likely spread to hotspots, which is already the case in Rwanda and Tanzania [12][13]. Studies in South East Cameroon, in Kenya and Malawi have substantiated that the withdrawal of chloroquine and consequently it pressure on the parasite lead to a gradual reduction in the proportion of circulating mutant genotypes of the *Pfcrt* and *Pfmdr1* genes. [14][15][16].

This study was carried out to determine the changes in the prevalence of chloroquine resistant between 2010 and 2021 following it replacement in 2005 with Artesunate-amodiaquine (ASAQ) and Artemether-lumefantrine (AL). The emergence of resistance to ACT and the gradual revert of chloroquine sensitive *Plasmodium falciparum* reported in some parts of Africa motivated us to investigate the situation of chloroquine sensitive *Plasmodium falciparum* in CAR. The reason being that chloroquine was safe, effective, widely available, and remarkably inexpensive with a long half-life that protecting against early relapses [17][18]. It could be given to pregnant women as well as nursing mothers. The benefits of chloroquine were enormous, so if it turns out that chloroquine sensitivity has returned to CAR, its return to the market could be a plus in the fight against malaria.

Method

Study site

This study was conducted in Bangui the capital of the Central African Republic. Bangui, is close to the country's southern border. It lies on the northern bank of river Ubangi and covers an area of 67 square kilometers. Bangui is the political and economic capital of the Central African Republic. The CAR is located in a rainy tropical zone, the vegetation consists of approximately 3.5 million hectares of forests in the South and a wooded savannah zone in the North. The average annual temperature is around 26°C, the rainfall indicates on average: 226 mm (July) and 5mm (December) for the rainy and dry seasons respectively [4][19].

Ethical consideration and sample collection

Children between the ages of 6 to 59 months who fulfilled all of the inclusion criteria and none of the non-inclusion criteria were enrolled in the study following consent obtained from their parents or guardians. Study participants were recruited at the Bédé Combattant urban health center (CSU) in Bangui, randomly selected in 2021, eleven years after the collection of the first samples with which we carried out our comparison. A malaria rapid diagnostic test was used to test for malaria in febrile patients with an axillary temperature of ≥ 37°C. This diagnosis were confirmed by microscopy. Finger prick blood was collected from those who were diagnosed positive for *Plasmodium falciparum* and used to prepare dried blood spots (DBS) on Whatman 3mm filter paper. The filter papers were air dried, stored with silica gel in zip luck bags and transported to the laboratory for DNA extraction and molecular analysis.

DNA extraction

The parasitic genomic DNA was extracted from the dried blood spots by chelex (Bio-Rad laboratories SIGMA) method as previously described by Plowe and colleagues [21]. The DBS was isolated from the filter paper and placed in a labeled micro-centrifuge tube and a 1 ml 0.5% saponin solution added to it and incubated overnight at 4°C. The saponin was then replaced with the same amount of phosphoric buffered saline solution (1X PBS) and incubated for about 30 minutes at 4°C. The filter paper was then transferred to another tube with 20% hot Chelex on a heating block. The supernatant following voltexing and centrifugation at 10,000 rpm for 2 minutes was transferred to new tubes and stored at -20°C.

Amplification of genomic DNA

The extracted parasitic DNA was used to amplify the *plasmodium falciparum* chloroquine resistant transporter gene (*Pfcrt*) and the *plasmodium falciparum* multidrug resistant gene 1 (*Pfmdr1*). A set of primers obtained from Inquaba Biotec (Pretoria, South Africa) were used in a nested PCR to amplify codon 76 and 86 of the *Pfcrt* and *Pfmdr1* genes respectively, in a T3 thermocycler (Biometra UK) [21]. A reaction mixture of 25µl was used. Each of these reaction mixture was made up of nuclease free water, 10 X thermopol buffers, 10mM dNTPs, Taq Polymerase and substrate. The *Pfcrt* and *Pfmdr1* genes were then amplified as previously describe in other studies [21][32]. The product of the first amplification was used as the substrate for the second amplification. The primers MDRIF and MDR2R were used for the first round amplification of *Pfmdr1* under the following conditions; pre-denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 s, primer annealing at 45°C for 30 seconds, extension at 65° C for 45 seconds and then final extension at 72°C for 5 minutes. The primers, MDR3F and MDR4R were used for the second round of *Pfmdr1* nested PCR amplification under the following conditions; Pre-denaturation was performed at 95°C for 3 minutes followed by 25 cycles of denaturation at 95°C for 30 seconds, primer annealing at 45°C for 30 seconds and extension at 65°C for 45 seconds with a final extension at 65°C for 3 minutes.

The primers CRTP1 and CRTP2 were used for the first round amplification of *Pfcrt* gene under the following conditions; pre-denaturation at 94°C for 3 minutes, 45 cycles of denaturation at 94°C for 30 seconds, primer annealing at 56°C for 30 seconds, extension at 60°C for 1 minute and then final extension at 60°C for 3 minutes. The primers, CRTD1 and CRTD2 were used for the second round of *Pfcrt* nested PCR amplification under the following conditions; Pre-denaturation was performed at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 45°C for 30 seconds and extension at 65°C for 45 seconds with a final extension at 65°C for 5 minutes 21.

Restriction fragment length polymorphism (RFLP) and gel electrophoresis.

Amplicons were evaluated for single nucleotides polymorphisms at the *Pfcrt* and *Pfmdr1* genes by subjecting them to restriction fragment length polymorphism (RFLP) using site-specific restriction enzymes (New England Biolabs). *Pfcrt* was incubated at 37°C with the restriction enzyme apoI. ApoI digest *Pfcrt-K76* (wildtype) and not *Pfcrt-76T* (mutant). *Pfmdr1* was incubated with the restriction enzyme Afl III at 50°C overnight. Afl III digest *Pfmdr1-N86* but not *Pfmdr1-86Y* [21][22]. Amplicons and products resulting from the restriction enzyme reaction were electrophoresed using a 2% agarose gel stained with 0.2 µl of ethidium bromide and visualized under ultraviolet light.

Results

A total of 138 samples were analyzed in 2021. The age range was 6 to 59 months with an average age of 27.44 months. Among the 138 participants enrolled, 56% were girls and 44% boys. *Plasmodium falciparum* DNA was successfully extracted from all samples. The genotyping rate of *Pfmdr1* and *Pfcrt* genes stood at 83.33% and 100% respectively. Of the 114 PCR positive *Pfmdr1*, 99.14% (114/115) were mutant alleles (*Pfmdr1-86Y*) and 0.86% (1/115) were wild types (Pfcrt-N86). Regarding studies conducted in 2010 by Nambei et al, a proportion of 1.72% of mutant alleles (*Pfmdr1-86Y*) and 98.24% wild types alleles (*Pfmdr1-N86*) were observed.

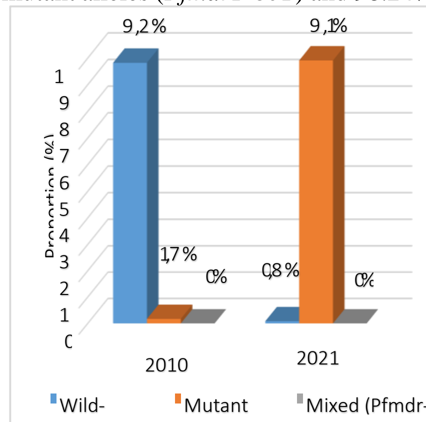

Figure 1 and table 1.

Figure I : Proportion of *Pfmr1* gene with wild type (*Pfmdr1 N86*), mutants (*Pfmdr1 86Y*) and mixed alleles.

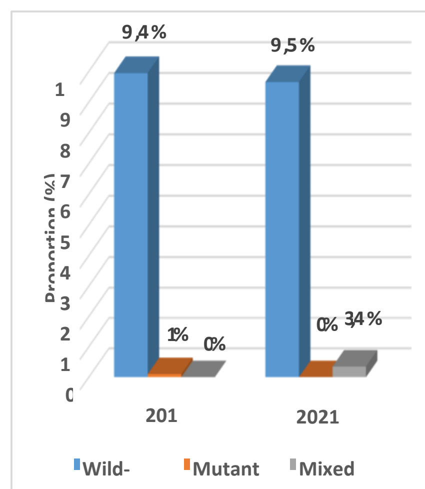

Figure II : proportions of *Pfcrt* gene with wild-type (*Pfcrt K76),* mutants (*Pfcrt 76T*) and mixed alleles (*Pfcrt-K76T)*

As concern the *Pfcrt* gene, 96.52% (107/115) were wild types (*Pfcrt-K76*), none of the isolates had mutant alleles. However 3.48% (8/115) of the isolates had mixed infection (*Pfcrt-K76T*). Isolates with both mutant and wild type alleles were considered to be having a mixed infection. In 2010 Nambei *et al* observed a 99.42% (171/174) isolates with wild types alleles (*Pfcrt-K76*). and a 1% (1/174) of mutant alleles (*Pfcrt-76T*). Figure 2, Table 1.

Table I: proportion of single nucleotides polymorphisms of the *Pfmdr1* and *Pfcrt* genes in 2010 and 2021

Gène	alleles	type	**2010**	**2021**	**P-value**
Pfmdr 1	76T	mutant	1,72% (3/174)	99,1% (114/115)	P < 0.001
	K76	Wild-type	98,27%(171/174)	0,86% (1/115)	
	K76T	mixed	0.0%(0/174)	3.48% (8/115)	
Pfcrt	86Y	mutant	0,57%(1/174)	3,48%(8/115)	P < 0.003
	N86	Wild-type	99,42%(173/174)	96,52%(107/115)	
	N86Y	mixed	0.0%(0/174)	0.0%(0/115)	

Key; T=Threonine, K=Lysine, N=Asparagine and Y=Tyrosine.

Discussion

The objective of this study was to compare the evolution of the prevalence of the chloroquine resistance markers *Pfmdr1* and *Pfcrt* between 2010 and 2021 as a means of investigating a possible reintroduction of Chloroquine in the Central African Republic. *Pfmdr1* and *Pfcrt* are molecular markers which have been demonstrated to be associated with *Plasmodium falciparum* resistant to chloroquine. The role chloroquine played in the fight against malaria before it withdrawal in 2005 due to resistant of the parasite and the current revert of chloroquine sensitive *Plasmodium falciparum* in other African countries, prompted us to investigate a possible revert in the Central African Republic.

Analysis of the single nucleotides polymorphisms (SNPs) of the genes revealed that they was no isolates with *pfcrt* mutant alleles (*pfcrt-76T*) in 2021 as compare to 2010 and a significant increase in the *pfmdr1* mutant alleles (*pfmdr1-86Y*). this study was similar to another study conducted in Malawi where the prevalence of *pfcrt* mutant allele was found to decrease from 85% in 1999 to 13% in 2000 and then to 0% in 2001 following a 100% chloroquine clearance of in vitro isolates of *Plasmodium falciparum* infections however the two study differ by the fact that, that of Malawi investigated the SNPs of *Pfcrt* only. [25]. This study is also consistent with another study in northern Cameroon were a similar trend in *pfcrt* and *pfmdr1* SNPs were observe even though the decrease in *pfcrt* mutant allele was non-significant [32]. The results clearly demonstrate a complete return of chloroquine sensitive circulating *Plasmodium falciparum* in Bangui given that no mutant *pfcrt* allele was observed and owing to the fact that SNPs in the *pfcrt* gene is an important determinant of chloroquine resistant *Plasmodium falciparum* one can suspect a possible complete revert of chloroquine sensitive *Plasmodium falciparum* in Bangui. This determinant resistant to chloroquine was demonstrated by Hamza *et al* were they also showed that they was a less significant association of chloroquine resistant *plasmodium falciparum* with mutant alleles of *pfmdr1* as compared to those *pfcrt* mutant alleles [28].These results differs from those of a similar studies conducted in south west and south-eastern regions of Cameroon where the decreases in *pfcrt* mutant alleles was accompany by a similar decrease in those of *pfmdr1* as opposed to our study where a decrease in the *pfcrt* mutant alleles were rather accompany by an increase in those of *pfmdr1*. This difference may be explained by the fact that artemether-lumefatrine may be over used in Central African Republic than in Cameroon since SNPs of *pfmdr1* gene has been proven to be associated with the resistant artemether-lumefatrine so it pressure may be a possible reason for this difference [14][25]. These results also differ from work conducted in Zambia and Senegal where the frequency of *Pfmdr* alleles was repopulated by the susceptible alleles (*pfmdr1-N86*) after many years of complete withdrawal of chloroquine [30][31].

Conclusion

This study showed that the replacement of chloroquine in Central Africa Republic led to the return of chloroquine sensitive plasmodium falciparum in Bangui. These findings is of public health importance in that chloroquine might be reintroduce in CAR may be as combine therapy with artemisinine after similar findings from other regions in the country

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Conflict of interest

The authors declare that they have no conflict of interest

REFERENCES:

1. WHO. World Malaria Report 2021. *Angewandte Chemie International Edition*, 6(11), 951–952. Vol. 13 (2021).
2. Gallup, J.L. & Sachs, J.D. The Economic Burden of Malaria Working Papers. Work. Pap. 52, 1–22 (2000).
3. Bi, Y. & Tong, S. Poverty and malaria in the Yunnan province, China. Infect. Say. Poverty 3, 1–4 (2014).
4. National Malaria Control Program. Assessment of the susceptibility of malaria vectors to insecticides in the Central African Republic. (2019).
5. Walker-Abbey, A. et al. Malaria in pregnant Cameroonian women: The effect of age and gravidity on submicroscopic and mixed-species infections and multiple parasite genotypes. Am. J. Too. Med. Hyg. 72, 229–235 (2005).
6. Delmont, J. et al. Evaluation of the chemo-resistance of Plasmodium falciparum to chloroquine in Central African children: assessment of five years (1984-1988) of studies by in vivo tests. Med. Afr. Black 37, 1988–1990 (1990).
7. Chinappi, M., Via, A., Marcatili, P. & Tramontano, A. On the mechanism of chloroquine resistance in *Plasmodium falciparum.* 1–16 (2010).
8. Saier, T. and M. H. The principal chloroquine resistance protein of *Plasmodium falciparum* is a member of the drug/metabolite transporter superfamily. *Microbiol. comment* 1–3 (2004) doi:10.1099/mic.0.26818-0.
9. Djimdé, Abdoulay, Kossoum kayentoa, Xin-zhuan su, *et al*. A Molecular marker for Chloroquine-Resistance Falciparum Malaria. *New English J.Med.* 344, 257–263 (2001).
10. Fontecha, G. et al. Assessment of Plasmodium falciparum anti-malarial drug resistance markers in pfcrt and pfmdr1 genes in isolates from Honduras and Nicaragua, 2018–2021. *Malar. J.* **20**, 1–8 (2021).
11. Das, S. et al. Double mutation in the pfmdr1 gene is associated with emergence of chloroquine-resistant Plasmodium falciparum malaria in eastern India. *Antimicrob. Chemother Agents*. 58, 5909–5915 (2014).
12. Bergmann, C. et al. Increase in Kelch 13 Polymorphisms in Plasmodium. *Emerg. Infect. Dis. • www.cdc.gov/eid* **27**, 5–7 (2021).
13. Ndwiga, L. *et al.* Drugs and Drug Resistance A review of the frequencies of Plasmodium falciparum Kelch 13 artemisinin resistance mutations in Africa. *Int. J. Parasitol. Drugs Drug Resist.* **16**, 155–161 (2021).
14. Ndam, N.T. *et al.* Reemergence of chloroquine-sensitive pfcrt K76 Plasmodium falciparum genotype in southeastern Cameroon. *Malar. J.* 16, 1–6 (2017).
15. Mehta, S.R. *et al.* Return of Chloroquine Antimalaria Efficacy in Malawi. *N.Engl. J.Med.* 687–696 (2015) doi: 355:1959-66.
16. Pelleau, S. et al. Adaptive evolution of malaria parasites in French Guiana: Reversal of chloroquine resistance by acquisition of a mutation in pfcrt. *Proc. Natl. Acad. Science. U.S.A*. 112, 11672–11677 (2015).
17. Mekonnen, S.K. et al. Return of chloroquine-sensitive Plasmodium falciparum parasites and emergence of chloroquine-resistant Plasmodium vivax in Ethiopia. *Malar. J.* 13, 1–9 (2014).
18. Ringwald, P., Same Ekobo, A., Keundjian, A., Kedy Mangamba, D. & Basco, L. K. Chemoresistance of P. falciparum in an urban setting in Yaounde, Cameroon. Part I: in vitro and in vivo monitoring of plasmodium falciparum resistance to chloroquine between 1994 and 1999 in Yaounde, Cameroon. *Trop. Med. Int. Heal.* **5**, 612–619 (2000).
19. Nambei W.S.1, Lango Yaya E.1, Pounguinza S.1, Achonduh O.2, Bogon A.1, Lengande R.1, Evehe M.-S.2, Ekollo Mbange A.H.2, M. W. . Efficacy and tolerance of combinations of antimalarials in the treatment of uncomplicated malaria in children in Bangui, Central African Republic. *Med Sante Trop 2013* **23**, 313–319 (2013).
20. Nambei W, U. Biago, O. Balizou, S. Pounguinza, M. Moyen, C. Ndoua, J. C. G. Monitoring the efficacy of Artemether-lumefantrine in the treatment of uncomplicated plasmodium falciparum malaria by studying mutations in Kelch 13 genes in Bangui, Central African Republic. *Med Sante Trop 2013* **23**, 313–319 (2013).
21. Plowe, C.V et al. Mutations in Plasmodium falciparum Dihydrofolate Reductase and Dihydropteroate Synthase and Epidemiologic Patterns of Pyrimethamine- Sulfadoxine use and resistance. *J. Infect. Deseases* **6**, 1590–1596 (1997).
22. Wang, P. et al. Resistance to antifolates in Plasmodium falciparum monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. *Mol. Biochem. Parasitol*. 89, 161–177 (1997).
23. WHO. World malaria report 2020. (2020). https://www.who.int/docs/default-source/malaria/world-malaria-reports/
24. Kublin, J.G. et al. Reemergence of chloroquine-sensitive Plasmodium falciparum malaria after cessation of chloroquine use in Malawi. *J. Infect. Dis.* **187**, 1870–1875 (2003).
25. Valderramos, S.G. & Fidock, D.A. Transporters involved in resistance to antimalarial drugs. Pharmacol Trends. Science. 27, 594–601 (2006).
26. Griffing, S. et al. pfmdr1 amplification and fixation of pfcrt chloroquine resistance alleles in Plasmodium falciparum in Venezuela. *Antimicrob. Chemother Agents.* 54, 1572–1579 (2010).
27. Moyeh, M.N. et al. Effects of Drug Policy Changes on Evolution of Molecular Markers of Plasmodium falciparum Resistance to Chloroquine, Amodiaquine, and Sulphadoxine-Pyrimethamine in the South West Region of Cameroon. *J. Malar. Res. Trait.* **2018**, 1–7 (2018).
28. Babiker, H.A. et al. High-level chloroquine resistance in Sudanese isolates of Plasmodium falciparum is associated with mutations in the chloroquine resistance transporter gene pfcrt and the multidrug resistance gene pfmdr1. J. Infect. Say. 183, 1535–1538 (2001).
29. Hanboonkunupakarn, B. and N. J. W. The threat of artemisinin resistant malaria in Southeast Asia. *Travel Med. Infect. Dis.* **14**, 548–550 (2016)
30. Mehta, S.R. et al. Return of chloroquine antimalarial efficacy in malawi. *N.Engl. J.Med*. 687–696 (2015).
31. Mbaye, A. et al. Selection of N86F184D1246 haplotype of Pfmrd1 gene by artemether-lumefantrine drug pressure on Plasmodium falciparum populations in Senegal. *Malar. J.* 15, 1–7 (2016).
32. Ngum, N. L. *et al.* Evolution of the Pfcrt and Pfmdr1 Markers and Revert of Chloroquine Sensitive Plasmodium falciparum in a Seasonal Malaria Chemoprevention Setting in Cameroon. *Fortune J Heal. Sci* **5**, 310–320 (2022).