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

Mulenga Chilumba Mwenda (mchmwenda_c@yahoo.com)
Program for Appropriate Technology in Health: PATH  https://orcid.org/0000-0002-9962-7027

Lungowe Sitali
University of Zambia

Ilincia Ciubotariu
Purdue University

Moonga B Hawela
Zambia: Ministry of Health

Busiku Hamainza
Zambia: Ministry of Health

Sungano Mharakurwa
Africa University Faculty of Agriculture and Natural Resources

Research

Keywords: Chloroquine resistance, Chloroquine sensitivity, malaria, mutation, genotypes

DOI: https://doi.org/10.21203/rs.3.rs-388401/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

**Background:** In 2002, Zambia withdrew chloroquine as first line treatment for *Plasmodium falciparum* malaria due to increased treatment failure and worldwide 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 haemozoin [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.

Amplication and Detection of the *Pfcrt* K76T SNP

To amplify amino acid codon 76 of the *Pfcrt* gene, a nested PCR was performed according to Djimde et al. using anking primers (CRTP1 5'-CCGTTAATAAACTAGCGC-3 and CRTP2 5'-CGGATGTTAACAATAGTTACC-3) as the first round primers which span 537 base pairs of the *Pfcrt* genes [8]. In summary, the PCR 25µl reaction contained 12.5µl of 2X PCR master mix (0.05u/µl Taq polymerase, reaction buffer, 4mM MgCl$_2$ and 0.4mM of each dNTP) from Thermo Fisher Scientific (Waltham, MA) 0.2mM of each primer, and 4µl of chelex extracted DNA. The amplication 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'-TGTGCTCATGTGTTTAAACTT-3' and CRTD2 5'-CAAACTATAGTTACCAATTTTG-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.

Amplication and detection of the *Pfmdr1* N86Y SNP

To amplify amino acid codon 86 of the *Pfmdr1* gene, a nested PCR was performed using anking primers MDR1 5'-ATGGGTAAAGAGCAGAAAGA-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 MgCl$_2$ and 0.4mM of each dNTP), 0.2mM of each primer, 2µl of chelex extracted DNA. The amplication 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 anking primer MDR 4 5'-ATAAACCTAAAAAGGAACTGG-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 *A III* 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 product form CRTD1/CRTD2 containing 86Y 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 *Pfcrt* and *Pfmdr1*, respectively. Among the 2016 isolates, 69 (65.7%) and 72 (68.6%) were successfully amplified for *Pfcrt* 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** | **Individuals screened** |
| Males | Males |
| 57 (50.4%) | 56 (49.6%) |
| Females | Females |
| 79 (61.7%) | 49 (38.2%) |
| **Median Age (in years)** | **Median Age (in years)** |
| 4.4 | 7.0 |
| **Mean Temperatures (°C)** | **Mean Temperatures (°C)** |
| 37.7 | 38.2 |
| **Slide positivity** | **Slide positivity** |
| 99 (48.5%) | 105 (51.5%) |
| **Mean Parasitaemia (Parasites/µl)** | **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 Pfcrt 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 Pfcrt 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 Pfcrt, 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 Pfcrt 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 Pfcrt 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 Pfcrt K76T mutation had decreased from 95–23% two decades post-chloroquine treatment withdrawal [26]. In Rwanda, the prevalence of wild type Pfcrt 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 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].

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; *Pfcr*. *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.

**Acknowledgements**

We wish to thank Eastern province (Katete District) office for supervision and facilitating the study. We would also like to thank the NMEC staff for their support.

**Funding**

This work was supported by SMUTH-MRU and the National Malaria Elimination Centre.

**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.

**Author details**

1. PATH Malaria Control and Elimination Partnership in Africa (MACEPA), National Malaria Elimination Centre, Ministry of Health, Chainama Grounds, Lusaka, Zambia
2. School of Health Sciences, Biomedical Sciences Department, Ridgeway campus, Lusaka, Zambia
3. School of Medicine and University Teaching Hospital Malaria Research Unit (SMUTH-MRU), Lusaka, Zambia
4. Purdue University, Department of Biological Sciences, West Lafayette, IN, USA
5. Malaria Elimination Centre, Ministry of Health, Chainama Hospital and College Grounds, Lusaka, Zambia
6. College of Health, Agriculture and Natural Sciences, Africa University, Mutare, Zimbabwe

**References**

1. Kamuliwo M, Chanda E, Haque U, Mwanza-Ingwe M, Sikaala C, Katebe-Sakala C, Mukonka VM, Norris DE, Smith DL, Glass GE, Moss WJ. The changing burden of malaria and association with vector control interventions in Zambia using district-level surveillance data, 2006–2011. Malar J. 2013;12:437.
2. Inambao ABKR, Hamainza B, Makasa M, Nielsen CF. Malaria incidence in Zambia, 2013 to 2015: observations from the health management information system. Health Press Zambia Bull. 2017;1(3):11–21.
3. Ministry of Health. National Malaria Control Programme Strategic Plan For FY 2011–2015. Lusaka: National Malaria Control Programme; 2011. https://extranet.who.int/countryplanningcycles/sites/default/files/planning_cycle_repository/zambia/zambia_malaria_nsp_2011-2015_.pdf.
4. Ministry of Health. National Malaria Elimination Strategic Plan 2017–2021. Lusaka: National Malaria Elimination Centre; 2017. https://static1.squarespace.com/static/58d002f017b9f399fe21889/t/5b28d7f1575d1ff0942d BCE1/1529403401067/National+Malaria+Elimination+Str Final_PRINT.pdf.
5. Masaininga F, Chanda E, Chanda-Kapata P, Hamainza B, Masendu HT, Kamuliwo M, Kapelwa W, Chimumbwa J, Govere J, Otten M, et al. Review of the malaria epidemiology and trends in Zambia. Asian Pac J Trop Biomed. 2013;3:89–94.

6. Siplianyambne N, Simon JL, Chanda P, Olumese P, Snow RW, Hamer DH. From chloroquine to artemether-lumefantrine: the process of drug policy change in Zambia. Malar J. 2008;7:25.

7. WHO. Guidelines for the Treatment of Malaria. Geneva: Roll Back Malaria, World Health Organization; 2006.

8. Djimdé A, Douombo OK, Cortese JF, Kayentao K, Douombo S, Diourté Y, Coulibaly D, Dicko A, Su XZ, Nomura T, et al. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med. 2001;344:257–63.

9. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. Nature. 2000;403:906–9.

10. Setthavadom C, Tan-ariya P, Sithitchot N, Khositnithikul R, Suwandittakul N, Leelayoova S, Munthgin M. Role of Plasmodium falciparum chloroquine resistance transporter and multidrug resistance 1 genes on in vitro chloroquine resistance in isolates of Plasmodium falciparum from Thailand. Am J Trop Med Hyg. 2011;85:606–11.

11. Zakeri S, Afsharbad M, Kazemzadeh T, Meh dizadeh K, Shaban K, Djadid ND. Association of pfcrt but not pfmdr1 alleles with chloroquine resistance in Iranian isolates of Plasmodium falciparum. Am J Trop Med Hyg. 2008;78:633–40.

12. Dom A, Vippagunta SR, Matile H, Jaquet C, Vennerstrom JL, Ridley RG. An assessment of drug-haematin binding as a mechanism for inhibition of haematin polymerisation by quinoline antimalarials. Biochem Pharmacol. 1998;55:727–36.

13. Laufer MK, Thesing PC, Eddington ND, Masonga R, Dzinjalama FK, Takala SL, Taylor TE, Plowe CV. Return of chloroquine antimalarial efficacy in Malawi. N Engl J Med. 2006;355:1959–66.

14. Kublin JG, Cortese JF, Njunju EM, Mukadam RA, Wirima JJ, Kazembe PN, Djimdé AA, Kouriba B, Taylor TE, Plowe CV. Reemergence of chloroquine-sensitive Plasmodium falciparum malaria after cessation of chloroquine use in Malawi. J Infect Dis. 2003;187:1870–5.

15. Kiarie WC, Wangai L, Agola E, Kimani FT, Hungu C. Chloroquine sensitivity: diminished prevalence of chloroquine-resistant gene marker pfcr7-76 13 years after cessation of chloroquine use in Msambweni, Kenya. Malar J. 2015;14:328.

16. Mekonnen SK, Aseffa A, Berhe N, Teklehaymanot T, Clouse RM, Gebru T, Medhin G, Velavan TP. Return of chloroquine-sensitive Plasmodium falciparum falciparum parasites and emergence of chloroquine-resistant Plasmodium falciparum vivax in Ethiopia. Malar J. 2014;13:244.

17. Bogreua H, Renaud P, Bouchiba H, Durand P, Assi SB, Henry MC, Gamotel E, Pradines B, Fusi A, Wode B, et al. Genetic diversity and structure of African Plasmodium falciparum populations in urban and rural areas. Am J Trop Med Hyg. 2006;74:953–9.

18. Mwanza S, Joshi S, Nambizi M, Chileshe J, Malunga P, Kabuya J-BB, Hachizov S, Manyando C, Mulenga M, Laufer M. The return of chloroquine-susceptible Plasmodium falciparum malaria in Zambia. Malar J. 2016;15:584.

19. Hamainza B, Masaininga F, Moonga H, Mwenda M, Chanda-kapata P, Chalwe V, Chanda E, Kamuliwo M, Babaniyi OA. Therapeutic efficacy of artemether-lumefantrine on treatment of uncomplicated Plasmodium falciparum mono-infection in an area of high malaria transmission in Zambia. Malar J. 2014;13:430–0.

20. Wang X, Mu J, Li, G, Chen P, Guo X, Fu L, Chen L, Su X, Wellems TE. Decreased prevalence of the Plasmodium falciparum chloroquine resistance transporter 76T marker associated with cessation of chloroquine use against Plasmodium falciparum malaria in Hainan, People's Republic of China. Am J Trop Med Hyg. 2005;72:410–4.

21. Antony HA, Das S, Parija SC, Padhi S. Sequence analysis of pfcrt and pfmdr1 genes and its association with chloroquine resistance in Southeast Indian Plasmodium falciparum isolates. Genom Data. 2016;8:85–90.

22. Sitali L, Mwenda MC, Miller JM, Bridges DJ, Hawela MB, Chizema-Kawesha E, Chipeta J, Lindtjorn B. En-route to the 'elimination' of genotypic chloroquine resistance in Western and Southern Zambia, 14 years after chloroquine withdrawal. Malar J. 2019;18:391.

23. Kublin JG, Cortese JF, Kayentao K, Doumbo OK, Eyong K, Misere P, Nomura T, et al. A molecular marker for chloroquine-resistant Plasmodium falciparum malaria. N Engl J Med. 2001;344:257–63.
31. Alam MS, Ley B, Nima MK, Johora FT, Hossain ME, Thriemer K, Auburn S, Marfurt J, Price RN, Khan WA. Molecular analysis demonstrates high prevalence of chloroquine resistance but no evidence of artemisinin resistance in Plasmodium falciparum in the Chittagong Hill Tracts of Bangladesh. Malar J. 2017;16:335.

32. Takala-Harrison S, Laufer MK. Antimalarial drug resistance in Africa: key lessons for the future. Ann N Y Acad Sci. 2015;1342:62–7.

Figures

**Figure 1**

Prevalence of chloroquine resistance among collected isolates. A) Prevalence of Pfcr (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.