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Genotyping for point mutations in selected codons of *pfcrt* and *pfmdr-1* genes of *Plasmodium falciparum* among patients with uncomplicated malaria in Mbita district Kenya.

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Abstract

Background

Malaria remains a leading cause of morbidity and mortality in Kenya, especially in young children and pregnant women. Due to widespread resistance of *Plasmodium falciparum* to drugs such as Chloroquine (CQ) and Sulfurdoxine-Pyrthamine (SP), Artemisisnin Combination Therapy (ACT) was adopted in Africa as a means of improving treatment efficacy and slowing the spread of resistance. The development of drug resistance by the parasites for the various malaria drug regimens that have been in use before has been attributed to point mutations within the parasite genome. Therefore this study investigated the prevalence of point mutations in selected codons of the *pfcrt* and *pfmdr-1* genes of *Plasmodium falciparum*. It is however unclear whether ACT will be effective in preventing the selection of resistant parasites in Africa, where parasite transmission rates are generally much higher with parts of Asia and Africa already reporting a reduction in sensitivity to ACT.

Methods

The dot-blot/probe hybridization technique was used to identify point mutations in codons 74, 75 and 76 of the *pfcrt* gene and codons 1034, 1042 and 1246 of the *pfmdr-1* gene in Mbita a malaria holoendemic site in Kenya. In the *pfcrt* gene, 76T mutation was found to be in 91 (79.83% CI 63.1-88.5) of 114 samples while the, the wild type allele 76K was present in 23 (20.17% CI 9.0-22.0) samples. Codons 1246 showed allelic variation with 1246D the wild type allele being 72.8% (CI 52.0-89.1). This was a significant increase in the 76K allele (*p*=0.001) in comparison to the year 2005 where prevalence of 76K was 6%.

Conclusion
There's an expansion of the wild-type allele 76K of the *pfcrt* gene and no significant difference in the 1246D allele of the *pfmdr1* gene, moreover the prevalence of 76T allele is still high in Mbita hence it's beneficial to continue using AL as treatment for uncomplicated malaria.
Keywords: Malaria, Drug Resistance, Point Mutations

Background

Malaria remains one of the leading causes of morbidity and mortality, globally. About a half of the world's population is at risk of malaria leading to 300- 500 million clinical cases and 600,000 deaths each year (1). At least 550,000 of the malaria deaths are attributable to *Plasmodium falciparum* infections (1). Sub-Saharan Africa accounts for over 90% of the malaria cases and deaths predominantly in children of age below five years and pregnant women (1). In Kenya, 28 million people are at risk of malaria infections and malaria accounts for 34% of outpatient hospital visits, 15% in-patient admissions and ~ 33,220 deaths. Therefore, malaria is an important health and socio-economic burden not only globally but also in countries of sub-Saharan Africa including Kenya (2).

The alarming increase in *P. falciparum* resistance to commonly used anti-malarial drugs represents a major public health threat (3). In high transmission areas, the clinical effects are mainly prolonged hence chronically increasing the risk of severe anemia (4). Mortality estimates from public health records in Africa generally suggest significant increases in malaria-associated mortality among children when resistance develops and spreads. Hospital attendances and admissions show similar trends (5). The development of drug resistance by the parasites for the various drug malaria regimens that have been in use before is attributable to point mutations within the parasite genome (6). WHO recommends that all countries experiencing resistance to conventional monotherapies use combination therapies, preferably those containing artemisinin derivatives for *falciparum* malaria to cope with the increasing resistance to antimalarial drugs (7).

Kenya changed its first line policy for uncomplicated malaria from SP to ACT, in 2006. Quinine became the treatment of choice for children below 5kg, pregnant women, and as the second line; SP was reserved only for intermittent preventive treatment in pregnancy; and Amodiaquine (AQ) (previous second line treatment) was no longer recommended (8). It is however unclear whether ACT will be successful in preventing the selection of resistant parasites especially in Africa, where parasite transmission rates are generally much higher. Studies have shown that elevated *P. falciparum* multi-drug resistance 1(*pfmdr1*) gene copy number is associated with up to 8-fold risk of ACT failure(9–11). There's therefore the need for continued molecular surveillance to determine point mutations in the parasite genome with the aim of reintroducing CQ as a combination drug. This help to guide selection of sentinel sites for *in vitro* and *in vivo* monitoring of anti-malarial drug resistance.

METHODOLOGY

Study Design

Laboratory-based cross-sectional descriptive study.

Study site

The study was conducted in Mbita (0° 30´ 0´´ South, 34° 15´ 0´´ East) is on the shores of Lake Victoria in Western Kenya. It is located in the heart of a malaria holoendemic area. The main malaria vectors in the area are *Anopheles gambiae*, *A. funestus*, and *A. arabiensis*.

Study Participants

At enrolment, a brief clinical examination was performed. Blood smears were collected on 2mm Whatman filter papers and stored in sterile polyethene bags containing Silica granules. The full dosage of co-formulated AL was given to the subjects on day 0 through to day 2. Subjects had follow-up visits at 3, 7, 14, 21, 28, 35, and 42 days after the date of enrolment. Participants who did not return on their own were actively sought out. At these follow-up visits, temperature was measured and thick blood smears were used to screen for peripheral parasitemia. The blood samples taken on day 0 were done before treatment but the rest were done after treatment of the patients with AL. The eligibility criteria for the study was age > 6 years, having uncomplicated *P. falciparum* mono-infection , having an initial parasite density of between 800 and 10,000 asexual parasites/μL, having a measured axilliary temperature 37.5°C, providing informed consent (by parent or guardian, when appropriate), and willing to return for follow-up, this was done at the Mbita sub-district hospital. Exclusion criteria was inability to take drugs orally, known hypersensitivity to any of the drugs given, reported treatment with antimalarial chemotherapy during the previous 2 weeks except for those experiencing persistent and severe malaria, evidence of chronic disease or of an acute infection other than with a malarial parasite and residence outside of the study area.

DNA Extraction from dried blood spots

Blood spots on the archived 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* (12). 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 in 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 (BD Triac™) differentially at 4°C leaving the pellet and supernatant transferred into 10% Jik, and then into 1ml of PBS 4°C for 30 minutes. The solution was centrifuged differentially at 4°C leaving the pellet and the supernatant placed again in 10% Jik with 50µl of 20% (w/v) chelex- 100® Bio-Rad solution and 100µl of DNase-free water (Sigma). The solution was heated at between 95 – 100 °C for 10 minutes with vortexing at 2 minutes intervals (Genie vortexer®). The solution (containing DNA) was then centrifuged at 11952 g for 2 minutes. The supernatant was transferred to a fresh tube and further centrifuged for 2 minutes at 11952 g then transferred into another fresh tube. The DNA preparation was subjected to spectrophotometry at optical density (O.D) of 260/280 nm giving values close to 1.8 conforming the purity of the double stranded DNA amplified and subsequently stored at -70°C.

Analysis of drug resistance loci *pfcrt* for point mutations at *pfcrt*- 74, 75 & 76

PCR amplification of the *pfcrt* gene

Oligonucleotide primer pairs CRTP1 and CRTP2 (table 1.1) were used as forward and reverse primers respectively for outer PCR. Nested PCR was performed using CRTD1 and CRTD2 as forward and reverse primers respectively (table 1.1). The primer sequences used in amplifying this gene were adopted from Sabah *et al* (4), the fragment amplified was expected to be 164bp. Control amplification using DNA from *P. falciparum* parasites clones 3D7 and HB3, from WHO/ International Atomic Energy Agency (IAEA), known to contain the possible different haplotypes at position 74, 75 and 76 of *pfcrt* gene were used to ensure specificity and sensitivity of the technique.

A master mix for the outer PCR was prepared composed of 16.36 µl nuclease-free water (Sigma), 3.0 µl of 10 × PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl) (Roche), 1.8 µl of 25mM Magnesium Chloride (Roche), 3.0 µl 2mM mixed dNTP's (dGTP, dATP, dTTP, dCTP) (Promega), 0.3 µl of each primers (10 µM) (Outer loop primers P1, forward, and P2, reverse) and 0.24 µl Taq polymerase enzyme (KEMTAQ™) (5 units/ µl) per PCR tube. DNA (5 µl) of was added to 25 µl master mix to a total volume of 30 µl. Amplification was performed using a MyCycler™ Thermal cycler PCR machine (BIORAD, USA). Primary denaturation was conducted for 3 minutes at 94°C followed by a 40 cycles of, denaturing for 30 sec at 94°C, annealing for 30 seconds at 56°C and extension for 1 minute at 60°C. Final extension was carried out for 3 minutes at 60°C then the reaction brought to a hold at 4°C. .

Nested PCR reaction was conducted on the PCR products of the first round reactions targeting internal segment of the amplicons. Briefly, a master mix was prepared composed of 16.36 µl nuclease-free water (Sigma), 3.0 µl of 10 × PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl) (Roche), 1.8 µl of 25mM Magnesium Chloride (Roche), 3.0 µl 2mM mixed dNTP's (dGTP, dATP, dTTP, dCTP) (Promega), 0.3 µl of each primers (10 µM) (Outer loop primers D1, forward, and D2, reverse) and 0.24 µl Taq polymerase enzyme (KEMTAQ™) (5 units/ µl) per PCR tube. 5 µl of outer PCR product, used as DNA template and was added to 25 µl master mix to a total volume of 30 µl. Amplification was performed using a MyCycler™ Thermal cycler machine (BIORAD, USA). Primary denaturation was conducted for 3 min at 94°C followed by a 30 cycles of, denaturing for 30 sec at 94°C, annealing for 30 sec at 56°C and extension for 1 min at 60°C. Final extension was carried out for 3 min at 60°C and the reaction brought to a hold at 4°C. 22.5 µg of nested PCR product was mixed with a 1 µl of 10× loading dye and analyzed by agarose gel electrophoresis on 1.5% agarose gel stained with Ethidium bromide (0.5 µg/ml) in Tris-acetate-EDTA (TAE) buffer. The electrophoresis was run at 80 volts for 50 minutes. DNA was visualized by ultraviolet transillumination and the expected nested PCR product band size, which is 164 base pairs, determined by comparison with a standard 100-base pair DNA ladder.

Preparation for dot blotting

Dot blots were prepared by methods of Abdel-Muhsin *et al* (13). 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×Sodium Saline Citrate (SSC) for 10 minutes and fitted on to dot blotting apparatus (Bio Rad). The membranes were re-hydrated with TE buffer and then dried using vacuum pump. Denature nested PCR products from field samples were individually loaded into the manifold containing the blots in duplicate. They were held for 30 min. The membranes were removed and neutralized in 2× SSC for 30-60 seconds, then 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. Denatured DNA was then cross-linked to the membrane by exposing to UV light at 0.120 joules

for 5 minutes in to fix it onto the membrane. The membrane was then wrapped using cling film and stored at -20°C until it was needed.

Labelling of oligonucleotide probes

Probes for all the possible alleles at codons 74, 75 and 76 of the *pfcrt* gene were labelled as MNK, MNT, IEK, IET, MEK, MET, INK and INT (MWG Biotech) as described by Abdel-Muhsin *et al* (13). Ten picomoles of each probe were labelled with 10µCi of [γ-^{32}P] 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, 1µl of [γ-^{32}P] dATP (Amersham Biosciences, UK: Redivue [γ-^{32}P] ATP, 3000 Ci/mmol: Cat No. AA00068) with a half-life of 14 days, 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 37ºC for 30 minutes in a programmable heating block (Eppendorf). Adding 5µl of 250mM EDTA, into the tube, stopped the reaction.

Removal of unincorporated [γ-^{32}P] ATP

Unincorporated [γ-^{32}P] 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 in a refrigerated centrifuge (BD Triac™) for 1 minute at 1077 g and 4° C (BD Triac™), in an eppendorf microfuge, to pack the sephadex resin. The column was placed in a new 1.5ml tube and all of the labelled 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 1077 g. The purified sample was collected at the bottom of the support tube. The column was discarded accordingly, as radioactive solid waste. The labelled purified probe was stored at –20°C shielded until required. Labelled probes were used within 1 week. Unused probes were disposed off as per standards IAEA for disposal of 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 20mls 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 depending on the hybridization temperature of every probe for 30 minutes with agitation making sure that the bottle was closed properly and the buffer did not leak. The hybridization buffer was pre-warmed at the exact temperature at which the probe hybridizes. Allele specific oligonucleotide probes 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 probes were already single stranded with varied lengths between 18 base pairs and 25 base pairs hence no denaturation was required. The blots were added into rotor bottles containing the pre-warmed buffer for another 30 minutes. A volume of 20µl of the labelled oligonucleotide probe (1µl for every 1ml of the hybridization buffer) was then added into the bottle contents. Hybridization at appropriate temperature depending on the temperature at which the probe hybridizes for at least 5 hours with agitation followed. 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. 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; and for 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. For ease of identifying autoradiograph orientation, the film was folded at bottom right corner. This allowed for accurate positioning of the autoradiograph 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 autoradiograph 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. The fixative was 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 and then scored.

Stripping the probe from the membrane

The probe was stripped off the membranes using excess of 0.1 M NaOH, for 15 minutes, at room temperature with agitation, followed by a brief wash with 5× SSC. The stripping was done to necessitate use of other probes on the denatured DNA on the nitrocellulose membrane. 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.

Analysis of drug resistance gene *pfmdr-1* for point mutations at *pfmdr-1* 1034, *pfmdr-1* 1042 and *pfmdr-1* 1246

PCR amplification of the *Pfmdr-1* gene

Oligonucleotide forward primers MDR/B1 and reverse MDR/B2 primers (table 1.2) were used in single outer PCR reaction. Using the PCR products generated from the primary reaction as templates, a nested PCR reaction was conducted using forward MDR/B3 and reverse MDR/B4 primers (table 1.2). The primer sequences used in amplifying this gene were adopted from (2); the fragment amplified was expected to be 860bp.

The master mix for the outer PCR consisted of 15.66 µl nuclease-free water (Sigma), 3.0 µl 10 × PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl) (Roche), 2.5 µl 25mM magnesium chloride (Roche), 3.0 µl 2mM mixed dNTP's (dGTP, dATP, dTTP, dCTP) (Promega), 0.3 µl of each primers (10 µM) (Outer loop primers B1, forward, and B2, reverse) and 0.24 µl Taq polymerase enzyme (KEMTAQ™) (5 units/ µl). DNA (5 µl) was added to 25 µl master mix to a total volume of 30 µl. Amplification was performed using MyCycler™ Thermal cycler PCR machine (BIORAD, USA). Primary denaturation was conducted for 3 min at 94°C followed by a 30 cycles of denaturing for 30 seconds at 94°C, annealing for 1 minute at 50°C and extension for 2 minutes at 65°C. Final extension was carried out for 3 minutes at 65°C and the reaction brought to a hold at 4°C.

Second round of PCR reactions was conducted on the PCR products of the first round reactions targeting internal segment of the amplicons. Briefly, a master mix was prepared composed of 15.66 µl nuclease-free water (Sigma), 3.0 µl 10 × PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl) (Roche), 2.5 µl 25mM magnesium chloride, 3.0 µl 2mM mixed dNTP's (dGTP, dATP, dTTP, dCTP) (Promega), 0.3 µl of each primers (10 µM) (Outer loop primers B3, forward, and B4, reverse) and 0.24 µl Taq polymerase enzyme (5 units/ µl). DNA (5 µl) was added to 25 µl master mix to a total volume of 30 µl. Amplification was performed using a MyCycler™ Thermal cycler PCR machine (BIORAD, USA). Primary denaturation was conducted for 3 minutes at 94°C followed by 30 cycles of denaturing for 30 seconds at 94°C, annealing for 1 minute at 50°C and extension for 1 minute at 65°C. Final extension was carried out for 3 minutes at 65°C and the reaction brought to a hold at 4°C.

22.5µg of nested PCR product was mixed with a 2 µl of 10× loading dye and analysed by agarose gel electrophoresis on 1.5% agarose gel stained with Ethidium bromide (0.5µg/ml) in Tris-acetate-EDTA buffer. The electrophoresis was run at 80 volts for 45 minutes. DNA was visualized by ultraviolet transillumination and the expected nested PCR product 864 base pairs band size determined by comparison with a standard 100-base pair DNA ladder. The rest of the dot blot/probe hybridization methods for *pfmdr-1* gene were done as described for *pfcrt*.

RESULTS

Patients, whose samples were used in the study, had an initial average parasite density of between 800 and 10,000 asexual parasites/µL and an axilliary temperature 37.5°C on day 0. Follow up was done to establish parasitemia on days 2, 3, 7, 14, 21, 28, 35 and 42 after administering the patients with the full Coartem® dosage. Most of the patients showed a parasitemia of zero asexual parasites/µl by day 3 with several others showing re-infections after day 14.

Point mutations in *pfcrt* codons 74, 75 and 76

PCR amplification

114 malaria positive DNA samples were amplified by PCR using both the outer and nested primers (table 1.1) specific for the codons 74, 75 and 76 of the *pfcrt* gene.

Genotyping *pfcrt*

Samples were genotyped by dot-blot hybridization on nitrocellulose membranes to identify the haplotypes in the *pfcrt* gene at codons 74, 75 and 76. The analysis involved the use of probes that identify MNK (wild type haplotype), MNT, MEK, MET, IET IEK, INK and INT.

The *pfcrt* K76T mutation is the definitive CQ resistance marker. That is; the presence of the amino acid Threonine in the place of Lysine has is associated to CQ resistance *in vitro* and treatment during clinical CQ efficacy studies. For purposes of statistical analysis all the samples containing *pfcrt* 76K (MNK and MEK) were grouped together as having wild type codon at codon 76 regardless of the haplotypes at codons 74 and 75 since codon 76 is the definitive codon for CQ resistance. Samples containing MNT, MET and the one sample that had

MNK/MEK/MET haplotypes were all grouped together as having *pfcrt*76T. Thus 23 (20.17%, [95% CI 8.0-27.0]) samples in total had the wild type 76K while 91 (79.83%, [95% CI 61.3-89.7]) had *pfcrt* 76T mutation. Table 1.3 shows the summary of mutation profiles for *pfcrt* in Mbita 2009.

Point mutations in *Pfmdr-1* codons 1034, 1042 and 1246.

PCR amplification.

114 malaria positive DNA samples were amplified by PCR using both the outer and nested primers (table 1.2) for the codons 1034, 1042 and 1246 of the *pfmdr-1* gene.

Genotyping *pfmdr-1*.

A total of 114 samples were analysed by dot blot hybridization for polymorphisms in *pfmdr-1* gene. All the PCR positive samples 95 (83.3%) hybridized with the Serine probe (Ser) the wild type probe for codon 1034 of the *pfmdr-1* gene (Table 1.4). None of the samples hybridized with Cysteine probe (Cys) the mutant probe for codon 1034. Ninety two (80.7%) of the PCR positive samples hybridized with the Asparagine probe (Asn) the wild type probe for codon 1042 of the *pfmdr-1* gene. None of the samples hybridized with Aspartate probe (Asp) the mutant probe for codon 1042. Eighty three (72.8%) of the PCR positive samples hybridized with Aspartate probe (Asp) the wild type probe for codon 1246 of the *pfmdr-1* gene (Plate 4-4). Nine (7.9%) samples hybridized with the Tyrosine probe (Tyr) the mutant probe for codon 1246. Three (2.6%) of the samples gave mixed signals by hybridizing to both the mutant probe Tyr as well as the wild type probe Asp. The prevalence of the *pfmdr-1* 1246D the wild type allele is therefore 72.8% (95% CI 59.0-81.5).

Analysis of Polymorphism in selected codons of *pfcrt* and *pfmdr-1*.

12 (10.5%) samples showed a D1246Y mutation in *pfmdr-1*, 3(2.6%) of which were mixed, that hybridized with both the mutant probe (Tyrosine) and the wild type probe (Aspartate) during dot blot hybridization. 91 (79.83 %) samples showed a K76T mutation, 9 (7.89%) of which were mixed hence hybridized with both the mutant probe (MNT) as well as other probes. In *pfcrt*, point mutations were found to be present in codons 75 and 76 while in *pfmdr-1*; point mutations were existent in codon1246 only. 11samples out of the 12 samples which showed D1246Y mutation in the *pfmdr-1* gene had K76T mutation in the *pfcrt* gene. One sample with a D1246Y mutation did not have K76T mutation. The student *t*-test statistic was used to determine the possible expansion of the 76K allele of the *pfcrt* gene and 1246D allele of the *pfmdr-1* gene. The student t-test for *pfcrt* stood at t_{114} (N= 114) = 2.864, (95% CI 0.001-0.683, p= 0.001). The critical value at t_{114} (N=114) =2.26 is lower than the tabulated value and hence statistically significant indicating a significant expansion of the 76K allele and its possible selection by AL The t-test for the *pfmdr-1* yielded a value of t_{114} (N= 114) = 1.988, 95% CI 0.034-0.087, p= 0.069). The critical value at t_{114} (N=114) =2.26 is higher than the tabulated value and hence statistically insignificant.

Discussion

AL is currently the combination therapy for treatment of uncomplicated *P. falciparum* malaria in Kenya. The drug has been in use as the drug of choice for treatment of uncomplicated malaria in Kenya since the year 2006. In 1997, the *pfcrt* 76T mutation prevalence stood at 100% (14) that is all the circulating parasites were resistant to CQ. In 2007 Sabah *et al* (4) reported a significant decrease in this mutation to 94% [*P*=0.04058]. These are data from two Kenyan malaria endemic sites of Oyugis and Mwea respectively. This study used them as baseline data for comparison with 2009 since both sites are reflective of the malaria status in holoendemic sites in Kenya, moreover Oyugis is a neighbouring district to Mbita and therefore most of the environmental factors are similar. The haplotype reported in 1997 was IET suggesting that all the three codons, 74, 75 and 76 had undergone mutation (14). Sabah *et al* (4) later reported a haplotype that was entirely MET suggesting that codon 74 was wild type but 75 and 76 were still mutant (4). This was for *P.falciparum* samples collected in the year 2005 in Mwea, central Kenya.

Four years later our study reports a *pfcrt* 76T mutation prevalence of 79.83%, a further decrease in comparison to 2005, the prevalence of the *pfcrt* 76K allele was found to be 20.17% (CI 9.0-27.0) at a *p* value of 0.001 indicating a significant expansion of this wild type allele. This study therefore reports a sharp decline in prevalence of 76T mutation is parasite from 94% (4) in 2005 to 79.83% in 2009, twelve years after cessation of CQ use as a first-line drug in treatment of malaria in Kenya. It is however important to note that the two malaria holoendemic sites are geographically different, much as Mwea is in the Kenyan highlands, Mbita is along the shows of Lake Victoria hence dynamics of transmission could likely be different. However this reduction is low compared to that reported in Malawi where prevalence of *pfcrt* 76T decreased from 83% in 1992 to 13% in 2000 (15). These findings were corroborated by Mita *et al* (16) who later established that this was due to expansion of

the wild-type *pfcrt* allele in *P.falciparum* populations in the absence of CQ pressure rather than a back mutation of K76T to K76 (16). 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 (17).

It further reports MNT as the predominant haplotype at 61.40% suggesting that codons 74 and 75 in the parasites circulating in Mbita have significantly higher wild type alleles than in codon 76.This is the first study to report these haplotypes in Kenya since the study by (4) found 75E in all isolates. The slow rate of expansion of the 76K allele in Kenya could be partly due that the fact that there could be compensatory mutations in the *pfcrt* gene that have maintained the integrity of the mutant protein lowering the cost to fitness with which the mutation comes with (18). Alternatively the withdrawal of CQ or related drugs with different brand names was not complete since there is no evidence suggesting surveillance to ensure a complete withdrawal of CQ from circulation(19). 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 (4). 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 *pfcrt* K76T mutation(20).

Overall, the fact that there is an observed expansion of the 76K allele implies that with time, complete absence of use of CQ may lead to elimination of the mutant parasite due to the high cost of fitness of fit (18) and further expansion of the 76K allele in parasite populations in Kenya. This may eventually allow reintroduction of CQ as a combination drug. Further the use artemisinin combination therapy in malaria treatment in has been shown to select for 76K mutations (21). This synergy will therefore ensure an accelerated expansion of the wild-type alleles and thus ensure complete sensitivity to CQ once again.

The expansion of the 76K allele could further be attributed to selection by the longer acting drug in AL combination. Mutant *pfcrt* is thought to efflux compounds out of the digestive vacuole into the cytoplasm. For CQ, which interferes with detoxification of heme moieties, resistance is thought to be achieved by mutant protein pfCRT-mediated exportation of the drug away from the hematin target (22). CQ resistance is critically dependent on the *pfcrt* K76T mutation. Parasites carrying the K76T mutation are more susceptible to lumefantrine (23) suggesting that these compounds might also be transported by the mutant protein pfCRT out of the digestive vacuole and consequently exert their action in the parasite cytoplasm. Furthermore CQ susceptibile *pfcrt* 76K and *pfmdr1* 86N alleles have been associated with decreased susceptibility to lumefantrinem (24).

Lumefantrine one of the partner drugs in AL has also been shown to select for the *pfmdr1* 86N allele(23). Several other mutations in *pfcrt* and *pfmdr1* are potentially involved in the AL reduced susceptibility, some of these mutations could be K76T and S163R for *pfcrt* and Y184F, S1034C, N1042D and D1246Y for *pfmdr1*(24). This study looked at the prevalence of some of these mutations mainly *pfcrt* 76T and *pfmdr1* S1034C, N1042D and D1246Y in Mbita. Single nucleotide polymorphisms (SNPs) in the *Plasmodium falciparum* multidrug resistance gene 1 have been associated with altered *in vitro* and *in vivo* parasite response to arylaminoalcohols a class in which lumefantrine falls. It has been suggested that the selection of *pfmdr1* 86N allele may represent a marker of tolerance to lumefantrine (25). Tolerant parasites being in the intermediate stage between sensitive and resistant, are killed by high drug levels during treatment but can withstand residual lumefantrine concentrations and proliferate in the blood earlier than sensitive parasites (26).

The genetic basis of *in vivo* resistance to AL is still unclear; however several factors of potential importance in development of AL tolerance/resistance are thought to be: the amplification of the *pfmdr1* gene selected in recurrent infections after AL treatment as it has been observed to influence mefloquine (27) and lumefantrine (28).

Of all the SNPs analyzed in this study, allelic variation in *pfmdr1* was observed in codon 1246 only, the remaining *pfmdr1* 1034 and 1042 showed the wild type allele. This similar observation was made by Sisowath *et al* 2009 where allelic variation in *pfmdr1* was reported in codons 184 and 1246 while codons 1034 and 1042 were wild type.

The *pfmdr1* 1246D prevalence in this study is 72.8% (95% CI 59.0-81.5) with a *p* value of 0.069 being statistically insignificant. This was based on the baseline data in 1997 by Omar (14) that reported a prevalence of 68% of the 1246D allele in Oyugis, Western Kenya. The study by Sisowath *et al* (21) analyzing the possible selection of 1246D in combination with 86N by lumefantrine reported a significant increase in the 1246D alleles in parasites that showed tolerance towards lumefantrine.

This study does not report expansion of the wild-type 1246D allele in the parasite populations since it was statistically insignificant, but it is important to note that studies have suggested that the increase in this allele in parasite populations could be an indicator to the onset of tolerance/resistance to lumefantrine (21). The insignificant difference in the prevalence of the 1246D allele in this study therefore cannot be linked to lumefantrine tolerance.

Conclusions

The prevalence of point mutations in the *pfcrt* gene is still high especially in codon 76, which recorded a prevalence of 79.83% in Mbita district. There's a significant increase expansion of the wild-type *pfcrt* 76K allele and hence increased sensitivity to CQ in Mbita district.

There's no significant difference in the wild-type *pfmdr-1* 1246D in the parasite isolates circulating in district in 2009 viz a viz 1997.

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Table 1.1: Primers used for the PCR amplification of the *pfcrt* gene

Primer name	Sequence (5' – 3')	T_m °C	Annealing Temperature °C
CRTP1	**CCGTTAATAATAAATACACGCAC**	**55.4**	**56**
CRTP2	**CGGATGTTACAAAACTATAGTTAC**	**56.7**	**56**
CRTD1	**TGTGCTCATGTGTTTAAACTT**	**49.3**	**56**
CRTD2	**CAAAACTATAGTTACCAATTTTG**	**57.1**	**56**

Table 1.2: Primers used for the PCR amplification of the *Pfmdr-1* gene

Primer name	Sequence (5' – 3')	T_m (°C)	Annealing Temperature (°C)
MDR/B1	**TGCATTTAGTTCAGATGATG**	**43.6**	**50**
MDR/B2	**AATGTTGCTACTTCTCTTC**	**50.1**	**50**
MDR/B3	**TGGTTTAGAAGATTATTTCTG**	**52.0**	**50**
MDR/B4	**AAATAACATGGGTTCTTGAC**	**47.4**	**50**

Table 1.3: Summary of mutation profiles for *pfcrt* in Mbita 2009.

Haplotype	M74I	N75E	K76T	n (114)	%	95% CI
MNK	-	-	-	19	16.67	7.8-23.0
MNT	-	-	+	70	61.40	47.6-70.4
MEK	-	+	-	1	0.88	0.4-1.8
MET	-	+	-	15	13.16	6.9-17.6
IEK	-	-	-	0	0	0
IET	-	-	-	0	0	0
MIXED	-	-	-	9	7.89	4.6-12.1
TOTAL				**114**	**100**	**100**

Table 1.4 Analysed polymorphisms in *pfmdr-1* gene at codons 1034, 1042 and 1246 by dot-blot hybridization for Mbita 2009 samples.

	***pfmdr-1* Codon 1246**			
Probe	**Wild Type (Asp)**	**Mutant (Tyr)**	**Mixed**	**Blank**
N	83	9	3	19

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