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Prevalence of *Plasmodium falciparum* Chloroquine Resistance Transporter (*Pfcrt*76T) Mutation Associated with Antimalarial Drug Resistance in Two Different Epidemiological Setting (Banfora and Saponé) in Burkina Faso Few Years after the Implementation of Artemisnine Based Combination Therapy (ACTs)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SN, IS, JS, SBS, SSS, SS, AD and FWD designed the study, wrote the protocol, performed the study analysis and wrote the first draft of the manuscript. Authors NBH, ESB, SAC, SMO and JMK ensure the participant follow up and collected field data. All authors read and approved the final version.

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ABSTRACT

Introduction: In spite of considerable progress, malaria remains a public health problem in many areas, particularly in sub-Saharan Africa. One major complexity of malaria disease is caused by the development and the spread of vector and parasite resistance to insecticides and antimalarial drugs respectively. The *Pfcrt7*6T gene mutation has been validated as a marker conferring resistance to chloroquine and other antimalarial drugs. The extension *of Plasmodium falciparum* resistance to commonly used antimalarial drugs (chloroquine, sulfadoxine-pyrimethamine) led to the adoption and the use of artemisinin-based combinations in Burkina Faso since 2005.
Aims: The present study was initiated to assess the prevalence of the *Pfcrt76T* mutation in two different malaria epidemiological setting after a decade of introduction of artemisinin-based combination therapies (ACTs) in Burkina Faso.
Methodology: The study population consisted of 181 uncomplicated malaria patients recruited in Banfora and Saponé health districts in 2012 and 2013. Blood samples were collected from finger prick on filter paper, dried and sent to the Molecular Biology Laboratory at *Centre National de Recherche et de Formation sur le Paludisme (*CNRFP) for molecular analyzes. DNA of *Plasmodium falciparum* was extracted with DNA extraction kit (Qiagen®) and the *Pfcrt76T* mutation was determined based on Polymerase Chain Reaction / Restriction Fragment Length Polymorphism technique (RFLP).
Results: The results of this study showed that the frequency of the *pfcrt*76T mutant allele (33.7%) was statistically lower than the *Pfcrt*76K wild-type allele (57.4%) in the study area. Moreover, the prevalence of *Pfcrt*76T mutation was neither associated with the patient age nor with the parasite density while a significant difference was observed between the two epidemiological setting, Banfora and Saponé.
Conclusion: The findings of this study has shown a drop in the prevalence of mutant parasites *Pfcrt76T* in both the study area eight years after the introduction of ACTs compared to previous studies.

Keywords: Plasmodium falciparum; antimalarial drugs; Pfcrt gene; CTAs; Banfora; saponé; resistance.

1. INTRODUCTION

Malaria is known to be the most deadly parasitic infection in the world. Indeed, malaria is responsible for many deaths every year, cripples economic growth especially in Africa and perpetuates the vicious circle of poverty [1]. In spite of considerable progress toward the control and fight against malaria, it still represents a public health concern in many areas, particularly in sub-Saharan Africa. Unfortunately, between 2015 and 2016, mortality rates stalled in the WHO regions of South-East Asia, the Western Pacific and Africa, and increased in the Eastern Mediterranean and America. That reconfirms we are of course to meet two critical 2020 milestones of the WHO Global Technical Strategy for Malaria 2016-2030: reducing case incidence and death rates by at least 40% from 2015 levels [2].

In Burkina Faso, malaria is endemic and constitutes the main cause of consultation, hospitalization and mortality in health facilities. Children under 5 years and pregnant women pay the highest tribute for this disease each year [3]. In 2016, this disease led to 45% of consultation, 45.6% of hospitalization and 25.2% of deaths [4]. Unfortunately, despite the considerable progress made over the last decade in search of a malaria vaccine, there is still no effective vaccine available ready against malaria. The most advanced RTS, S vaccine is still at an implementation phase in Ghana, Kenya and Malawi [5]. The current struggle against malaria is based on vector control. The main way of preventing and reducing the transmission of malaria; the prevention and treatment with specific and effective antimalarial drugs. However, one of the major concern in malaria treatment (curative or prophylactic) is currently the progression of resistance of *Plasmodium falciparum* to antimalarials, in general, threatening the Artemisinin-based combination therapy (ACTs) considered to be more effective against malaria *Plasmodium falciparum* [6]. Indeed, the emergence of a strain of malaria that is resistant to artemisinin-based combination therapy (ACT) in Southeast Asia (the currently recommended first-line treatment for malaria) is a

concern because it threatens to stop or reduce the progress that's been made against malaria-causing parasites in recent years [7,8].

This resistance is characterized by a decrease in the sensitivity of ACTs observed in Southeast Asia. No alternative therapeutic solution is currently available. Moreover, it was shown from the analysis of common mutant resistance genes as Pfcrt (Plasmodium falciparum chloroquine resistance transporter) and Pfdhfr (Plasmodium falciparum dihydrofolate reductase) a genetic origin links with the parasite from the Thailand-Cambodia border [9,10]. However, up to date, there is no clear evidence that artemisinin resistance parasites have reached Africa yet [11]. In that context of the damage caused by resistance, it is, therefore, necessary to continue to carry out studies that will assuredly make it possible to evaluate drug resistance for monitoring resistance to current antimalarials drugs. Thus, several studies have been conducted around the world in general and particularly in Africa, where several mutations associated with resistance to antimalarials, including the Pfcrt76T gene mutation in the parasite, resistance to chloroquine and therapeutic failures have been identified [12]. These phenomena of resistance to the previous antimalarials led several countries to change their malaria treatment policy, particularly with the introduction of artemisinin-based combination therapies, further to consultation with the World Health Organization [13]. However, in Burkina Faso, ACTs are known to be effective on *Pfcrt76T* parasite and have been used in the treatment of malaria since 2005. Unfortunately, to our knowledge, there are no molecular markers which demonstrated any correlation with resistance to the used ACTs. Therefore, it is still recommended to continue monitoring the prevalence of existing known molecular markers such as *Pfcrt*, as a signal of the evolution of parasite resistant population. The present study was initiated in the framework of the characterization of the *Pfcrt76T* mutation associated with antimalarial drug resistance in the treatment of Plasmodium falciparum malaria in Banfora and Saponé in the western and central part of Burkina Faso, more than eight years after the introduction of ACTs.

2. MATERIAL AND METHODS

2.1 Study Area

The study was conducted in two different epidemiological settings (Banfora and Saponé) from September 2012 to September 2013 as part of two antimalarial drugs clinical studies.

The first study area, Banfora Health District area, in the province of Comoé, is located at 441 km west of Ouagadougou, the capital of Burkina Faso. Malaria transmission is markedly seasonal and intense during the rainy season (May - November). The cumulative annual entomological inoculation rate varies from 55 to 400 infective bites/person/year. Plasmodium falciparum is the main parasite present in more than 90% of infections.

The second study area was the Saponé Health district, located in - the province of Bazéga, a rural area at 50 km south-west of Ouagadougou where malaria is mesoendemic. Malaria transmission in this district is permanent with a peak during the intense and seasonal rainy season from June to November.

2.2 Study Participants and Samples Collection

About 181 participants, from both male and female, aged from 6 months and above were included in this study. For each patient included in the study, blood samples were taken from the finger prick to prepare blood spots on filter paper and blood smears for malaria diagnosis. The filter paper spots were each kept in a plastic bag containing silica gel to keep the medium dry, then inserted into an envelope and transported to the Molecular Biology Laboratory at CNRFP (Centre National de Recherche et de Formation sur le Paludisme) waiting for molecular analysis of *Pfcrt76T* mutation.

As selected from the clinical studies, the only participant having a complete demographic data (age, sex) and analyzable samples (blood smears for microscopy, filter paper for parasite genotyping) were included in the study for subsequent molecular analysis.

2.3 Asexual *Plasmodium falciparum* Parasites Density

Thick and thin blood smears were used for microscopic diagnosis of a parasite count in the parasitology laboratory at CNRFP. Thick and thin blood smears were stained with Giemsa. The number of asexual *Plasmodium falciparum* parasites was estimated against 200 leucocytes and parasite density was calculated assuming a mean of 8000 leucocytes per µl. A sample was declared negative after examining 200 thick film fields without observing any asexual parasites.

2.4 DNA Extraction of *Plasmodium falciparum*

DNA of *Plasmodium falciparum* was extracted from the blood spots using DNA extraction kits (Qiagen®) according to the manufacturer's instructions. Briefly, the blood was first dissolved in a solution and the red blood cells burst by osmotic shock by mixing it with a hypotonic solution (ATL buffer). These were then introduced into the resulting mixture, a detergent (buffer AL) to destroy Plasmodium membranes and proteinase K to digest proteins associated with DNA. The parasite DNA was precipitated with ethyl alcohol (100%) and retained in the extraction columns followed by a double washing successively with the solutions (buffer AW1 and AW2). Finally, the eluent (buffer AE) is used to collect the DNA of *Plasmodium falciparum* in final tubes. The DNA was stored at -20°C until used.

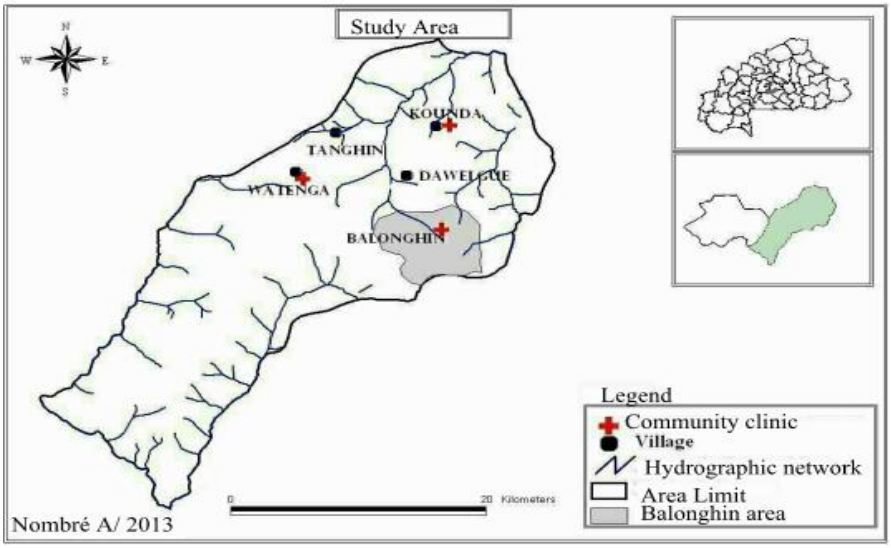

Fig. 1. Study areas of the Banfora Clinical Research Unit. Source: NOMBRE.A (CNRFP / Dec 2013)

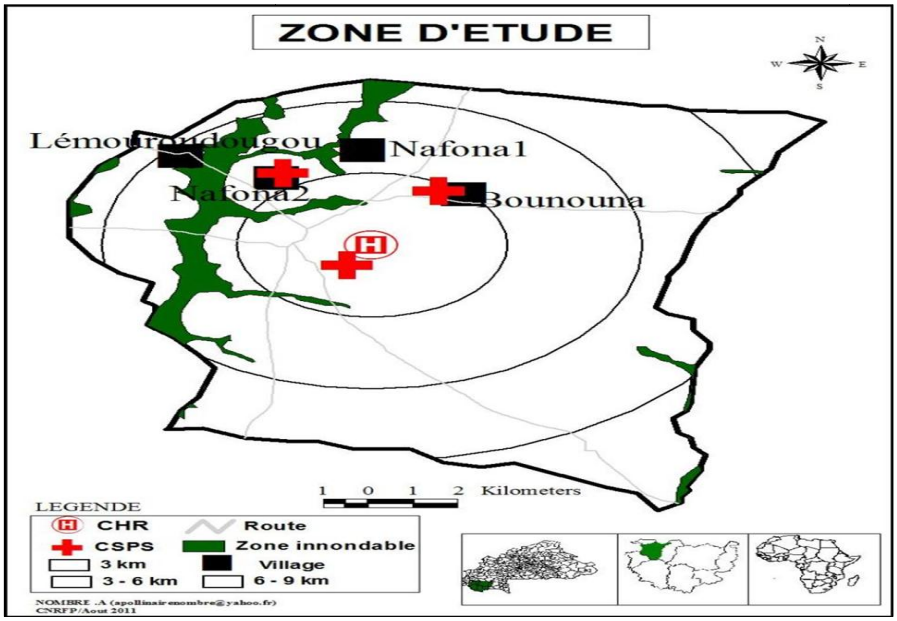

Fig. 2. Study Areas of the Balonghin Vaccinology Unit. Source: NOMBRE.A CNRFP / Dec 2013)

2.5 DNA Amplification (PCR) and Genotyping of *Pfcrt* gene par RFLP

DNA extracted from *Plasmodium falciparum* was amplified and used for the *Pfcrt* 76T single nucleotide polymorphisms genotyping by PCR-RFLP based on two major steps, namely the amplification step of the specific DNA sequences by PCR and the step of the enzymatic digestion of the DNA with a restriction enzyme, ApoI.

DNA amplification: The first amplification was performed in a reaction volume of 25 µL composed of 2.5 µL of Buffer (1X) 0.75 µL of MgCl2 (20 Mm), 0.25 µL of nucleotides (0.2 mM), 2.5 µL of outer P1 primer (1 µM), 2.5 µL outer P2 (1 µM), 0.2 µL Taq polymerase, 11.3 µL of distilled water and 5 µL of parasite DNA to amplify a large portion of the variable region of the desired gene, *Pfcrt*. And the second amplification was carried out from the product of the first of the same reaction volume and the same component except with two inner pairs of primers (D1 and D2) for amplifying a more precise region of the *Pfcrt* gene. This pair of primers (D1, D2) is specific to the region containing the K76T mutation.

Enzymatic digestion of PCR product using PCR-RFLP: A given volume of the samples of the second amplification (about 5 µL) was taken and introduced into the reaction mixture of the digestion containing the restriction enzyme (ApoI). The reaction mixture of this enzymatic digestion was performed in a reaction volume of 20 µL composed of 0.2 µL of BSA (10X), 2 µL of NEB Buffer (1x) 0.125 µL of ApoI 12.675 µL of distilled water and 5 µL of DNA of the second amplification.

The tubes were then placed into the thermocycler for digestion at 50°C for 6 hours followed by agarose (2%) gel electrophoresis.

2.6 Visualization of *Pfcrt* gene Mutations by Agarose Gel Photography

After the electrophoresis, the mutations were then visualized by a device under UV.

A successful reaction is reflected by the presence of specific expected sized bands. The expected band sizes were given concerning the molecular marker and confirmed by the positive control. The sample containing a single 134 bp band P*fcrt* 76T) is considered as a mutant while the sample with both 100 bp and 34 bp (*Pfcrt* 76 K) bands is considered as a wild type. Mixed infections samples are represented with 134 bp, 100 bp and 34 bp with the presence of both *Pfcrt* 76T and *Pfcrt* 76 K).

Two samples out of 181 gave negative PCR of 1.10%.

2.7 Statistical Analyses

The data were double entered into a database using an Access 2007 programme and analyzed using the software Stata IC version 13. Pearson $\chi 2$ tests and Fisher's exact test were used for comparison of proportions. P-value ≥0.05 was used as the threshold significance for the different statistical tests.

3. RESULTS

3.1 Socio-demographic Characteristics

The study population consisted of 50.83% (92/181) of female and 48.9% (89/182) of male both aged from 1 to 59 years with a sex ratio of 1.03 (F / M). The mean age was estimated at 17.57 ± 16.2 years. The Children under 5 years old accounted for 31, 50% of the study population while participant more and those aged between 5 and 12 years while those of older age > 15 years accounted for 51.93% of the study population (Table1).

3.2 Prevalence of the *Pfcrt 76* Alleles in the Study Population

The frequency of alleles carriage was estimated at 33.70%, 57.40% and 7.80% respectively for the mutant allele (pfcrt76T), the wild type allele (pfcrt76K) and mixed alleles or heterozygous type (*Pfcrt*76T and *Pfcrt*76K) in the study population (Fig. 1).

3.3 Prevalence of *pfcrt*76T Mutation According to Study Areas

The distribution of both mutant and wild type carriers was analyzed according to the two study sites (Table 2). The frequencies of the *pfcrt*76T mutation carriage in Banfora and Saponé were estimated at 45.90% and 27.5% respectively. While there was no statistical difference between the wild type and mutant allele carriage in Banfora Health District area (p=1), the prevalence of mutant allele *Pfcrt* 76T was significantly higher in Banfora (45.9%) compared to Saponé (27.96%) (p= 0.01).

Table 1. The basic characteristic of the study population

Characteristics	Female	Male	Total
Age (year)			
≤ 5, n (%)	28(30.44%)	29(32.60%)	57(31.50%)
5-15, n (%)	11(11.96%)	19(21.35%)	30(16.58%)
> 15, n (%)	53(57.61%)	41(46.07%)	94(51.93%)
Total	92(50.83%)	89(49.17%)	181(100.00%)

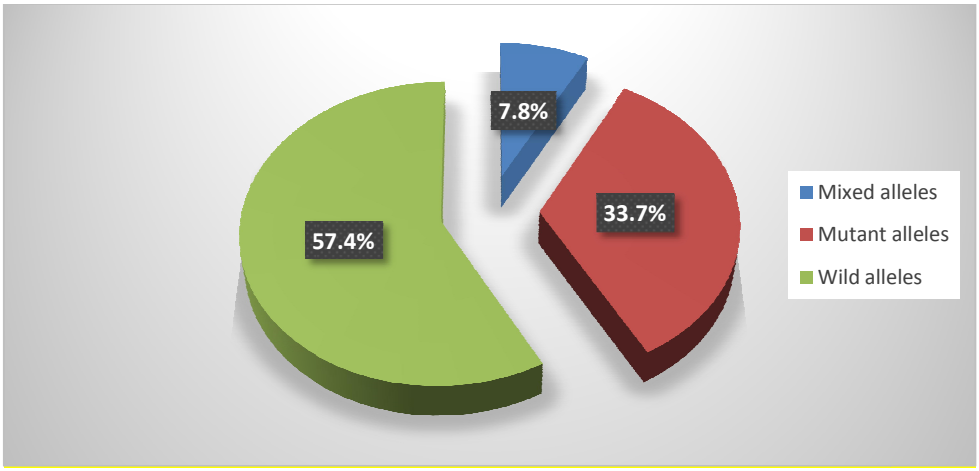

Fig. 3. Distribution of *pfcrt* alleles in the study population. Mutant (*pfcrt*76T), wild (*pfcrt*76K) and mixed (*Pfcrt*76T + *Pfcrt*76K)

Table 2. Distribution of mutant and wild alleles according to study sites

Genes	Banfora Health District area n (%)	Saponé Health District area n (%)	P
Homozygous pfcrt 76 T	28 (45.90 %)	33 (27.96 %)	0.01
Homozygous pfcrt 76K	28 (45.90%)	76 (64.41%)	0.03
Heterozygous *pfcrt* 76K/T	5 (8.2%)	9 (7.63 %)	0.87

3.4 Prevalence of Mutant Alleles According to Age

There was no statistically significant difference when comparing the prevalence between *Pfcrt*76T (mutant) and *Pfcrt*76K (wild) as a function of age (p = 0.08) (Fig. 2). However, the prevalence of the wild type *Pfcrt* 76K alleles carriage was statically higher than the mutant ones in patients for more than 10 years (p<0.05).

3.5 Prevalence of Different Alleles of *Pfcrt* According to Parasitaemia

The prevalence of different *Pfcrt* alleles was analyzed as a function of parasite density considering three population groups based on the clinical malaria definition from several clinical studies (Table 3) didn't showed any statistically significant difference when comparing the frequencies of the mutant (p = 0, 71), the wild type (p = 0.60) as well as *Heterozygous* (p = 0.56) according to the different parasite density ranges.

4. DISCUSSION

4.1 The Prevalence of the pfcrt76T Mutation in the Study Population

The *Pfcrt* alleles distribution assessed in our study in both study areas Banfora and Saponé Health District area showed a reduced rate compared to the previous studies carried out in Burkina Faso in the last few years. Indeed, with an overall prevalence of 34% in the study population, the mutant allele *Pfcrt* 76T presents a reduction compared to the 61.4%, 65%, obtained

by TINTO et al., in 2000 [14] and 2005 [15] in Bobo Dioulasso respectively. The findings of this study were lower than the prevalence of 48.8% and 50% obtained in 2008 by MAHAMAT et al. [16] in Gourcy and Dandé located at the North and western part of Burkina Faso respectively. The overall significance of this study indicated a clear reduction of the chloroquine resistance parasite since the introduction of the ACTs in 2005 in Burkina Faso. This conforms with the results obtained by Some et al. in 2016 as well as [17]. This re-affirmed the efficacy of the ACTs on the chloroquine-resistant parasite as described previously. Looking at this decrease in the prevalence of the *Pfcrt*76T mutation over the years, the reduction to the zero rates of the *Pfcrt*76T resistant allele in the long term would be a possibility, as observed in Malawi [18]. However, the proportion of the circulating resistant *Pfcrt* 76T alleles is timely and geographically varying in malaria-endemic countries as confirmed by different results in Niger in 2006 (50%) and Malaysia in 2012 (52%) and 2016 (97.7%) [19].

During the PCR analyses, negative results for PCR were obtained (without bands of genes). This could be explained by a very small amount of parasitic DNA in these samples as we did not get the DNA quantified before amplifying. Indeed, a minimum amount of DNA is needed to amplify it. An additional possible explanation may be related to the occurrence of reading errors of slides declaring a patient positive while it is negative.

4.2 Prevalence of *pfcrt*76T According to Age

In this study, there was no statistically significant difference when comparing the prevalence between age groups and the frequency of alleles (mutant and wild) in our study population. The lack of significant difference with age was also observed in a study by DJIMDE et al. in Koro (Mopti), Mali in 2008 [20]. However, our results are in contradiction with those obtained by Inas et al. in 2005 in Burkina Faso and this could be explained by the difference in the number of age distribution with ours (age <10 years and ≥ 10 years) [21].

4.3 The Prevalence of the Mutation According to Parasitaemia

Our results did not find any relationship between the *Pfcrt*76T mutation and the parasitaemia. Our results corroborate with those obtained by MAHAMAT et al., in (2008), in Burkina Faso [16]. However, some literature reviews note that high parasite densities favour the appearance and selection of mutations under the influence of drug pressure [22]. This contradiction with ours could be explained by the fact that the test was carried by in vitro and giving only an overall response of the parasite population.

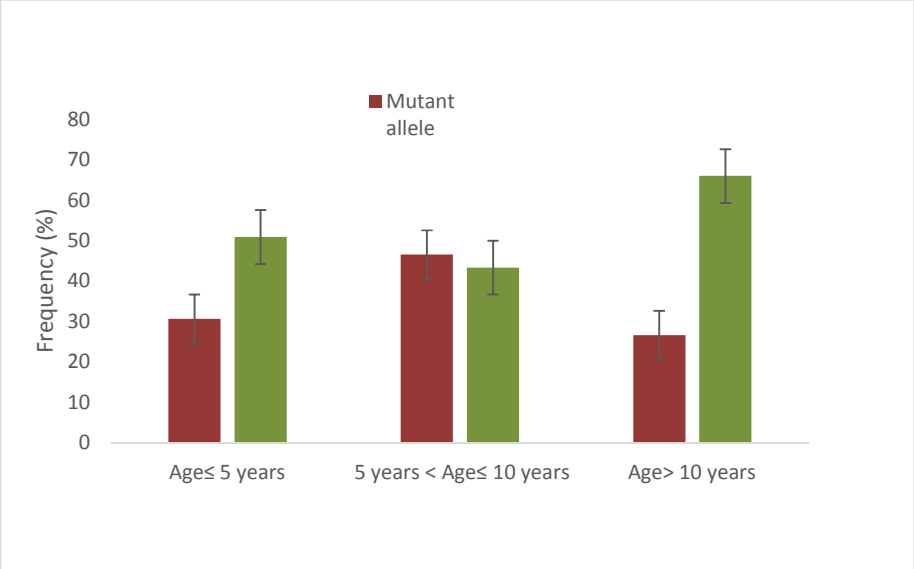

Fig. 4. Distribution of mutant and wild alleles according to age

Table 3. Distribution different allele's *Pfcrt* according to Parasitaemia.

Parasitaemia (parasites /µl)	<5000 n (%)	[5000-10000] n (%)	≥10000 n (%)	P
Mutant alleles	16 (39,02)	4 (30,77)	41 (32,28)	0,81
Wild alleles	21(51,22)	7(53,85)	76 (59,87)	0,60
Mixed alleles	3 (7, 32)	2(15,38)	9 (7, 09)	0,52

4.4 Prevalence of *pfcrt*76T Mutation According to Study Areas

Our study showed that the *Pfcrt*76T mutation is a function of the study areas. Our results are different from those obtained by MAHAMAT et al., in (2008), in the West (Dandé) and North (Gourcy) with *Pfcrt*76T respectively 48.8% and 50% in Burkina Faso [16]. This could be explained by the difference in epidemiological setting but mainly by the effect of the ACTs on the chloroquine-resistant parasites as the previous studies were conducted very close to the introduction of those news treatments. One additional explanation could also be the sample size for those previous studies used to characterize the mutation of the *Pcrt*76T gene, 47 samples, lower than that in our study. Also, the areas of Banfora and Saponé originate from two neighbouring epidemiological setting facies (equatorial stratum and tropical stratum) compared to that of Gourcy and Dandé (equatorial stratum and Sahelian stratum). However, our results corroborate with those obtained by HASTINGS and MACKINNON in the United Kingdom, in 1998 [23] and those generated by MOLYNEUX et al., in 1999 [24].

The frequency of the *Pfcrt*76T mutation at Banfora and Saponé was 45.90% and 27.5%, respectively. It was previously argued that strong transmission may contribute to the emergence of resistant parasites strains in our study.

4.5 Influence of the Introduction of CTAs on the Prevalence of the Pfcrt76T Mutation

This study assessed the prevalence of the mutation associated with antimalarial resistance eight years after the introduction of CTAs in Burkina Faso. Indeed, the prevalence of *Pfcrt* 76T mutation before the introduction of CTAs was estimated at 60% in Burkina Faso [15]. With the studies conducted in 2008 and 2016, the overall results showed a prevalence of *Pfcrt*76T mutation at 50% [16]; 24.78% [17] respectively; This was a representative of a decline in *Pfcrt*76T mutation prevalence after a decade of the ACTs used in Burkina Faso as a proof of the efficacy against the chloroquine-resistant parasites [25]. Our study data are contributing as an additional confirmation of such reduction in Burkina Faso. However, the emergence of *Plasmodium falciparum* resistance to artemisinin derivatives as presented in Cambodia [7], consists of a serious complexity in the control and elimination of malaria in the world. The spread of parasite resistance from Asia to Africa has already been well described previously with chloroquine and Sulfadoxine Pyrimethamine [9]. Then, this decrease in the sensitivity of Plasmodium to CTAs could jeopardize the progress in the fight against malaria.

5. CONCLUSION

This study showed a decrease in the prevalence of *Pfcrt*76T mutation about a few years after the introduction of Artemisinin-based Therapeutic Combinations in Burkina Faso. However, even the trends in the reduction of the chloroquine-resistant parasite are effective and will result in the possible efficacy of chloroquine, the spread of the resistant parasite to artemisinin derivative may represent a very important threat for malaria treatment in Africa. Therefore, the monitoring of ACTs efficacy remains an important activity in Africa to delay the generalization of their resistance.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the personal efforts of the authors.

CONSENT

Written informed consent was obtained from participant and the parents or legal guardians of

all children participants before enrolment. All case of health concerns was treated free of charge according to the local standards of care.

ETHICAL APPROVAL

Risks associated with drawing blood include minimal discomfort and/or bruising. Infection, excess bleeding, clotting or fainting is also possible, although unlikely. A local certified phlebotomist will collect blood samples in the clinic following standard protocols of the CNRFP.

This study was approved by the Ethical Committee of the Ministry of Health of Burkina Faso.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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ANNEXE 1

Table 4. Primer sequences and amplification programme of *pfcrt*76T

Gene	PCR reaction	Primers and sequences	Programme of amplification
Pfcrt76T	PCR Nº1	P1: CCGTTAATAATAAATACACGCAG P2: CGGATGTTACAAAACTATAGTTACC	94°C x 3 min; (94°C x 30s; 56°C x 30s; 60°C x 1min) x 30; 60°Cx 3 min
	PCR Nº 2	D1: TGTGCTCATGTGTTTAAACTT D2: CAAAACTATAGTTACCAATTTTG	95°C x 5 min; (92°C x 25s; 65°C x 1 min) x25-30; 65°Cx 3 min48°C x 30s

ANNEXE 2

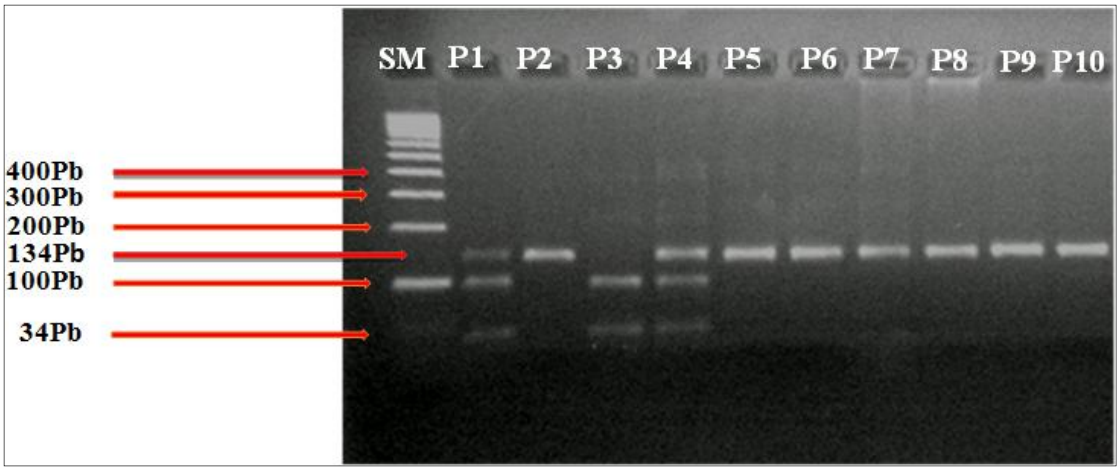

Fig. 5. Electrophoresis migration after amplification product digestion: Mutant and wild type *P. falciparum* detection

Standard Molecular Marker (SM), P2, P5…P9= pfcr76T Mutant allele, P3 = pfcrt76K Wild-type allele, P1 and P4 =Mixed infection (mutant and wild type strain) cases

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