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## Molecular marker of *Plasmodium falciparum* resistance to chloroquine (*Pfcr*) in an area with long history of antimalarial resistance

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### Abstract

#### Background

High levels *Plasmodium falciparum* resistance to Chloroquine (CQ) compelled Tanzania to replace CQ with Sphadoxine-pyrimethamine (SP) as first-line antimalarial in 2001 which was however replaced with Artemether Lumefantrine (AL) in 2006. Studies in Malawi have shown sufficient recovery of CQ-sensitivity after its withdrawal warranting re-using CQ in combination with other antimalarials in the future. This paper assessed the level of CQ resistance at molecular level in an area with long history of antimalarial resistance in North-eastern Tanzania.

#### Materials and Methods

Samples were obtained from patients recruited in a clinical trial to assess *in vivo* efficacy of AL at Mkuzi health centre in Muheza district, North-eastern Tanzania. DNA was extracted from venous blood using Qiagen extraction midi kit. The samples were analyzed for single nucleotide polymorphisms (SNPs) in the *P. falciparum* CQ resistance transporter gene (*Pfcr*; codons 72–76) using polymerase chain reaction (PCR) and sequence-specific oligonucleotide probe (SSOP) enzymelinked immunosorbent assay (ELISA). Prevalence of *Pfcr* haplotypes before and after treatment samples was compared.

## Results

A total of 104 microscopically positive samples were genotyped for the *Pfcr*t haplotypes. Of these, 78 (75%) samples contained wild-type (CVMNK) haplotype, 21 (20.2%) contained resistant (CVIET) haplotype while 5 (4.8%) samples had mixed (CVMNK/CVIET) infections. There were no SVMNT haplotype among the samples. The prevalence of the *Pfcr*t wild-type CVMNK haplotype was high in the study area reaching over 76%. No significant selection of the *Pfcr*t wild-type CVMNK haplotype after treatment with AL was observed ( $p > 0.05$ ).

## Conclusions

Compared to the previous studies in the study area, the prevalence of CQ sensitive parasites has increased in the study area. However the rate of sensitivity restoration in this study site with long history of antimalarial drug resistance was slower than rates reported from other parts of Tanzania. These findings suggest complete CQ sensitivity restoration and hence re-introduction of CQ (e.g. in a drug combination) in the study area will most likely take longer than previously anticipated.

**Key words:** *Plasmodium falciparum*, Malaria, *Pfcr*t, Chloroquine, Drug resistance, CVMNK-haplotype, CVIET-haplotype

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## Introduction

Control of malaria depends greatly on prompt diagnosis and treatment using effective antimalarial drugs, besides use of insecticide impregnated bed-nets and other preventive measures [1, 2]. However, *Plasmodium falciparum*; the main causative agent of malaria in Sub Saharan Africa has developed significant resistance to the common and affordable drugs monotherapies such as chloroquine (CQ) and sulphadoxine/pyrimethamine (SP) [3] thus posing a great challenge in malaria control efforts.

For more than three decades, CQ had been a cheap and effective antimalarial drug particularly in Africa and raised hopes to eradicate malaria [4]. However, the hopes were shattered by the emergence of chloroquine resistance (CQR) in the late 1960s which spread on the African continent leading to high level of treatment failures and increased morbidity and mortality associated with malaria [5]. CQR was first reported in Tanzania in early 1980s [6] and by the end of 1990s, CQ was replaced with SP together with amodiaquine (AQ) (as second line drug) in many African countries. SP was used in large-scale before it was also withdrawn in the mid 2000s due to widespread resistance which was associated with high treatment failures. SP and other antimalarial monotherapies have now been replaced with artemisinin combination therapy (ACT) in nearly all malaria endemic countries [7].

In 2001, Tanzania replaced CQ with SP as first line treatment and AQ as second line for the treatment of uncomplicated malaria. This change was an interim strategy due to high rates of CQ treatment failures and increased malaria mortalities particularly among children [8]. In 2006 following the World Health Organisation (WHO) guidelines [9] suggesting the use of ACT for treating uncomplicated malaria, Tanzania replaced SP with Artemether lumefantrine (AL) as first line therapy against uncomplicated malaria [10].

The usefulness of molecular markers of drug resistance in monitoring drug resistance is based on the detection of single nucleotide polymorphisms (SNPs) in target genes of *P. falciparum* which have been confirmed to be linked to *in-vivo* and *in-vitro* parasite resistance to a given drug. CQR has been linked to mutations in the *P. falciparum* chloroquine resistance transporter (*Pfcr*) gene [11, 12] and mutations in the *P. falciparum* multidrug resistance *Pfmdr1* gene [13]. However, the most important determinant of CQR is the mutation that cause lysine to threonine amino acid substitution in the wild-type (CVMNK) at codons 72-76 of the *Pfcr* gene leading to several resistant haplotypes, the most common being CVIET which predominates in Africa and SVMNT which occurs in Asia but rarely in Africa [14, 15]. CVIET haplotype is thus regarded as a suitable molecular marker to monitor CQR in Africa.

Previous studies that assessed *P. falciparum* drug resistance at molecular level in Tanzania have shown that withdrawal of CQ resulted in increased wild-type *Pfcr* (CVMK) haplotype frequency in Tanzania [16, 17] indicating restoration of CQ sensitivity as previously reported in Malawi [18, 19]. In addition, treatment with AL has been associated with selection of CQ sensitive parasites (at codon 76K) which results into increased level of CQ sensitivity, though possibly leading to increased tolerance to ACT [20].

The present study was conducted to assess the prevalence and frequency of parasite genotypes in the *Pfcr* and hence the restoration of CQ sensitivity in Muheza district. The study area has historically recorded among the highest levels of parasite drug resistance associated with treatment failures to CQ in Tanzania and Africa at large [21].

## Materials and Methods

### Study design and data collection

Samples were obtained from an open label non randomised clinical trial carried to assess the efficacy and safety of Artemether Lumefantrine (AL) at Mkuzi health centre, Muheza district, North-eastern Tanzania. This area has previously recorded high levels of antimalarial drug resistance in Tanzania; including CQ [22, 23], AQ [24], pyrimethamine [25], and SP [26-29].

Briefly, 88 children aged 6 months to 10 years with confirmed uncomplicated falciparum malaria were recruited, treated under direct observation and followed up for 28 days. Venous blood was collected depending on age ( $\leq 5$ ml for under fives and up to 7ml for those aged  $\geq 5$  years) in EDTA vacutainer for parasite genotyping and molecular analysis on the day of enrolment and the day of failure (late clinical failure or late parasitological failure). Parasite DNA was extracted from whole blood samples using QIAamp DNA blood midi kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracted parasite DNA was stored at  $-20^{\circ}\text{C}$  before analysis. All specimens were labelled anonymously using patients study number together with the code of study site and the date of enrolment. The results for the efficacy and safety of AL have been presented elsewhere [30].

### Polymerase chain reaction

Forward and reverse primers (Eurofins MWG Operon, Inc) were used to amplify the chloroquine resistance transporter gene, with a biotin modification of the reverse primer at the 5' end. Each 20  $\mu\text{L}$  outer PCR reaction consisted of 2 $\mu\text{l}$  of DNA template in a master-mixture containing 2 $\mu\text{l}$  of double distilled PCR water (ddH<sub>2</sub>O), 0.8  $\mu\text{l}$  25mM MgCl (Qiagen), 2 $\mu\text{l}$  of forward and reverse (P1 and P2) primers (5 $\mu\text{M}$ ), and 10 $\mu\text{l}$  of tempase hot

start master mix. Reaction conditions were 94°C for 15 minutes followed by 45 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for one minute, with a final extension at 72°C for eight minutes. Each 20 µL nested PCR reaction contained 1µl of outer PCR product, 4.2µl of ddH<sub>2</sub>O, 4µl of forward and reverse (TCRD1&2-Biotin) primers 5µM, and 10µl of tempase hot start master mix. Amplifications were performed in 96-well PCR plates using BIO RAD S1000 thermocycler<sup>TM</sup> (Singapore). Reaction conditions were 94°C for 15 minutes followed by 30 cycles of 92°C for 30 sec, 48°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for ten minutes. PCR products were confirmed by electrophoresis on a 1.5% agarose gel.

### **SSOP-ELISA to detect *Pfcr* haplotypes**

The method has been adopted from the work by Alifrangis *et al* [31]. Briefly, ELISA plates (NUNC<sup>TM</sup> - Immunoplate, Denmark) were coated with streptavidin in phosphate buffered saline (PBS) (1 g/mL), covered, and left overnight at 4°C. Prior to use, the plates were washed three times in washing buffer (1x PBS containing 0.05% Tween 20). The PCR products were diluted 1:5 in water in a 96-well PCR plate, denatured at 95°C for five minutes, and immediately cooled to 4°C until use. The 3'-end digoxigenin-conjugated SSOPs for the haplotypes of interest (CVMNK, CVIET or SVMNT) were diluted to a 4nM concentration in tetraethyl ammonium chloride (TMAC; Sigma Aldrich, Dorset, UK) solution heated to 53°C, and 100 µL was then added to each well of the ELISA plate. Two microlitres of the diluted PCR products were subsequently added. Replicate ELISA plates were made to enable simultaneous probing with SSOPs targeting all three *Pfcr* haplotypes. The plates were incubated in a hybridization oven (Thermo Electron, Hybaid 'n' Stack, USA) at 53°C on a shaking device for one hour and washed three times in washing buffer. This was followed by two rounds of washing and incubation (10 minutes per

round) in TMAC solution at 60°C. To remove TMAC, the plates were then washed three times in washing buffer. Peroxidase-conjugated anti-digoxigenin antibody in dilution buffer (1:1,000) (Roche Diagnostics, GmbH) was then added to each well and incubated for one hour at room temperature. The plates were then washed three times in washing buffer. 100µl solution of OPD tablets (Dako, Glostrup, Denmark) solution in ddH<sub>2</sub>O and 30% of H<sub>2</sub>O<sub>2</sub> was added (to each well) and incubated for 5-20 minutes to allow for the reaction to occur. The reaction was stopped by adding 0.5M H<sub>2</sub>SO<sub>4</sub> and the optical density (OD) values at 492nm were measured in an ELISA reader (VERSAmax™ tunable microplate reader, USA).

### **Ethical Considerations**

The study which donated samples for this analysis obtained ethical clearance from the National Medical Research Coordination Committee of the National Institute for Medical Research (NIMR-MRCC). Permission to conduct the study in Muheza district was sought in writing from the relevant regional and district medical authorities. Oral and written informed consent was obtained from parents or guardians of all patients before they were screened for possible inclusion into the study.

### **Data Analysis**

OD values were entered in a Microsoft Excel sheet and the haplotype of each positive sample was determined. Haplotype frequencies were calculated by excluding infections with mixed genotypes and comparisons were made between infections containing pure wild-type and mutant haplotype only, while prevalence of haplotypes included mutants, wild-type and mixed infections. Proportion test was used to compare the differences in prevalence of *Pfcr* haplotypes between the pre-treatment samples and post-treatment samples. Statistical

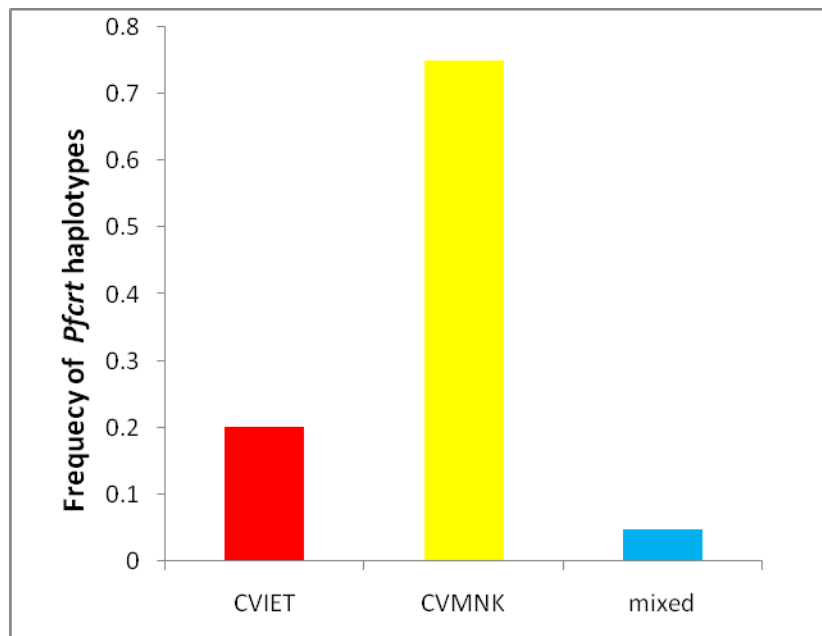


analyses were performed using Stata for Windows, version 11 (Stata Corporation, TX-USA). For all statistical tests, p-value was set at 0.05.

## Results

### Distribution of the SNPs in the *Pfcr* gene in the study site

A total of 104 microscopically positive samples were successfully genotyped for the *Pfcr* haplotypes (success rate 97.2%). Of these, 78 (75%) samples contained wild-type (CVMNK) haplotype, 21 (20.2%) contained resistant (CVIET) haplotype while 5 (4.8%) samples had mixed (CVMNK/CVIET) infections. There were no SVMNT haplotype among the samples (Fig. 1).

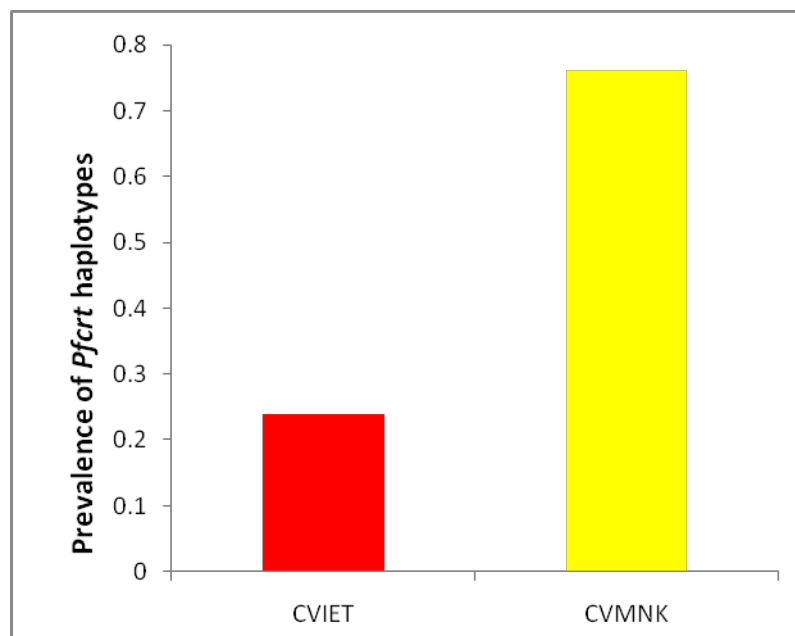


**Figure 1: Distribution of *Pfcr* haplotypes in the study site.**

CVMNK=haplotype containing wild-type genotypes at codons 72-76 of *Pfcr* and  
CVIET= haplotype with mutant genotypes at codons 72-76 of *Pfcr* gene  
Mixed= haplotype with co-infection of CVMNK/CVIET

### Prevalence and frequency of *Pfcr* wild-type parasites (haplotype; CVMNK) in the study site

By excluding the mixed infections, the frequency of *Pfcr* wild-type parasites (haplotype; CVMNK) was 78.8% and when the mixed (CVMNK/CVIET haplotype) infections were included, the calculated prevalence of *Pfcr* wild-type parasites (haplotype; CVMNK) was 76.1%. Conversely, the prevalence of mutant CVIET haplotype including mixed haplotype infections was 23.9% (Fig. 2).



**Figure 2: Prevalence of *Pfcr* CVIET and CVMNK haplotypes.**

CVMNK=haplotype containing wild-type genotypes at codons 72-76 of *Pfcr* and  
CVIET= haplotype with mutant genotypes at codons 72-76 of *Pfcr* gene

### Comparison of *Pfcr* mutations in pre-treatment and post-treatment samples

A total of 85 pre-treatment samples were successfully genotyped for mutations in the *Pfcr* gene (success rate 96.6%) and the prevalence of wild-type (CVMNK) haplotype was 80%. After treatment with AL, 19 samples were successfully genotyped on day 21 and 28 of which

15 (79 %) samples carried wild-type (CVMNK) haplotype. The difference in prevalence of the wild-type haplotype between the pre-treatment samples and post-treatment (re-infections) was not statistically significant (Table 1).

**Table 1: Prevalence of wild-type haplotype in pre-treatment and post-treatment samples**

	Wild-type (CVMNK)	Percentage	<i>p</i> -value
Pre-treatment (85)	68	80	
Post-treatment (19)	15	79	0.6557

## Discussion

*P. falciparum* resistance to antimalarials is one of the most important challenges to the malaria control efforts. Following widespread drug resistance to monotherapy antimalarials in all malaria endemic countries, Tanzania changed malaria treatment policy replacing CQ with SP in 2001 [8] and later SP with AL in 2006 [10] as first-line antimalarial. With the recent emergence of *P. falciparum* resistance to artemisinin in Great Mekong sub-region, and given the potential of spreading to other malarial endemic regions [32], alternative approaches for malaria control are imperative. While developing new drugs is a viable approach, the possibility of re-introducing withdrawn antimalarials such as CQ deserves considerable exploration. Withdraw of CQ from the health system in Malawi was accompanied by a significant a reduction in the prevalence of the CQR (*Pfcr*t T76 allele) from 85% to 13% in a period less than ten years [18].

Regular monitoring of efficacy of antimalarials as recommended by WHO [33] aims to

provide early warning of emergency of parasite tolerance/resistance to the drugs in use and to guide review of treatment policy in malaria endemic countries. Monitoring of prevalence of known molecular markers of resistance to drugs in use as well to withdrawn drugs facilitates an understanding of mechanisms of evolution of parasite resistance to antimalarials.

This study assessed the level of drug resistance at molecular level to CQ after several years withdrawal as first line antimalarials in Muheza; an area well renowned for having high levels of CQ drug resistance [21]. In comparison with previous studies in the study area [34], where the average frequency and prevalence of *Pfcr* wild-type in 2009 were 52% and 68.7% respectively, the findings from the present study have shown that parasite infections carrying CQ wild-type haplotypes have continued to increase reaching an overall prevalence of 76.1%. The prevalence of mutant allele in the study area was 54.8% in 2010 [34] compared to the present prevalence of 23.9%. This is in support of the fact that CQ resistant parasites are less fit than the wild-type parasites in the absence of CQ drug pressure [35]. These findings parallel to results in other studies in Tanzania [16] and neighbouring countries like Malawi [19] and Kenya [36] suggest CQ might regain full sensitivity and be re-introduced (in combination with other antimalarials). However, in general the restoration of CQ sensitivity in Kenya and Tanzania has been slower than in Malawi possibly due to introduction and continued use of AQ as second line drug along with SP during the policy changes which withdrew CQ. It is important to note that Muheza villages in North-eastern Tanzania are known to have recorded high levels of antimalarial drugs resistance in Tanzania and Africa at large [21]. High levels of resistance to antimalarials in Muheza villages in comparison to other parts of Tanzania could possibly be resulted from the participation of communities in these villages in various antimalarials clinical trials. During such trials, various antimalarial drugs including CQ, SP, and AQ [24, 37] were introduced in these communities leading to

high drug pressure which eventually resulted to high levels of drug resistance in the area. The prevalence of the mutant CVIET haplotype in Muheza vilages at the time of CQ withdrawal was high and almost fixed [24]. This observation may partly explain the difference in prevalence of wild-type CVMNK haplotype reported in this study compared to the country-wide average of 85.7-93.5% prevalence recently reported in a study from six representative regions of Tanzania [16].

Treatment with AL has been associated with selection of CQ sensitive parasites (at codon 76K) which may result into increased level of CQ sensitivity, though possibly leading to increased tolerance to ACT [20]. However, it is known that even before official adoption of AL, recovery of CQ sensitivity had already been reported in Malawi [18]. It is thus not known whether it is the use of AL, or withdraw of CQ or both factors acting in synergy that causes selection of CQ susceptible parasites. The present study showed no significant selection of *Pfcr*t wild-type haplotype between pre-treatment and post-treatment samples ( $p > 0.05$ ). This could probably be explained by the high prevalence of the wild-type CVMNK haplotype at baseline (76.1%) in the study area. Admittedly, this could also be attributed to the small sample size in the present study. These findings are however comparable to other studies in Tanzania which have reported no significant selection of wild-type CVMNK haplotype after treatment with AL [38, 39].

## Conclusions

This study has shown prevalence of CQ wild-type haplotype has increased reaching over 76% indicating an increase in CQ sensitivity in this area with long history of antimalarial resistance in North-eastern Tanzania. Compared to the nearly fixed CQ resistance before CQ withdrawal in 2001, this sensitivity restoration is relatively rapid and affirms the fact that

resistant parasites have less fitness cost than the wild-type parasites in the absence of drug pressure. However this recovery is slower than the rates recently reported from other parts of Tanzania. Full CQ sensitivity restoration and hence re-introduction of CQ (e.g. in a drug combination) in the study area will most likely take longer than anticipated.

Thus when considering re-introducing CQ (e.g. in combination with other antimalarials), local variations of parasite CQ sensitivity must be observed.

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