Expansion of the wild-type \textit{pfcrt} 76K allele in \textit{Plasmodium falciparum} populations in Mbita, Kenya.

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**SUMMARY**

**Background and Methodology:** Chloroquine resistance in \textit{P. falciparum} is conferred by mutations in the \textit{pfcrt} gene with K76T mutation being definitive in as far as chloroquine resistance is concerned. The prevalence of point mutations at codons 74, 75 and 76 of \textit{P. falciparum} of the \textit{pfcrt} gene was determined by dot–blot/probe hybridization analysis in 121 samples collected in May 2009 twelve years after chloroquine cessation in Kenya.

**Results:** We found out that 80.17% of the field isolates from Mbita harbor the T76 mutation while 19.83% have the wild type allele K76 [T test, \(P=0.108\) (2005 versus 2009)]. Majority of the isolates had MNT haplotype at codons 74, 75 and 76.

**Conclusion:** The study showed a significant expansion of the \textit{pfcrt} 76K allele and thus expansion of parasite populations that are sensitive to chloroquine.

**Key words:** \textit{Plasmodium falciparum}; Dot–blot/probe hybridization; Chloroquine resistance.

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**Introduction**

The major problem with effective malaria therapy and control has always been the resistance of malaria parasites to anti–malarial drugs with \textit{Plasmodium falciparum} being the most problematic. To date it is believed that the most devastating resistance of any anti–malarial drug is still chloroquine resistance owing to the fact that it was the most widely used drug. Its resistance caused an increase in mortality and morbidity with Africa being the hardest hit [1].

Chloroquine falls in the class of compounds referred to as 4–aminoquinolines which have the amino group at
position 4 of the quinoline. A number of 4–aminoquinolines exhibit anti–malarial activity including Hydroxychloroquine and Amodiaquine. Chloroquine acts in the digestive vacuole of plasmodium parasites binding hematin and consequently preventing detoxification of by–products produced in the haemoglobin digestion by the parasite [2].

*P. falciparum* chloroquine transporter protein (pfCRT) which is encoded by the chloroquine transporter gene (*pfcrt*) is found in the parasite’s digestive vacuole membrane. Its mutated form is believed to act as an exporter protein, transporting chloroquine out of the vacuole compromising the prevention of detoxification [3, 4].

The *pfcrt* gene is located on chromosome 7 of the parasite [5–8]. Chloroquine resistance in *Plasmodium falciparum* is as a result of mutations in the *pfcrt* gene which in turn encodes for the mutant protein. The K76T mutation at codon 76 of the *pfcrt* gene is definitive for chloroquine resistant parasites. A cross between chloroquine sensitive and resistant clones from different geographical places also associates the *in vitro* progeny to the K76T mutation [8]. Codons 74 and 75 are associate variants of the definitive codon 76, thus several haplotypes can possibly exist with IET being the pure mutant while MNK being the wild–type haplotype [9]. The M74I mutation usually occurs at codon 74 while the N75E at codon 75 selective drug pressure being the main reason associated with all these mutations [10]. In the absence drug pressure, the wild–type chloroquine sensitive parasites tend to reemerge [9].

The World Health Organization (WHO) recommends the use of artemisinin based combination therapy (ACT) in the first line treatment for un–complicated malaria following the worldwide spread of *P. falciparum* resistant to chloroquine and Sulfadoxine–Pyrimethamine (SP). ACTs have been effective in treating un–complicated malaria, but their efficacy has recently come into question. A recent study in French Guyana and Senegal on *P. falciparum* field isolates have shown reduced *in vitro* sensitivity to artemether [11]. Moreover, reports from Cambodia indicate a slight reduction in *in vitro* sensitivity to artesunate and prolonged parasite clearance [12, 13].

Parasites having started developing resistance to ACTs and with the raising numbers of ACT failures reported after a short time of their use unlike in the case of chloroquine where resistance started developing after long use, there is need to continue monitoring the prevalence of molecular markers for chloroquine resistance with the aim of re–introducing chloroquine in combination with another drug. We therefore determined the prevalence of point mutations in codons 74, 75 and 76 of the *pfcrt* gene from field isolates collected in Mbita a malaria endemic site in Western Kenya 12 years after cessation of chloroquine use as first line drug in malaria treatment and compared with earlier studies done in other endemic areas in Kenya.

### Material and Methods

#### Sample Collection

The eligibility criteria for this study was age > 6 years, having uncomplicated *P. falciparum* Malaria with no other *Plasmodium* species present, having an initial parasite density of between 800 and 100,000 asexual parasites/µL, having a measured auxiliary temperature 37.5°C, providing informed consent (by parent or
guardian, when appropriate), and willing to return for follow-up, this was be done at the Mbita sub-district hospital. At enrolment, a brief clinical exam was performed. Blood spot samples and clones collected on a fresh 3mm Whatman filter paper (Whatman®) were excised from filter paper and DNA extracted using Chelex-100® Bio–Rad method as described by Warhurst et al [14]. Briefly, scalpels, forceps and glass plate were sterilized with 5M HCl, 5M NaOH, and distilled water. Blood spots in filter papers were scalpel excised on glass and the paper was transferred into 1ml of 0.5% saponin in 1 x PBS to a sterile 1.5ml microfuge tube (Eppendorf®). The tube was inverted several times to mix, and placed at 4°C overnight. The contents were centrifuged and supernatant transferred into 10% bleach, and then into 1ml of PBS 4°C for 30 minutes. The solution was centrifuged and placed again in 10% bleach with 50µl of 20% (w/v) chelex– 100® Bio–Rad solution and 100µl of DNase-free water (Sigma). The solutions were heated at 95 – 100°C for 10 minutes with vortexing at 2 minutes intervals (Genie vortexer®). The solutions (DNA containing) were centrifuged at 10,000 for 2 minutes and the supernatant transferred to a fresh tube, and further centrifuged for 2 minutes at 10,000 and transferred into another fresh tube, and subsequently stored at -70°C.

DNA extraction, PCR amplification and product analysis

Molecular analysis of filter paper–preserved blood samples was done in the Malaria Molecular Lab in the Centre of Biotechnology Research and Development, at the Kenya Medical Research Institute. The blood spot samples and clones collected using filter papers were excised from the filter paper and the DNA extracted using Chelex-100® Bio–Rad method as described by Plowe et al [15]. Oligonucleotides primer pairs P1 and P2 were included in a single outer PCR reaction. The outer amplification reaction volume was 30 µl containing a sector of prepared 5 µl DNA from chelex extraction, 0.25µM of each outer primer, 1× standard PCR buffer (1.5mM MgCl2, 50mM KCl, 10mM Tris HCl (pH8.3), 0.5% DMSO), 200 µM of each of the dNTPs, and 1 unit of Taq polymerase (KEMTAQ®). The reaction was allowed to proceed for one cycle at 94°C for 3 minutes, then 40 cycles at 94°C for 1 minute, 50°C for 2 minutes, 72°C for 2 minutes and finally 1 cycle at 72°C for 10 minutes. Using the PCR products generated from the primary reaction as templates, a nested PCR was then performed using primers based on conserved sequences, so that the PCR product included codons 74, 75 and 76 in pfcrt. D1 and D2 nested primers were used for codon 74, 75 and 76. The nested reaction consisted of 0.25µM of each nested primer, standard PCR buffer, 200 µM of each of the dNTPs, 1 unit of Taq polymerase, and 5 µl of nest 1 PCR products as template. Tubes were briefly centrifuged and placed in a thermocycler (Bio–Rad / MJ Research). The reaction was cycled 40 times at 94°C for 1 minute, 45°C for 1 minute, 72°C for 2 minutes and a final extension step of 72°C for 10 minutes.

Dot blot analysis

Preparation for dot blotting

Dot blots were prepared by methods of Abdel Muhsin [16]. Briefly, 20 µl each of PCR product were denatured in 10 mM EDTA and 0.4M NaOH at 100°C for 10 minutes to a final volume of 30µl, and then neutralized in an equal volume of 2M–ammonium acetate, pH 7. Nitrocellulose membranes (Millipore)
cut to exact size to fit manifolds were pre-wetted by soaking in 2× SSC for 10 minutes and fitted on to dot blotting apparatus (Bio Rad). The membranes were rehydrated with TE buffer and then dried using vacuum pump. Denature samples were individually loaded in duplicate into the manifold containing the blots, and were held for 30 min. The membranes were removed and neutralized in 2× SSC for 30–60 seconds and washed in 0.4M NaOH for 30–60 seconds to denature immobilized DNA. The membranes were rinsed in neutralizing solution (1M Tris–HCL, 1.5M NaCl, and pH 8) for 30 seconds then UV cross-linked at 0.120 joules for 5 minutes to fix the PCR products onto the membrane. The membrane was then wrapped using cling film and stored at −20°C until it was needed.

**Labeling of oligonucleotide probes**

Probes for all the possible alleles at codons 74, 75 and 76 of the *Pfcrt* gene were labeled as MNK (5′–TAA TGA ATA AAA TTT TTG–3′), MNT (5′–TAA TGA ATA CAA TTT TTG–3′), IEK (5′–TAA TTG AAA AAA TTT TTG–3′), IET (5′–TAA TTG AAA CAA TTT TTG–3′), MEK (5′–TAA TGG AAA AAA TTT TTG–3′), MET (5′–TAA TGG AAA CAA TTT TTG–3′), INK (5′–TAA TTA ATA AAA TTT TTG–3′) and INT (5′–TAA TTA ATA CAA TTT TTG–3′) (MWG Biotech). Ten picomoles of each probe were labeled with 10µCi of [γ⁻³²P]dATP using 5 units of polynucleotide kinase. This was prepared by adding 1µl probe, 1µl T4 Polynucleotide kinase (5 units/µl) (USB, Cat 70031), 5µl T4 Polynucleotide kinase 10× buffer and 42µl nuclease free water to a tube. The contents of tube were then mixed by pipetting up and down briefly.

From this stage all procedures were carried out in a radiation containment room/area, using beta-shields such as 1cm acrylic for protection from the radiation, and wearing appropriate personal radiation monitors such as film badges. Solid and liquid wastes were disposed off according to the advice of the local Atomic Energy Agency/radiation protection advisors. To the reaction mix of 1µl of [γ⁻³²P] dATP (Amersham Biosciences, UK: Redivue [γ⁻³²P] ATP, 3000Ci/mmol: Cat No. AA00068) was added and mixed gently. These were then spun briefly in a microfuge to collect the contents at the bottom of the tube followed by incubation at 37°C for 30 minutes in a programmable heating block (Eppendorf). Adding 5µl of 250mM EDTA, into the tube, stopped the reaction.

**Removal of unincorporated [γ⁻³²P] ATP**

Unincorporated [γ⁻³²P] dATP was removed using G–25 Micro spin columns (Amersham Pharmacia Biotech, UK. Cat 27–5325–01). These were prepared by re-suspending the resin in the column by vortexing gently. The column was then placed in 1.5 ml screw cap microfuge tube for support, and pre–spun for 1 minute at 3000 rpm, in an eppendorf microfuge, to pack the sephadex resin. The column was placed in a new 1.5ml tube and all of the labeling mixture applied to the center of the angled surface of the compacted resin bed, being careful not to disturb the resin. The column was spun for 2 minutes at 3000 rpm. The purified sample was collected at the bottom of the support tube. The column was discarded accordingly, as radioactive solid waste. The labeled purified probe was stored at −20 °C shielded until required. Labeled probes were used within 1 week. Unused probes were disposed off as per radioactive items.
Hybridization and stringent washes

The prepared blots were unwrapped and placed into a rotor bottle, making sure that there were no overlapping areas, into which 20 mls hybridization buffer were added (0.25–0.125ml per cm$^2$ of membrane). The hybridization buffer consisted of a final volume of 5× SSPE, 5x Denhardt’s reagent, 0.5% SDS; 0.02mg/ml sonicated salmon sperm DNA (Gibco) in DNase–free Water. This was then pre-warmed at different temperatures for each probe in a hybridization oven for 30 minutes with agitation making sure that the bottle was closed properly and the buffer does not leak. MNK, INK and INT were hybridized at 36°C, IEK, IET, MNT, and MEK were hybridized at 37°C while MET was hybridized at 38°C.

The blots were added into rotor bottles containing the pre-warmed buffer for another 30 minutes. A volume of 20µl of the labeled oligonucleotide probe (1µl for every 1ml of the hybridization buffer) was then added into the bottle contents. Hybridization at appropriate temperature for at least 5 hours with agitation followed. Overnight hybridization was preferred especially when the labeled probe was over 2 weeks old. The hybridization solution was poured off and disposed of accordingly and stringent washes were carried out.

An excess (at least 1ml/cm$^2$ blot) of wash buffer 1 (2 × SSC) was added at the corresponding temperature and incubated with agitation in the oven for 10 minutes at the same temperature. The washes were repeated twice using excess (at least 1ml/cm$^2$ blot) wash buffer 2 (1× SSC/0.1% SDS) at the same temperature and incubated with agitation for 5 minutes for IEK, IET, MNK and MNT while 10 minutes for MEK, MET, INK and INT. The washing solutions were poured off and disposed of accordingly then the blot sealed by wrapping in cling film without allowing the blot to dry out.

Autoradiography

The sealed blot was taped right side up (DNA–side up) into an autoradiography cassette (Kodak) with intensifying screens. To avoid problems with autorad orientation, the film was folded at bottom right corner and this allowed for accurate positioning of the autorad after developing. Blots were exposed on Kodak® (Rochester, NY) X–Omat film for 12–24 hours at –70ºC in the freezer (Revco®).

The films were then removed from the cassette and developed to score the sample against the controls. If any of the controls showed non–specific hybridization, an extra stringent wash was carried out. The autorad was obtained by developing the image in a developer solution (Kodak) for 5 minutes followed by a brief rinse in clean water and finally fixing the developed image in a fixative solution (Kodak) for 5 minutes and the fixative rinsed off with clean tap water. These processes were done in a dark room as the films are light sensitive. The films were then air dried then scored.

Stripping the probe from the membrane

The membranes were then stripped off the probe using excess of 0.1 M NaOH, for 15 minutes, at room temperature with agitation, repeating the process followed by a brief wash with 5× SSC. The blot was probed again or stored, after sealing the blot by wrapping in a cling film, at –20ºC or dried and store at room temperature sandwiched and taped between two pieces of clean filter paper.
Figure 1: Gel showing PCR products of \textit{pfcrt} gene

The lanes denote the following DNA PCR products; Lane 1 = 100 base pair molecular marker, Lane 2 and 4 = \textit{P. falciparum} positive controls, Lane 3 = Negative control which was nuclease free water, Lane 5 to 15 = Mbita 2009 samples positive for \textit{Pfcrt} gene. Samples amplified by P1/P2 and D1/D2 primers. The PCR products were resolved in 1.5% agarose gel stained with Ethidium bromide in Tris-acetate-EDTA buffer. The electrophoresis was run at 80 volts for 30 minutes. The expected nested PCR product band size, which is 164 base pairs, was determined by comparison with a standard 100–base pair DNA ladder.

The amplicons were analyzed by dot–blot hybridization to identify the haplotypes in the \textit{pfcrt} gene at codons 74, 75 and 76. Analysis involved the use of probes that identify MNK (wild type haplotype), MNT, MEK, MET, IET and IEK.

Figure 2: Autorads of dot blot hybridization

DNA samples amplified by P1/P2 and D1/D2 primers was blotted on a nitrocellulose membrane and hybridized to \textit{pfcrt} haplotype probes for position 74, 75

Results

Total of 121 samples were amplified by PCR.
and 76. Autorad A represents an autoradiograph for 2009 samples from Mbita (MNT) after two stringent washes. Autorad B represents a dot blot autoradiograph of a second set of for samples from Mbita (MNT probe). A1 had 3D7 positive control (MNK); A2 had PCR negative control which was nuclease free water while A3 had Dd2, negative control. MNK is the haplotype of \textit{pfcrt} at position 74, 75 and 76 present in all chloroquine sensitive strains of \textit{P. falciparum}.

21 samples corresponding to 17.36% had MNK haplotype, 73 samples (60.33%) had MNT, 1 sample (0.83%) had MEK, 16 samples (13.22%) had MET, 3 samples (2.47%) had both MNK and MET haplotypes, 6 samples (4.96%) had MEK and MET haplotypes and one sample (0.83%) had a mixture of MNK, MEK and MNT haplotypes. The results are as shown in the figure below.

**Figure 3: Graph showing different \textit{Pfcrt} haplotypes in field isolates from Mbita**

The \textit{pfcrt} K76T mutation is the definitive resistance marker. That is; the presence of T mutation has been associated to chloroquine resistance \textit{in vitro} and treatment during clinical chloroquine efficacy studies. For purposes of statistical analysis all the samples containing pf\textit{crt} 76K (MNK and MEK) were put together as having wild type codon at position 76 regardless of the haplotypes at codons 74 and 75. Samples containing MNT, MET and the one sample that had MNK/MEK/MET haplotypes were all grouped together as having pfc\textit{rt}76T. Thus 22 (18.03%) samples in total had the wild--type 76K while 99 (81.97%) had \textit{pf}\textit{crt} 76T mutation. This differed from findings by Omar et al (2007) at a different malaria endemic site in which they reported 6% of the samples as having 76K and 94% had 76T. Unpublished data from a similar analysis in
samples collected in 1997 (the year of cessation of chloroquine use as first line drug in malaria treatment) indicate that the prevalence of \textit{pfcrT} 76T was 100%. A student t-test gave a \( p \) value of 0.108 suggesting there has been a significant reversal.

\textbf{Discussion.}

Chloroquine resistance has been associated to Lysine-Threonine mutation at codon 76 in the transporter \textit{pfCRT} protein found the vacuole membrane. In the wild-type, the protein is thought to transport chloroquine into the food vacuole where chloroquine interferes with breakdown of haemoglobin by preventing polymerization of toxic heme to non-toxic haemozoin. This leads to accumulation of heme in the parasite food vacuole. Heme then disrupts the parasite cell membrane resulting in death.

The \textit{pfcrT} K76T mutation is thought to be the definitive mutation as far as chloroquine resistance is concerned. This mutation exists with other mutations both upstream and downstream position associated with chloroquine resistance. These mutations either modulate chloroquine resistance or maintain the functional integrity of the membrane protein. Codon M74I and N75E mutations have previously been found together with K76T mutation in mutant parasites in Asian and African isolates. However work done by Sabah \textit{et al} (2007) found that isolates from an endemic site in Kenya harbored MET haplotype instead of IET as expected. This indicated that the wild-type alleles in the parasite population were reemerging and that the mutant parasites were not fit enough to survive in the absence of chloroquine due to a high cost of fitness of fit. In a study by Muregi \textit{et al} [18] the effect of mutations on the survival was shown clearly. The wild-type parasites showed a 3-fold faster growth rate than the resistant lines in the absence of the drug. Other studies from a number of pathogens including bacteria, \textit{Toxoplasma gondii} and viruses indicate that mutations associated with drug resistance confer a fitness cost. The mutant forms of organisms are therefore likely to be less fit than the wild-type strains in the absence of selection [19–23].

This study, established that the prevalence of \textit{pfcrT} K76T mutation is 81.97% in samples collected from Mbita, a malaria endemic region in 2009 using dot blot hybridization. This method has been found to be more sensitive than RFLP [10, 17]. This indicates a further decline in prevalence of K76T mutation is parasite from 94% [10] in 2005 to 81.97% in 2009, twelve years after cessation of chloroquine use as a first-line drug in treatment of malaria in Kenya. However this reduction is low compare to that reported in Malawi where prevalence of \textit{pfcrT} 76T decreased from 83% in 1992 to 13% in 2000 [9]. These findings were corroborated by Mita \textit{et al} [24] who later established that this was due to expansion of the wild-type \textit{pfcrT} allele in \textit{P.falciparum} populations in the absence of chloroquine pressure rather than a back mutation of K76T to K76 [25]. The rate of decline is also lower than that reported from the Chinese Island Hainan where the prevalence 76T from 90% in 1978 to 64% in samples collected between 2002 and 2004 in which 36% of the isolated were found to have the wild type genotype [26–28].

The dot blot results revealed that 94 (77.68%) parasite isolates had wild-type genotypes in codon 74 and 75
that is the MNT haplotypes. This is the first study to report this haplotypes in Kenya since the study by Sabah et al (2007) found 75E in all isolates.

Kenya switched from chloroquine to SP as first–line drug in 1997. The slow rate of expansion of the 76K allele in Kenya could be partly due that the fact that there could be compensatory mutation that in the \textit{pfcrt} gene that have maintained the integrity of the mutant protein lowering the cost to fitness of fit with which the mutation come [18]. Alternatively the withdrawal of chloroquine or related drugs with different brand names was not complete since there is no evidence suggesting surveillance to ensure a complete withdrawal of chloroquine from circulation [24]. Both situations could be possible and may have played a major role in maintaining mutant parasites in the population. Furthermore the samples were collected from a highly endemic zone with high rates of transmission [10]. This region is likely to have many individuals who have developed immunity to malaria. These individuals can survive parasite inoculation without developing clinical symptoms of malaria or may resolve plasmodium infections without drug intervention. Either way such individuals would serve as parasite reservoirs in the population and allow continuous persistence of the \textit{pfcrt} K76T mutation.

Overall, the fact that there is an observed expansion of the 76K allele implies that with time, complete absence of use of chloroquine will lead to elimination of the mutant parasite due to the high cost of fitness and further expansion of the 76K allele in parasite populations in Kenya. This may eventually allow re-introduction of chloroquine as a combination drug.

Further the use artemisinin combination therapy in malaria treatment in has been shown to select for 76K mutations [29]. This synergy will therefore ensure an accelerated expansion of the wild–type alleles and thus ensure complete sensitivity to chloroquine once again.

**Conclusion**

The study showed a significant expansion of the \textit{pfcrt} 76K allele and thus expansion of parasite populations that are sensitive to chloroquine. If this trend is replicated and continues to be observed over time, there is a possibility of re–introducing chloroquine treatment in combination with another drug.

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