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Dynamics of Pfcrt K76T and Pfmdr1N86Y fifteen years after the withdrawal of chloroquine in Mali

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Abstract: Background: In Mali, Chloroquine has been abandoned in 2005 because of the high in vivo and in vitro resistance rate of *Plasmodium falciparum* to this molecule. Artemisinin-based combination therapies (ACT) are currently recommended to treat uncomplicated malaria. Few antimalarials are in development. Assessing the prevalence of known antimalarial drug resistance markers might help in designing new combination regimens. This study aims to measure the dynamics of molecular markers of chloroquine in Kolle before, during and after chloroquine withdrawal. **Method:** Dried blood spot samples collected from previous drug efficacy studies conducted in Kolle between 2001 and 2015 were selected and *Pfcrt* and *Pfmdr* genes were genotyped for SNPs conferring resistance to chloroquine. **Results:** A total of 652 samples were analyzed. The overall prevalences of the mutant alleles *Pfcrt* 76T and *Pfmdr1* 86Y were 72.9% and 20%, respectively. The yearly prevalence rate for both mutant alleles remained constant from 2001 to 2015 ($p > 0.05$). **Conclusion**: The prevalence of the *Pfcrt* mutant allele remained high from 2001 to 2015 in Mali, although the prevalence of the *Pfmdr*1 mutant allele was low.

Keywords: *Plasmodium falciparum*; molecular markers; *Pfcrt*; *Pfmdr*; Mali

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1. Background

Malaria is a parasitic disease that constitutes a significant health problem in sub-Saharan countries. In a 2022 world malaria report, Malaria remained a leading cause of infectious diseases, with 247 million cases and 619,000 deaths worldwide [1]. The number of cases and deaths declined slightly from 2020 to 2022. Between 2019 and 2021, there were 63,000 deaths that were due to disruptions to essential malaria services during the COVID-19 pandemic. In addition, in some countries, the prevalence of malaria infection has become stable or increased since 2015 [2].

In Mali, according to the national health information system, 2,614,104 confirmed malaria cases and 1,001 deaths were recorded in 2018. Malaria has been reported to account for between 44.5% and 39% of the reasons for consultations among patients seeking care in health services from 2012 to 2020 [3].

Artemisinin-based combination therapies (ACTs) are currently recommended for the treatment of uncomplicated malaria [4]. However, recent studies have shown a decrease in parasite sensitivity to these ACTs [5,6]. Due to the small number of new antimalaria drugs under development worldwide, there is a critical need to protect existing drugs and reassess those that have been withdrawn [7].

When old malaria treatments including chloroquine, amodiaquine and sulfadoxine-pyrimethamine became less and less efficient, malaria became more and more difficult to cure. The increasing therapeutic failures and the emergence and wide spread of the resistant *P. falciparum* strain to chloroquine led to the withdrawal of chloroquine by the WHO in 2005 [8]. Chloroquine resistance is driven by a single nucleotide mutation in the *P. falciparum* chloroquine-resistant transporter (*Pfcrt*) gene located on chromosome 7 [9], specifically leading to the replacement of Lysine (K) by Threonine (T) at amino acid position 76 of the protein. The prevalence of this *Pfcrt* K76T mutation is often used to predict the treatment failure of chloroquine in vivo [10]. Furthermore, another single nucleotide mutation on the *P. falciparum* multi-drug-resistant gene 1 (*Pfmdr*1 N86Y) located on chromosome 5 was also shown to be associated with chloroquine resistance [11].

Studies conducted in Malawi and Zambia in 2010 and 2016, respectively, have shown that removing chloroquine from malaria treatment regimens resulted in the re-emergence of chloroquine-sensitive strains [12,13]. These findings suggest that the return of chloroquine susceptibility is not the result of a back mutation in a formerly resistant parasite or a new selective sweep. Chloroquine-susceptible parasites that predominate in Malawi likely represent a re-expansion of the susceptible parasites that survived in the population despite widespread drug pressure in the region. The re-emergence of these chloroquine-sensitive parasites would suggest a future possibility of using chloroquine with the possibility of combining it with other antimalarial drugs.

Assessing the prevalence of known antimalarial drug resistance markers might help understanding the extend of resistance and the risk of treatment failure that could be associated with such mutations. This study was carried out to measure the dynamics of *Pfcrt* K76T and *Pfmdr*1 N86Y mutation before, during and after the withdrawal of chloroquine in a malaria endemic area, in Mali.

2. Methods

2.1. Study site

The study was carried out in Kolle, where malaria transmission is seasonal, with a hyperendemic peak from August to November. The estimated plasmodic index (PI) varied between 40% and 50% during the dry season and 70% to 85% in the rainy season. The entomological inoculation rate (EIR) during the high transmission season is 5.2 infective bites per person per month [14].

2.2. Sample collection

All well-preserved dried-blood spots (DBS) from previous drug efficacy trials conducted in Kolle between 2001 and 2015 were retrospectively selected for this study. Samples from 2001 to 2004 are those before chloroquine withdrawal, samples from 2005 are those corresponding to the year of withdrawal and samples from 2007 to 2015 are those after withdrawal.

2.3. DNA extraction

Genomic DNA was extracted from selected DBS using a Qiagen mini kit (Qiagen, Valencia, CA, USA) according to the manufacturers recommendations. This is a fast and easy method for DNA extraction and purification. Briefly, proteinases and special lysis and elution buffers were used following rounds of centrifugation.

2.4. Genotyping of PfcrtK76T and Pfmdr1 N86Y

Pfcrt and *Pfmdr* genes were genotyped as previously described in a previous study. Briefly, nested PCRs followed by endonuclease digestion were used to determine the *Pfcrt* codon 76 and *Pfmdr* 86 genotypes [9,15]. During the interpretation of the results, samples which had both the wild type and the mutant allele concomitantly were considered as mixed cases.

2.5. Ethical approval

All the studies have been approved by the Biomedical Research Ethics Committee of the Faculty of Medicine, Pharmacy and Dentistry of the University de Bamako.

2.6. Statistical analysis

An independent Students t test was performed for variables comparison. The prevalence of molecular markers of drug resistance was compared using the Chi Squared test. All statistical analyses were carried out using Stata version 16.0 (Stata Corp., College Station, TX, USA).

3. Results

A total of 652 samples were selected for the genotyping of the molecular markers of chloroquine-resistance *Pf*crtK76T and *Pf*mdrN86Y. The prevalence of the mutant *Pfcrt* 76T allele varied between 58.3% and 88.6% (Table 1). The prevalence of *Pf*crt76T was comparable from 2001 to 2015 ($p > 0.05$). The prevalence of mixed alleles reached 10% in 2012 and 2015.

Table 1: Prevalence of *Pfcrt* 76 polymorphism in Kolle.

Prevalence of *Pfcrt* K76T					
Years of Collection	**Wild %(n)**	**Mixed %(n)**	**Mutant %(n)**	**Total**	**Collection Periods**
2001	31(18)	3.5(2)	65.5(38)	58	Before withdrawal
2002	26.8(15)	3.6(2)	69.6(39)	56	
2003	16.6(6)	5.6(2)	77.8(28)	36	
2004	25(14)	5.4(3)	69.6(39)	56	
2005	26.4(14)	3.8(2)	69.8(37)	53	During withdrawal
2007	31.8(14)	0(0)	68.2(30)	44	After withdrawal
2008	22.2(14)	0(0)	77.8(49)	63	
2010	23.5(16)	4.4(3)	72.1(49)	68	
2012	31.7(19)	10(6)	58.3(35)	60	
2013	12.9(8)	1.6(1)	85.5(53)	62	
2014	6.8(3)	4.6(2)	88.6(39)	44	
2015	17.2(5)	10.4(3)	72.4(21)	29	
Total	22.7(146)	4.4(26)	72.9(457)	629	

The prevalence of the mutant *Pfmdr*1 86Y allele varied between 13.8% and 26% (Table 2). There was no statistical difference between *Pfmdr*1 mutant allele prevalence from 2001 to 2015 ($p > 0.08$). The prevalence of mixed alleles reached 23.7% in 2002.

Table 2: Prevalence of *Pfmdr*1 86 polymorphism in Kolle.

	Prevalence of *Pfmdr1* N86Y				
Years of Collection	**Wild %(n)**	**Mixed %(n)**	**Mutant %(n)**	**Total**	**Collection Periods**
2001	81.5(53)	4.6(3)	13.9(9)	65	Before withdrawal
2002	60.5(23)	23.7(9)	15.8(6)	38	
2003	64(32)	10(5)	26(13)	50	
2004	70.7(41)	10.3(6)	19(11)	58	
2005	62(36)	13.9(8)	24.1(14)	58	During withdrawal
2007	68.3(28)	7.3(3)	24.4(10)	41	After withdrawal
2008	78.5(51)	7.7(5)	13.8(9)	65	
2010	70.7(53)	4(3)	25.3(19)	75	
2012	73.7(42)	5.3(3)	21(12)	57	
2013	80.8(42)	0(0)	19.2(10)	52	
2014	76.6(49)	7.8(5)	15.6(10)	64	
2015	63(17)	15(4)	22(6)	27	
Total	71(467)	9(54)	20(129)	650	

The prevalence of *Pfcrt* and *Pfmdr*1 mutant alleles remained high and constant throughout the study. The dynamic of mixed alleles for both *Pfcrt* and *Pfmdr*1 was lower compared to mutant alleles from 2001 to 2015 (Figure 1).

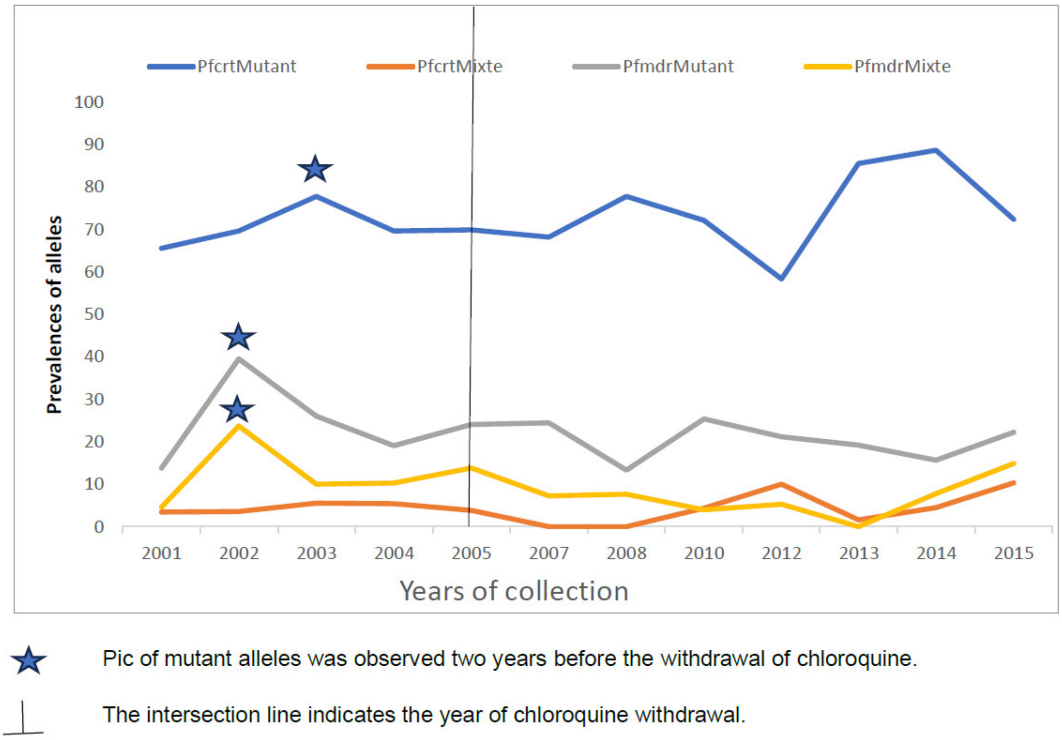

Figure 1: Dynamics of *Pfcrt* K76T and *Pfmdr*1 N86Y mixed and mutant alleles prevalence over the years of collection in Kolle.

4. Discussion

This current study showed that the prevalence of the *Pfcrt* mutant allele remained high from 2001 to 2015 despite the withdrawal of chloroquine from the national therapeutic policy in 2005. The results of this study are similar to previous studies in Mali, Senegal, Guinea and Malaysia [8,9,16–20]. However, the prevalence of the *Pfcrt* mutant allele in this study was higher than the prevalence obtained in Burkina Faso [21]. Chloroquine resistance prevalence was reported to be constant in several places in Africa. This phenomenon could be explained by the pressure of certain artemisinin-based combination therapy partner drugs such as amodiaquine and piperaquine. Also, the high prevalence of the *Pfcrt* mutant allele could be explained by the pressure exerted by the molecules of seasonal malaria chemoprevention (SMC), which contain amodiaquine. Amodiaquine, which is amino 4 quinoline, would have a similar mechanism of action as chloroquine and could lead to a cross-selection of molecular markers of resistance. The prevalence of the *Pfcrt* mutant allele in this study is different from the results of previous studies conducted in Malawi and Zambia, which reported a drastic decrease, respectively, of 9.2% and 0% after 9 years of chloroquine withdrawal. This difference in the Zambian and Malawian studies could be explained by the absence of use of analogous drugs in these areas, which can lead to cross-selection of *Pfcrt* mutant alleles. Also, these previous studies were conducted about a decade ago, and the prevalence would have changed in the meantime.

The prevalence of mixed *Pfcrt* K76T alleles is lower than that observed in Cameroon in 2002 [22], while this prevalence is comparable to that observed in Uganda in 2017 [23]. The prevalence of mixed

alleles shows the dynamics of change of wild type alleles into pure mutant alleles. Some studies showed an association between asymptomatic malaria and the prevalence of mixed alleles [23]. In most published studies, the frequencies of mixed alleles are added to those of mutant alleles because it is assumed that mixed alleles will become mutants in the future. This way of counting can be biased because mixed alleles can also revert to wild alleles without drug pressure [12].

The overall prevalence of the mutant *Pfmdr*1 86Y was relatively high in Kolle from 2001 to 2015. The dynamics of the prevalence of this marker did not change throughout the study period and the comparisons between the prevalence have shown no statistical difference ($p = 0.08$). These results are similar to those observed in a previous study conducted in Angola in 2008 [24]. The prevalence of mutant *Pfmdr*1 is slightly higher than that observed in Senegal in 2012, which found a prevalence of 16% of the mutant *Pfmdr*1 86Y [25]. The overall prevalence of the mutant *Pfmdr*1 86Y is higher than that obtained in a study in Bobo-Dioulasso in 2020 in Burkina Faso, which found 10.1% [26]. However, our data found a significantly lower prevalence of mutant allele than those observed in two different studies conducted in Mali in 2001 and 2009, which found, respectively, 50% and 63.6% mutant *Pfmdr*1 prevalence [9,16]. This difference could be explained by the pressure due to lumefantrine, which started to be used in 2006 and selects the wild type allele and decreases the frequency of the mutant allele [27]. The prevalence of mixed *Pfmdr*1 N86Y is lower than that observed in Uganda in 2017 [23].

5. Conclusions

The prevalence of the mutant *Pfcrt* K76T allele has remained high and constant from 2001 to 2015, although the prevalence of the *Pfmdr*1 mutant allele was low. This high prevalence of *Pfcrt* 76T indicates that it is very unlikely to currently use chloroquine for the treatment of malaria even in combination.

Author Contributions: A.A.D. designed the study, wrote the protocol, and participated in the scientific writing. S.D. wrote the first draft of the manuscript. B.D., A.B., A.K. (Aly Kodio), D.D., A.N. and M.D. (Moussa Doumbia). participated in molecular analysis and scientific writing. M.T., A.O., B.F., H.M., A.K.K., O.T.D., S.D., A.D., A.K. (Aminatou Kone), A.M.L., B.S. and N.D. participated in the conduct of the in vivo study, in the sample collection and in the scientific writing. M.D. (Moussa Djimde) and B.F. performed statistical analysis and scientific writing.

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Conflicts of Interest: The authors have declared that no conflicts of interest exist.

Study Limitation: Samples for the years 2006, 2009 and 2011 were not available. This study is retrospective and demographic databases of all studies to which these samples belong to were no longer available.

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