

Decreased Prevalence of the *Plasmodium Falciparum* *Pfprt* K76T and *Pfmdr1* N86Y Mutations Post- Chloroquine Treatment Withdrawal in Katete District, Eastern Zambia

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Abstract

Background: In 2002, Zambia withdrew chloroquine as first line treatment for *Plasmodium falciparum* malaria due to increased treatment failure and world-wide spread of chloroquine resistance. The artemisinin combination regimen artemether-lumefantrine replaced chloroquine as first choice malaria treatment. The present study determined the prevalence of chloroquine resistance molecular markers in the malaria parasite *Pfcr* and *Pfmdr1* genes in Eastern Zambia at nine and thirteen years after the removal of drug pressure.

Methods: We assayed by polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) the prevalence of the genetic mutations, K76T on the *Pfcr* gene and N86Y on the *Pfmdr1* gene in samples collected from Katete District during drug therapeutic efficacy assessments conducted in 2012 and 2016.

Results: A total of 204 *P. falciparum* positive samples from 2012 and 2016 were further analysed for *Pfcr* K76T and *Pfmdr1* N86Y. 112 *P. falciparum* infected samples collected in 2012 were successfully amplified for *Pfcr* and *Pfmdr1*, while 69 (65.7%) and 72 (68.6%) samples from 2016 were successfully amplified for *Pfcr* and *Pfmdr1*. In 2012, the prevalence of *Pfcr* 76K was 97.3%, 76T was 1.8%, and 0.8% had both 76K and 76T codons. The prevalence of *Pfmdr1* 86N was 97.9% and 86Y was 2.1%. In the 2016 samples, the prevalence of the respective parasite genotypes was 100% *Pfcr* 76K and *Pfmdr1* 86N.

Conclusion: This study shows that there was a complete recovery of chloroquine-sensitive parasites by 2016 in Katete District, thirteen years following the withdrawal of CQ. These findings add to the body of evidence for a fitness cost in chloroquine-resistant *P. falciparum* in Zambia and elsewhere. Further studies are recommended to explore the feasibility of integration of the former best antimalarial in combination therapy in the future.

Background

Malaria continues to be endemic throughout Zambia and thus still remains a significant public health concern. Over the past two decades, however, significant strides have been made in reducing overall malaria mortality and morbidity in the country. Multiple successes such as a 50% reduction in malaria cases and deaths between 2000 and 2015 have been observed, largely due to scale-up of vector-control interventions and malaria treatment as recommended by the World Health Organization (WHO) [1]. Additionally, the incidence of malaria in Zambia decreased to 335 cases per 100 population in 2015 from 407 cases per 1000 population as recorded in the previous year [2]. Based on these recent successes, the National Malaria Elimination Programme pledged to eliminate malaria in Zambia through the continued use of these effective vector control interventions, in combination with sustained and prompt case management, health promotion, and surveillance [3, 4]. However, development of drug resistance in the *de facto* malaria parasite in Zambia, *Plasmodium falciparum*, continues to threaten these successes. This was evidenced in the country, where anti-malarial medicine resistance developed to chloroquine and sulphadoxine-pyrimethamine [5]. Following recommendations set forth by the WHO after widespread treatment failures and the spread of CQ resistance were observed, Zambia withdrew Chloroquine (CQ) as the first-line drug for uncomplicated *falciparum* malaria and replaced it with artemether-lumefantrine (AL), an artemisinin-based combination therapy (ACT) regimen in 2002 [6]. AL is recommended for the treatment of uncomplicated cases as it results in the rapid reduction of malaria parasite load [7].

Early studies have suggested that a single mutation which results in the replacement of lysine (K) by threonine (T) at amino acid codon position 76 of the *Plasmodium falciparum* chloroquine resistant transporter (*Pfcr*) gene (chromosome 7), is responsible for chloroquine resistance [8]. This K76T mutation was established as the most useful prognostic marker for treatment failure [9]. Moreover, this point mutation has been linked to chloroquine resistance in isolates collected worldwide. Furthermore, there is another point mutation N86Y of the *P. falciparum* multi drug resistant gene 1 (*Pfmdr1*) located on chromosome 5 that also appears to play a role in CQ resistance [8]. Some studies have shown that there is an association between the *Pfcr* K76T mutation and the mutation on amino acid codon 86 of the *Pfmdr1* gene in chloroquine resistance, although others refute the association [10, 11].

CQ efficacy is thought to lie in its ability to interrupt haematin detoxification in malaria parasites as they grow in their host blood cells. Specifically, CQ acts against the trophozoite and schizont stages of the *P. falciparum* parasites. Haematin is released in large amounts as the parasite consumes and digests haemoglobin in its digestive food vacuole. Haematin normally is detoxified by polymerisation into innocuous crystals of heamozoin [12]. CQ does not allow for the proper detoxification of haematin by forming a drug: haematin complex. The disruption of the haematin detoxification process by quinolines has destructive consequences for the parasites [12].

In recent years, most malaria endemic countries including Zambia, have reported the re-emergence of chloroquine susceptible parasites in regions where there has been a sustained withdrawal of chloroquine, which has mostly resulted from the re-expansion of the wild type after the removal of drug pressure [13–18]. Thus, the present study was conducted to ascertain the prevalence of the *Pfcr* K76T and *Pfmdr1* N86Y mutations in *P. falciparum* parasites in Katete District, in Eastern Province, Zambia after the withdrawal of chloroquine as first line treatment for uncomplicated malaria.

Methods

Study site and Design

This study utilized samples from a larger cross-sectional study conducted on samples collected during the routine artemether-lumefantrine therapeutic efficacy studies (TES) completed between April-June 2012 and 2016 in Katete District, Eastern Zambia [19]. TES is an *in vivo* routine study conducted bi-annually in different selected study sites in Zambia to assess the efficacy of first line treatment drugs that are in use for treatment of malaria. The current work was conducted on purposively selected day-0 or pre-TES enrolment of 2012 and 2016 at a primary health facility in Katete District. The samples were collected from the routine clinic visits. All clients with a positive blood smear and/or *P. falciparum* positive rapid diagnostic test (RDT) were included in the study, yielding a total of 241 for further analysis.

DNA Extraction

P. falciparum DNA was extracted from air-dried blood spots using the chelex method. Briefly, three 3 mm-sized punches were incubated in 1 ml 1% saponin in phosphate buffered saline (PBS) at room temperature for 10 minutes. The samples were then centrifuged at 14,000 rpm for 2 minutes and the supernatant was discarded. A 1 ml volume of (PBS) was added and the samples were centrifuged at 14,000 RPM for another 2 minutes again discarding the supernatant. A volume of 150 µl nuclease free water and 50 µl of 20 % chelex suspension in nuclease-free water were then added to the filter paper and boiled for 10 minutes. Finally, samples were centrifuged at 14,000 RPM for 1 minute and 100 µl of the supernatant was stored at -20°C until utilised for PCR.

Amplification and Detection of the Pfprt K76T SNP

To amplify amino acid codon 76 of the *Pfprt* gene, a nested PCR was performed according to Djimde et al. using flanking primers (CRTP1 5'-CCGTTAATAATAATACACGCAG-3 and CRTP2 5'-CGGATGTTACAAAACCTATAGTTACC-3) as the first round primers which span 537 base pairs of the *Pfprt* genes [8]. In summary, the PCR 25µl reaction contained 12.5µl of 2X PCR master mix (0.05u/µl Taq polymerase, reaction buffer, 4mM MgCl₂ and 0.4mM of each dNTP) from Thermo Fisher Scientific (Waltham, MA) 0.2mM of each primer, and 4µl of chelex extracted DNA. The amplification was performed under the following conditions: a 3 minute initial denaturation step at 94°C, followed by 45 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 65°C for 1 minute, and lastly a single 3 minute extension step at 65°C. This was followed by a nested round using internal primers (CRTD1 5'-TGTGCTCATGTGTTAACTT-3' and CRTD2 5'-CAAACTATAGTTACCAATTTG-3) in a 25µl reaction, the reaction contained 12.5 µl Thermo scientific PCR master mix, 4µl of the template from the primary reaction was used as template, The PCR conditions for nested reaction were, a five minute initial denaturation at 95°C, then followed by 30 cycles of 92°C for 30 seconds, 48°C for 30 seconds, and 65°C for 30 seconds and finally a single 3 minute final extension step 65°C. Dd2 and 3D7 laboratory strains were used as controls for chloroquine resistance and chloroquine sensitivity, respectively. Digestion was performed using the enzyme *Apo I* (New England Biolabs, Ipswich, MA). A 15µl reaction was prepared containing 2.5 units of *Apo I* restriction enzyme and 8µl of the nested product, the reaction was incubated at 37°C for 12 hours. The enzyme recognizes and cuts the 145bp product form CRTD1/CRTD2 containing 76K codon but does not cut the products containing codon 76T found in chloroquine resistant parasites.

Amplification and detection of the Pfmdr1 N86Y SNP

To amplify amino acid codon 86 of the *Pfmdr1* gene, a nested PCR was performed using flanking primers MDR1 5'-ATGGGTAAGAGCAGAAAGA-3' and MDR2 5'-AACGCAAGTAATACATAAAGTCA-3' as the first round primers which span a 537 base pair fragment [20, 21]. The Taq PCR Kit NEB E5000s (New England Biolabs, Ipswich, MA) (0.02 U Taq polymerase, reaction buffer, 1mM MgCl₂ and 0.4mM of each dNTP), 0.2mM of each primer, 2µl of chelex extracted DNA. The amplification was performed under the following conditions: a 3 minute initial denaturation step at 94°C, followed by 35 cycles of 94°C for 20 seconds, 49°C for 25 seconds and 60°C for 45 seconds and finally a single 5 minute final extension step at 60°C. This was followed by a nested round using an internal MDR3 5'-TGGTAACCTCAGTATCAAAGAA-3 and flanking primer MDR 4 5'-ATAAACCTAAAAGGAACTGG-3' in a 25µl reaction. Dd2 and 3D7 laboratory strains were again used as controls for chloroquine resistance and chloroquine sensitivity, respectively. A 15µl reaction was prepared containing 2.5 units of *Afl III* (New England Biolabs, Ipswich, MA) restriction enzyme and 8µl of the nested product, the reaction was incubated at 37°C for 12 hours. The enzyme recognizes and cuts the 521bp nested PCR product containing the N86Y mutation found in chloroquine resistant parasites.

Results

A total of 241 slide-positive and slide-negative samples of *P. falciparum* were processed from Katete District, Zambia. Among the 2012 samples processed by PCR, 112 (82.4%) and 94 (69.1%) isolates were successfully amplified for *Pfprt* and *Pfmdr1*, respectively. Among the 2016 isolates, 69 (65.7%) and 72 (68.6%) were successfully amplified for *Pfprt* and *Pfmdr1* respectively.

Baseline Characteristics of the study population

Table 1
Baseline Characteristics of the study population from Katete in 2012 and 2016.

	Year 2012	Year 2016
Individuals screened	Males	Males
	57 (50.4%)	56 (49.6%)
	Females	Females
	79 (61.7%)	49 (38.2%)
Median Age (in years)	4.4	7.0
Mean Temperatures (°C)	37.7	38.2
Slide positivity	99 (48.5%)	105 (51.5%)
Mean Parasitaemia (Parasites/µl)	31813	64946

Table 1 shows the demographic characteristics of the study participants associated with these samples collected in 2012 and 2016. In total, there were 113 males (46.9%) and 128 (53.1%) females screened, with 136 individuals in 2012 and 105 individuals in 2016. The median age was 4.4 in 2012 and 7.0 in 2016.

Prevalence of chloroquine resistance

Overall, in 2012 the prevalence of *Pfcr* 76K wildtype was 97.3% (109/112), followed by 1.8% for 76T mutant (2/112), and 0.9% for K76T (1/112) mixed, which contains both the sensitive and resistant markers (Fig. 1A). The prevalence of *Pfcr* 76K wildtype increased to 100% in among isolates included from 2016 (69/69), with complete removal of the mutant and mixed resistance mutations. For the *Pfmdr1* gene, the prevalence of 86N wildtype was 97.9% (92/94) and of 86Y mutant was 2.1% (2/94) (Fig. 1B). Like was the case for *Pfcr*, the prevalence of the wildtype *Pfmdr1* 86N increased to 100% (72/72) among included samples from 2016, with the complete reduction in the prevalence of marker associated with chloroquine resistance.

Discussion

The data from the current study show a decrease in the prevalence of molecular markers associated with chloroquine resistance in Katete District, Eastern Zambia between 2012 and 2016, or nine and thirteen years post-chloroquine treatment withdrawal. Specifically, the prevalence of *Pfcr* 76T and *Pfmdr1* 86Y both decreased from 1.8% and 2.1%, respectively, in 2012 to 0% in 2016. These data are consistent with the results of other studies conducted in different locations within Zambia [18, 22]. Additionally, these data further suggest that the withdrawal of antimalarial drug pressure from the parasite population seemed to have resulted in the re-expansion of the wild type parasites carrying the K76 and N86 residues on the *Pfcr* and *Pfmdr1* genes, respectively. This observed recovery of sensitive strains seems to have occurred over a long period of time as seen in the prevalence of resistant strains in the isolates collected in 2012.

Malawi, Zambia, and Zimbabwe are three African countries that have reported 100% prevalence of wild type codon K76 carrying *P. falciparum* parasites after official cessation of chloroquine use but also general trend towards restoration of chloroquine sensitivity has been reported for the continent [3, 23, 24]. Malawi was the first country to report the return of chloroquine-susceptible *P. falciparum* parasites nine years after the withdrawal of chloroquine as treatment [14]. The return of chloroquine-susceptible parasites must have resulted from the re-expansion of chloroquine-sensitive parasites that had a survival fitness advantage over the CQ-resistant strains [25].

Kenya reported that the frequency of the *Pfcr* K76T mutation had decreased from 95–23% two decades post-chloroquine treatment withdrawal [26]. In Rwanda, the prevalence of wild type *Pfcr* K76 was reported to be at 50% around 14 years after cessation of CQ use while Tanzania reported a similar K76 allelic prevalence between 85.5–93% ten years after the discontinuation of chloroquine from treatment guidelines with regional variabilities [27, 28].

The trends in the re-emergence of chloroquine-susceptible genotypes seen in Zambia, Malawi, and Zimbabwe can be attributed to the fact that chloroquine was completely withdrawn as treatment for uncomplicated *falciparum* malaria. The replacement for chloroquine, AL, has a different mode of action on the parasite and selects for chloroquine-sensitive *P. falciparum* [29, 30]. Furthermore, there was no chloroquine drug pressure on the *P. falciparum* population which allowed the more fit CQ sensitive parasites to thrive. This is not the case in Southeast Asia and South America, where the parasites carrying the mutation have become fixed in the population due to continued use of chloroquine in the treatment of *vivax* malaria [23, 31].

Malaria epidemiology may also have contributed to the re-emergence of chloroquine-susceptible *P. falciparum*. Malaria transmission intensity, which impacts both human host immunity and the rate of parasite recombination in the arthropod vector, contributes to the spread of *Plasmodium* parasite. Different models have shown that high malaria transmission intensity results in high host immunity due to repeated exposure to malaria infections. Additionally, there is a high rate of parasite recombination in high transmission areas when compared to low transmission areas due to multiplicity of infections. Eastern Province was classified as a high malaria transmission area at the time when the samples were collected. Therefore, the chloroquine-sensitive parasites which already have a high fitness advantage over the resistance parasites will quickly increase in the population once the drug pressure is removed. In low transmission areas, however, there are usually unique parasite population characteristics, and each individual receives one infectious bite with a single genotype. In such cases, this single genotype is taken up in the blood meal by an *Anopheles* mosquito. Consequently, during the sexual reproductive stage of the parasite in the mosquito midgut, there is little opportunity for genetic recombination resulting in a single fixed genotype in the parasite population [32].

This study had limitations and the results should be interpreted with caution, keeping in mind the following reasons; Firstly, the sample size for 2016 was smaller than that from 2012, which may have introduced a selection bias. Therefore, more studies should be conducted with a larger sample size to acquire more accurate estimates of prevalence. Secondly, this study was conducted in one province in Eastern Zambia, so the results cannot be generalized to the whole country because of differences in epidemiologic patterns in the countries. As such, prevalence cannot be compared between provinces, so further studies should examine potential differences between areas of low and high transmission status. Finally, not all of the samples that were considered positive by microscopy were also amplified by PCR, so there was a possibility of amplification bias, which could lead to an under- or over-estimation of the prevalence.

Conclusion

The withdrawal of chloroquine from use as first line drug has resulted in the recovery of chloroquine sensitive parasite in Katete District, Zambia. The results obtained from this study agree with previously published data showing the recovery of chloroquine sensitive strains in the parasite population. Additionally, this study suggests that routine national surveillance of resistance markers should be regularly implemented at larger levels as the country aims to achieve malaria elimination.

Abbreviations

CQ: chloroquine; ACT: artemisinin-based combination therapy; AL: artemether-lumefantrine; PBS: phosphate buffered saline; PCR: polymerase chain reaction; *Pfcr1*: *Plasmodium falciparum* chloroquine resistant transporter gene; *Pfmdr1*: *P. falciparum* multi drug resistant gene 1; RFLP: restriction fragment length polymorphism; RDT: rapid diagnostic test; TES: therapeutic efficacy studies; WHO: World Health Organization

Declarations

Author contributions

SM and MCM conceived the study idea. MCM and LS processed the samples. MCM and IIC drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets acquired and analysed for this study will be available by request from the corresponding author.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

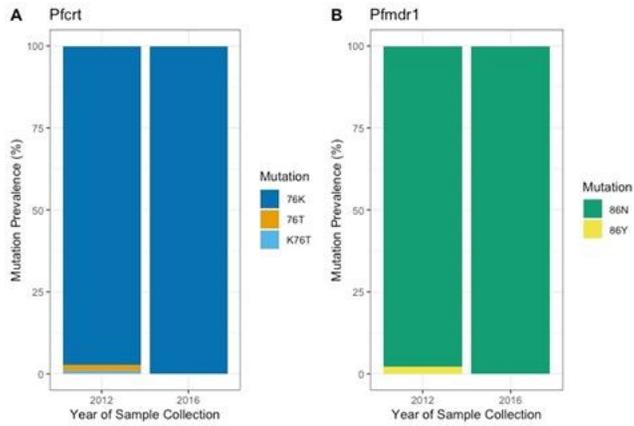


Figure 1

Prevalence of chloroquine resistance among collected isolates. A) Prevalence of *PfCRT* (K76T) mutations in 2012 and 2016 among samples included in study. B) Prevalence of *PfMDR1* (N86Y) mutations in 2012 and 2016 for samples included in analysis.