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Genetic Polymorphisms in *Plasmodium falciparum* Chloroquine Resistance Gene, *pfcrt* in Massakory (Chad)

Mahamat Moussa Hassane Taïsso[1, 2, *], Ako Aristide Berenger[2], Issa Mahamat Souleymane[3], Koui Stéphane Tossea[2], Kouman Kouamé Bouatini Angelo[2], Hamit Mahamat Alio[1], Mahamat Saleh Issakha Diar[3], Djiddi Ali Sougoudi[4], Djimadoum Mbanga[1], Pascal Ringwald[5], Djimrassengarh Honoré[6], Issa Ali Haggar[4], Hassoumi Manah[4], Hassan Ahmat Mihedi[3], N'garadoum Olivier[3], Brahim Boy Otchom[1], David Koffi[2], Dosso Mireille[2], Djaman Allico Joseph[7], Offianan André Touré[2]

[1]Faculty of Science and Human Health, University of N'Djamena, N'Djamena, Chad

[2]Department of Parasitology Mycology, Institute Pasteur, Abidjan, Côte d'Ivoire

[3]Chad National Malaria Control Program, N'Djamena, Chad

[4]Ministry of Health, Public and Human Solidarity, N'Djamena, Chad

[5]World Health Organization, Geneva, Switzerland

[6]World Health Organization, N'Djamena, Chad

[7]Clinical and Fundamental Biochemistry Department Institute, Pasteur, Cote d'Ivoire

Email address:

taissomahamat@gmail.com (M. M. H. Taïsso), taissomahamat@icloud.com (M. M. H. Taïsso), Issa.mahamatsoul@gmail.com (I. M. Souleymane), Andre_offianan@yahoo.fr (O. A. Touré)

[*]Corresponding author

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Abstract: *Background and Objective*: In 2005, Chad, like several other WHO countries, withdrew chloroquine as a first-line treatment for *Plasmodium falciparum* malaria in response to WHO recommendations related to the reason for the increase in treatment failures and the global spread of chloroquine resistance. Artemisinin-based combination therapy (ACTs), Artemether-lumefantrine, has replaced chloroquine as the first-choice treatment for malaria. The present study assessed *pfcrt* polymorphism in *Plasmodium falciparum* isolates in Massakory. *Methodology and Results*: Blood samples for PCR analysis were collected on Whatman 3MM filter paper in Massakory during a therapeutic efficacy study (TES) conducted from December 14, 2019 to March 14, 2020. Genomic DNA was extracted from 113 dried blood spots with the QIAamp DNA Micro Kit (Qiagen, Valencia, CA) as per manufacturer's protocol and amplified by nested-PCR with *pfcrt* specific primer. The amplification products were revealed by electrophoresis on 2% agarose gel and then sequenced according to Sanger method. A total of 71 sequences were readable. The *pfcrt* analysis showed that of the 71 readable sequences, high mutation prevalence: 66 (92.96%) *IET*, 2 (4.22%) *IDT* and 3 (4.22%) *MNK* wild *pfcrt* isolates. *Conclusion*: These results challenge the highest health authorities in the country. The government, through the Ministry of Public Health and National Solidarity and the National Malaria Control Program, must raise awareness for the effective withdrawal of chloroquine. This action will promote on the one hand the re-emergence of parasites sensitive to chloroquine, and on the other hand make possible the reintroduction of chloroquine in the treatment of simple malaria after the suppression of drug pressure.

Keywords: Malaria, *pfcrt*, Chloroquine, *Plasmodium falciparum*, Massakory, Chad

1. Introduction

Malaria is still a serious public health problem today, despite the achievements made over the past decade in the fight against this parasitosis. According to the latest world health organization (WHO) report, in 2019, there were an estimated 229 million cases of malaria worldwide with an estimated 409,000 deaths [1]. To combat malaria, WHO currently recommends a range of interventions including the use of Insecticide-Treated Nets (ITNs), Intermittent Preventive Treatment (IPT) in pregnant women, and timely and effective case management with Artemisinin-based Combination Therapies (ACT) for simple oral malaria and artesunate, artemether and quinine parenterally for severe malaria.

The progress made in recent years has been possible thanks to the combination of two major strategies, including rapid and efficient case management by ACT and prevention through the massive use of Long-Acting Impregnated Mosquito Nets (LLINs). Indeed, since the emergence and spread of resistance of *P. falciparum* to common antimalarials including chloroquine and Sulfadoxine-pyrimethamine, ACT has been recommended in the therapeutic management of simple malaria in almost all malaria-endemic areas under the impetus of the WHO.

In 2005, Chad, like several other WHO countries, withdrew chloroquine as a first-line treatment for *Plasmodium falciparum* malaria in response to WHO recommendations related to the reason for the increase in treatment failures and the global spread of chloroquine resistance. The National Malaria Control Program (NMCP) guidelines recommend Artesunate-Amodiaquine (ASAQ) as the first line for treatment of simple malaria and as a second line in case of treatment failure, contraindication or intolerance to ASAQ, Artemether-Lumefantrine (AL). In pregnant women in the first trimester, management is done with quinine in oral form, ACT being recommended only from the second trimester of pregnancy. The therapeutic management of malaria cases is now hampered by the resistance of *P. falciparum* to artemisinin derivatives and their partner molecules including amodiaquine, lumefantrine, piperaquine (DHA-PPQ) etc.

Indeed, clinical resistance to artemisinin, manifested by delayed parasite clearance, was first reported in Pailin, western Cambodia in 2009 [2]. This resistance has spread to all countries in the Greater Mekong subregion [3, 4]. The presence of resistance to ACT partner drugs caused treatment failure with DHA-PPQ, AL, etc. [5, 6].

Monitoring the effectiveness of antimalarial drugs is an essential part of the fight against malaria. Indeed, resistance to antimalarials can have devastating consequences including an increase in morbidity and mortality. Resistance to artemisinin derivatives in Southeast Asia in recent years is seriously undermining the control gains of ACT. In Chad, recent studies have shown the effectiveness of ASAQ [7] and absence of resistant mutations in the *k13 propeller* gene, an artemisinin resistance gene [8].

Chloroquine is the first synthetic antimalarial. From 1944, this active ingredient will be used in prophylaxis and for the treatment of un complicated malaria attacks. Inexpensive, it will quickly become the most used antimalarial during the twentieth century. Once in the digestive vacuole, chloroquine inhibits the detoxification of heme into hemozoin. Given fairly high levels of resistance observed in malaria-endemic areas, this molecule is no longer recommended in the management of cases of simple malaria a *P. falciparum* even in combination with artemisinin derivatives. However, chloroquine continues to be used for the treatment of uncomplicated *Plasmodium falciparum* malaria in areas where resistance to chloroquine is zero or low [9] as well as to treat *Plasmodium vivax* malaria [10].

Indeed, most of the chloroquine-resistant strains of *P. falciparum* studied showed a deficiency in the concentration of the drug in the digestive vacuole, more likely in connection with a rapid efflux of chloroquine-resistance out of the vacuole, than with a decrease in the penetration of the product [11].

The study of the genome of resistant parasites has allowed the identification of a gene linked to resistance to chloroquine: *pfcrt (Plasmodium falciparum* chloroquine resistance transporter).

Numerous research studies have demonstrated the importance of the K76T mutation in the pfcrt gene in chloroquine resistance [12]. Epidemiological studies revealed the presence of several additional mutations (about 8 mutations) to K76T in the *pfcrt* gene, defining several mutant haplotypes. It has also been shown that the expression of some of these haplotypes modifies the level of sensitivity to other lysosomotropism used alone or in combination, in the treatment of simple or severe malaria attacks, such as quinine, mefloquine, halofantrine, amodiaquine or artemisinin [13, 14]. Thus, in accordance with their ability to modify the physiology of the digestive vacuole, *pfcrt* alleles modulate sensitivity to first-line ACTs [15]. A meta-analysis of the therapeutic efficacy of the two widely used CTAs, artesunate-amodiaquine (AS-AQ) and artemether-lumefantrine (AL), revealed a reduction in reinfection time for parasites encoding the mutant *Pfcrt* [16].

The polymorphism of the pfcrt gene has been little studied in Chad while drug pressure seems to be strong probably related to the use of chloroquine in self-medication.

2. Materials and Methods

2.1. Site and Study Period

The study was conducted in the Massakory I Health Center, Hadjer Lamis Provincial Health District (DSP). The Massakory I Health Center is located about 160 km away at the northern exit of N'Djamena. Map link Latitude: 13.0016804 Longitude: 15.7286011. The study was conducted from December 14, 2019 to March 14, 2020 at the

Massakory I Health Center.

The blood collected in Massakory on Whatman filter paper was sent to Malariology Unit of Institut Pasteur of Côte d'Ivoire for PCR analysis. The study involved patients aged 6 to 59 months, who came to the Massakory health center for fever and other signs presumptive of malaria.

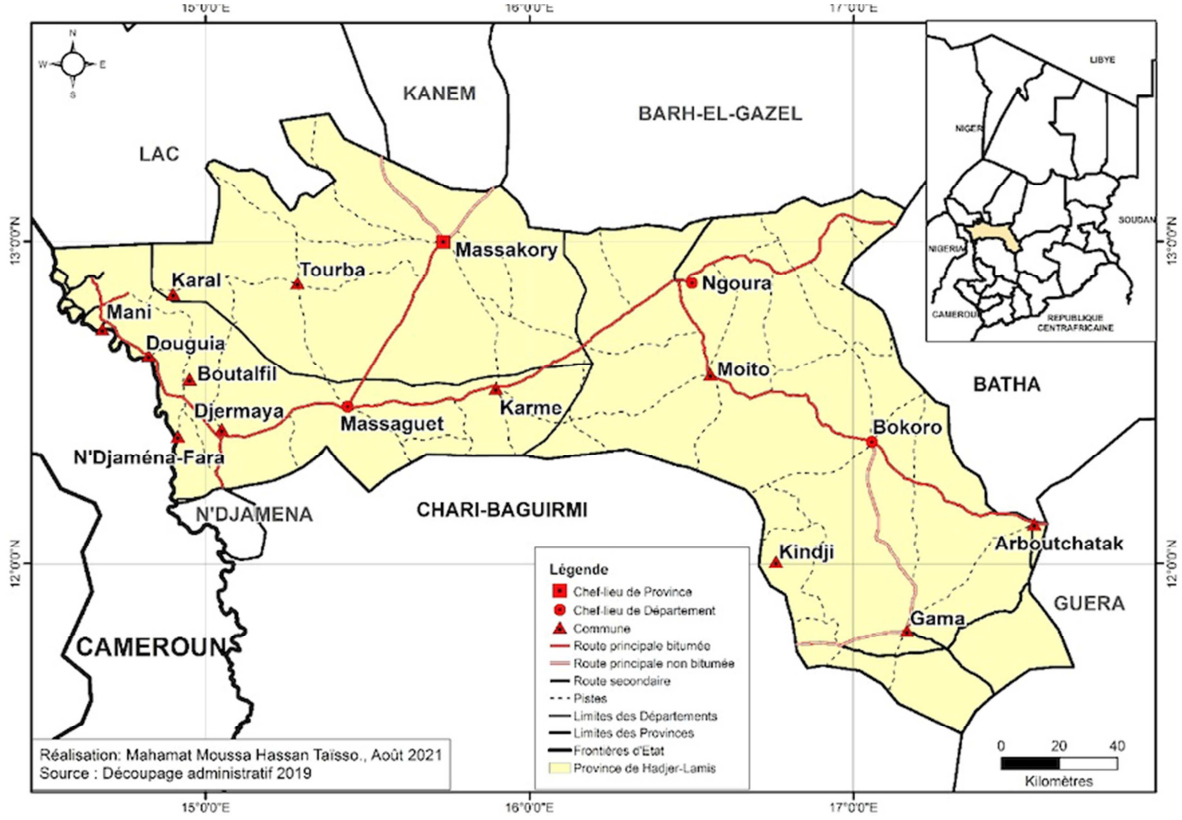

Figure 1. *Location of the study site (Administrative division 2019).*

2.2. Collection of Blood Samples

Blood samples for PCR analysis were collected on Whatman 3MM filter paper. A total of 3 drops of blood were spotted on filter paper. Dried at room temperature (25°C) and wrapped in desiccant-containing plastic bags, the filter paper was stored dry for genomic DNA extraction. The dried blood spot were then sent to Malariology Unit of Institute Pasteur of Cote d'Ivoire for DNA extraction and amplification.

2.3. DNA Extraction

DNA extraction and amplifications: Genomic DNA was extracted from dried blood spots with the QIAamp DNA Micro Kit (Qiagen, Valencia, CA) as per manufacturer's protocol. Briefly, filter paper was cut, and then denatured with Proteinase K. The lysate was washed twice with buffers through a silicate-containing membrane. Lastly, DNA was eluted with ultra-pure water.

2.4. Amplification of the pfcrt Gene

For plasmodial species typing, the targeted gene was that of the 18S subunit of the ribosomal RNA of *Plasmodium falciparum*, by the protocol of Snounou et al in 1993.

For the detection of point mutations on the *pfcrt* gene, conventional gene amplification was performed. The reactive medium was composed of MasterMix enzyme *5X FIREPol MasterMix RTL with 12.5 mM* $MglC_2$ from the company *Solis Biodyne* (enzyme, $MgCl_2$, DNTPs, buffer), molecular biology quality water and primers *SecIF _GGTAAATGTGCTCATGTGTTTAAACTTATT, SecIR_TTACTTGAATTTCCCTTTTTATTTCCATTTCCA*.

The amplification program began with a denaturation of the DNA strands at 95°C for 10 minutes followed by 31 cycles distributed as follows: a cyclic denaturation of 15 seconds at 95°C, hybridization at 60°C for 2 minutes and a cyclic elongation of one minute at 72°C. After the cycles, a final elongation of 7 minutes at 72°C was performed. The amplicons obtained were revealed by electrophoresis on 2% agarose gel. The images were viewed using *gelDoc* software. The size of interest of the amplicon was 241 bp.

The *Qiagen QIAquick Gel Extraction* kit was used to carry out the purification of the products obtained after conventional gene amplification. According to the manufacturer's protocol; After purification, 1 microliter of the purified product was used to read the optical density to check the nucleic acid concentration. The purified amplicons were automatically sequenced by the Sanger method at the Sanger CRCHU Sequencing Platform in Quebec City, Canada. The samples packaged according to the recommendations of the platform were sent via an international delivery company.

2.5. Analysis of Sequencing Data and Identification of Mutations

The raw sequencing data was cleaned using MEGA 11 software. All sequences were then aligned using BioEdit 7.2.5 software using as the reference sequence of the *pfcrt* gene the *PF3D7_0709000.1* sequence available on *PlasmoDB (http://www.ncbi.nlm.nih.gov).*

2.6. Ethical Considerations

Before the start of the study, the protocol received the approval of the Ministry of Public Health as well as the authorization of the ethics committee through a research authorization from the Ministry of Higher Education, of Research and Innovation of Chad. All parents/guardians of the children were given a clear explanation of the study and informed consent was obtained prior to any study procedure being performed.

3. Results

A total of 113 febrile children aged 6 to 59 months had been recruited into the Massakory study. The age of the patients ranged from 6 months to 59 months. The parasites density on D0 ranged from 2100 to 35000 globality, and 2080 to 35000 (ET 7921.20) for the AL group compared to 2100 and 29080 (ET 8471.26) for ASAQ. Clinical and parasitological results had been published elsewhere [17].

Molecular Characterization of the Pfcrt Gene

A total of 113 samples were used for DNA extraction by Qiagen Kit following the manufacturer's recommendations. DNA concentrations ranged from 1.58 to 22.65 ng/µL with an average of 8.58 ng/µL. Molecular diagnosis of the species by real-time PCR confirmed that all isolates were of the species *Plasmodium falciparum.* For the *pfcrt* gene 113 samples or 100% were amplified (Figure 3). However, only 80 samples were sent for sequencing. These samples were chosen randomly.

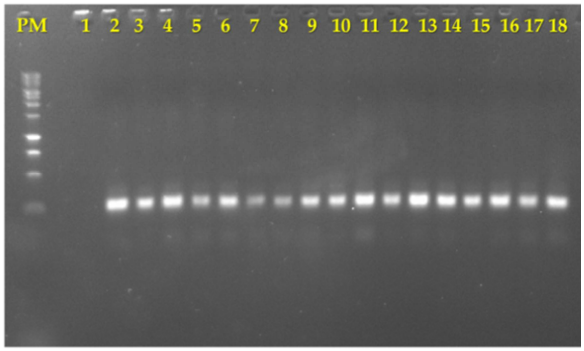

Figure 2. *Electrophoretic profile of the pfcrt gene.*

PM =Molecular weight marker (1kb)
1 = negative control
2= Positive control
3-18= Amplified samples with pfcrt primers (SecIF/SecIR)
Amplicon size = 241 bp.

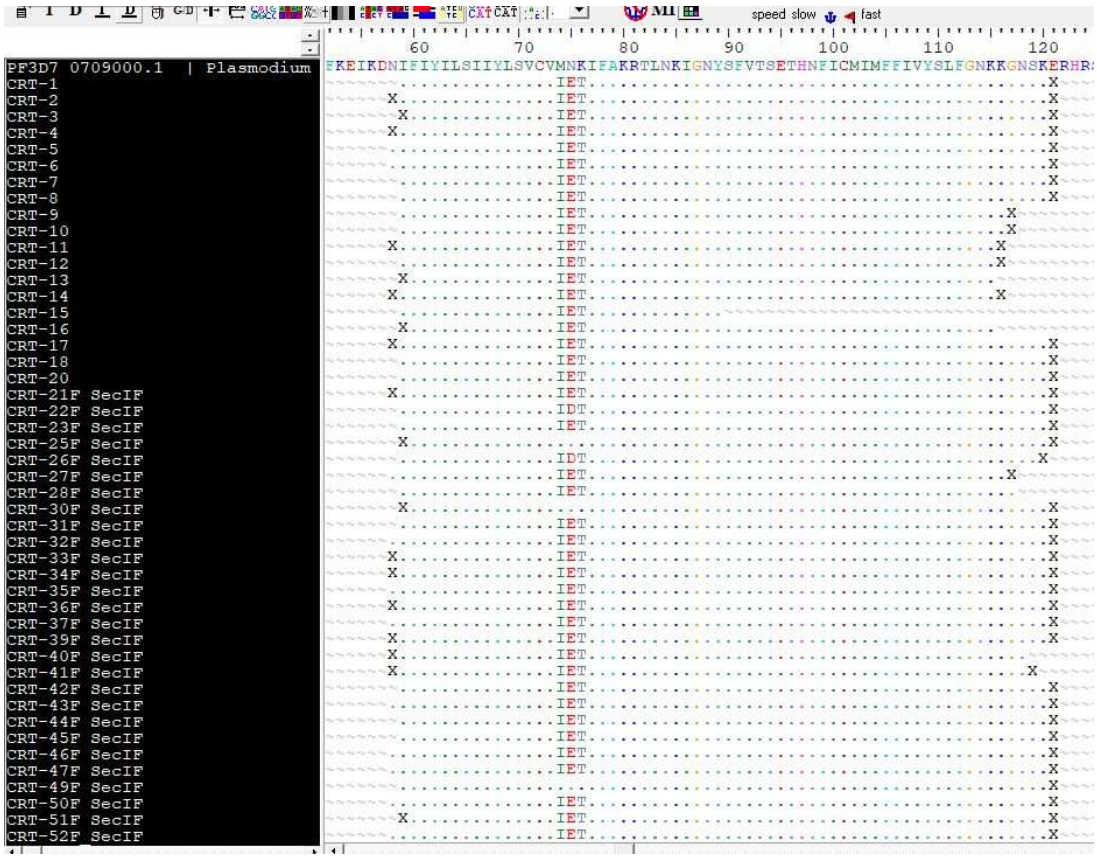

Figure 3. *Alignment of the sequences of the pfcrt gene (1).*

Figure 4. *Alignment of pfcrt gene sequences (2).*

The *pfcrt* analysis showed 71 readable sequences (Figures 4 a, b). The table 1 indicate the distribution of pure mutations. Of the 71 readable sequences, 68 contained the three *pfcrt* IET/IDT mutation points. The results showed 3/71 wild *pfcrt* isolates M-74/N-75/K-76, 68 pure mutants with 66 triple mutant isolates M74I/N75E/K76T and 2 triple mutant isolates M74I/N75D/K76T (Table 2).

Table 1. *Distribution of pure mutations in the pfcrt gene for n=71.*

Position	Allele	Effective (%)
74	I	68 (95.77)
75	E	66 (92.96%)
	D	2 (2.82%)
76	T	68 (95.77)

Table 2. *Prevalence of pfcrt genotypes.*

Gene	Genotype	Effective (n=71)	%
Pfcrt	MNK	03	4.22
	IET	66	92.96
	IDT	2	2.82
	Total	71	100

4. Discussion

Resistance of *Plasmodium falciparum* to antimalarial drugs has hampered efforts to eradicate malaria. The higher proportion of clinical isolates carrying the mutant allele Thr-76 of the *pfcrt* gene had led several countries including Chad to withdraw chloroquine in 2005. The informal use of chloroquine has been observed in most parts of Chad, including the town of Massakory. Nowadays no study on the polymorphism of the *pfcrt* gene has been carried out in this city in Chad.

Analysis of the 71 readable sequences on the 80 sequenced samples, indicated a frequency of 92.96% of IET genotypes.

According to the report of the Ministry of Public Health and INSEED on malaria indicators in Chad in 2017, Chad has three bioclimatic zones that determine three epidemiological profiles or facies of malaria (north, center and south). The city of Massakory located between the desert north, where 2.6% of the country's total population lives, is made up of oases and concentrates the majority of imported malaria cases and the center, whose climate is of the Sahelian type corresponds to unstable malaria, due to short seasonal transmission.

Despite the government's declaration on the official withdrawal of chloroquine, Massakory is one of the cities that informally use this molecule hence the maintenance of drug pressure. The use of chloroquine for the treatment of malaria would have led to a high prevalence of these mutant strains in this city because if its withdrawal had been respected it could be accompanied by a drastic decrease or decrease in these mutant strains as reported by Paul Sondo et al. in 2021 in Burkina Faso [18]. Indeed, when the drug pressure decreases, the proportion of sensitive parasites increases and that of resistant parasites decreases [19]. This is not the case found during this study in Chad.

The government through chad's Ministry of Public Health and National Solidarity must increase awareness or lobbying for an effective withdrawal of chloroquine. Such a decision was taken in Malawi where a reintroduction of chloroquine for the prevention and treatment of malaria is demonstrated possible. Indeed, Malawi was the first African country to stop using chloroquine due to widespread resistance, less than a decade after the removal of drug pressure, the chloroquine-resistant molecular marker of malaria had disappeared and the drug was found to have excellent clinical efficacy [20].

Recent studies in malaria-endemic countries had reported the re-emergence of chloroquine-sensitive parasites in areas where there has been sustained withdrawal of chloroquine, which has mainly resulted from the re-expansion of the wild type after the removal of drug pressure. This is the work of Mwanza et al, 2016 in Zambia who reported a 100% prevalence of chloroquine-sensitive strains (CVMNK) [21], Mekonnen et al., 2016 in Ethiopia with a frequency of susceptible strains of 95.9% [22]. William et al, 2015 in Kenya had also reported a decline in the prevalence of K76T to 41% after the 3 years of discontinuation of official chloroquine use [23].

In Nigeria Ikegbunam *et al.* in 2019, reported 94.54% CVIET haplotype after analysis of 55 samples of *Plasmodium falciparum* [24]. This high prevalence was explained by the continued use of Chloroquine in Nigeria despite its with draw al decided by the government. Massakory a crossroads where the supply of general merchandise and pharmaceutical products including chloroquine is from Nigeria. This could explain the possibility of chloroquine supply to the population of

Massakory. Oladipo *et al.* in 2015 also reported in their work that 91.6% (109/119) of children with *P. falciparum* infection harbored parasites with the mutant pfcrt haplotype CVIET compared to 4.2% of wild type (CVMNK) [25]. Similar results were reported by Djaman et al. [26]. Recent work by Dagnogo et al in Côte d'Ivoire reported a predominance of MNK genotype (wild type) with a frequency of 62% [27]. In contrast, single mutant, double mutant and triple mutant genotypes were observed with prevalence of 12%, 6% and 18% respectively. The low prevalence of mutants proves the effective removal of chloroquine from the therapeutic arsenal.

According to Rovira Graels *et al.*, there would be explanatory factors related to the host namely the level of premunition and pharmacokinetics of the molecule of the study [28]. Genetic background as well as diet could also be factors in the variation in therapeutic responses. The epidemiological facies of the city of Massakory, would favor and maintain a significant Anophelian pressure favorable to the circulation of resistant strains as reported by other works [29]. Indeed, in an environment where the Anophelian density is strong and in which the drug pressure to the usual molecules such as chloroquine is high, the risk is great that the infective bites transmit the parasites carrying the resistance mutations.

5. Conclusion

Despite the many efforts made to eradicate it, malaria remains a serious public health problem.

The general objective of this work was to determine the polymorphism of the *pfcrt* gene.

Results showed a prevalence of 92.96% of triple mutant IET**.** This high prevalence reflects the continued use of chloroquine in the study area, although it has been removed from the therapeutic arsenal since 2005.

Ultimately, this study generated clinical and biological data and reported the existence of chloroquine-resistant strains in Massakory. These results challenge the highest health authorities in the country. The government, through the MSPSN and the PNLP, must raise awareness for the effective withdrawal of chloroquine. This action will promote on the one hand the re-emergence of parasites sensitive to chloroquine, and on the other hand make possible the reintroduction of chloroquine in the treatment of simple malaria after the suppression of drug pressure.

The high prevalence of IET mutants being restored is alarming, however future work with a large number of samples is recommended for confirmation. This will enable the Government of Chad to find appropriate solutions for the effective treatment of chloroquine in Chad.

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