J. Protozool. Res. 21, 20-29 (2011)

Chloroquine resistance status a decade after: Re-emergence of sensitive *Plasmodium falciparum* strains in malaria endemic and epidemic areas in Kenya

Wangai, L.N[2, 3]., Kimani, F.T[1, 2]*., Omar, S.A[1]., Karanja, S.M[3]., Nderu, D.W[4]., Magoma, G[3]., Mutua, D[1].

[1]Kenya Medical Research Institute, Centre for Biotechnology Research and Development (KEMRI, CBRD), P.O. Box 54840-00200, Nairobi, Kenya,
[2]Institute of Tropical Medicine and Infectious Diseases (ITROMID), P.O. Box 54840-00200, Nairobi, Kenya,
[3]Jomo Kenyatta University of Agriculture and Technology (JKUAT), Biochemistry Department, P.O. Box 62000-00200, Nairobi, Kenya,
[4]Kenyatta University, P.O. Box 43844-00100, Nairobi, Kenya.
*Corresponding author: Kimani, F.T., E-mail:kimanift@yahoo.com or fkimani@kemri.org

ABSTRACT

Development and spread of chloroquine (CQ) resistance led to its withdrawal in most malaria endemic countries. In Kenya, this occurred in 1998 when clinical efficacy dropped below 50%. Less than a decade after CQ was removed from routine use in Malawi, the drug has reversed to activity and is again effective for first-line treatment of uncomplicated malaria. There is a probability of a similar reversed activity in Kenya for more 10 years of its absence in uncomplicated *Plasmodium falciparum* malaria treatment. The present study was aimed at establishing the CQ resistance status in the country, 10 years after its withdrawal, by looking at high malaria transmission zone, Mbita, a malaria endemic area and some malaria epidemic areas of the Kenyan highlands. The prevalence of T76 and Y86 *P. falciparum* molecular markers for CQ resistance in *Pfcrt* and *Pfmdr*1 genes were investigated by PCR-RFLP and dot blot analysis in 64 samples collected in March to May 2007 in the endemic area and 38 samples collected in April to July the same year in the epidemics. The study shows that 67.3% of field isolates from the endemic site still harbor Y86 mutation in *Pfmdr*1 while 32.7% have the wild type allele N86 compared to the 94% and 6 % prevalence observed in Mwea, an endemic area, in 2004 (χ^2=10.08, P=0.00015, 95% CI=2.085-27.8). In the epidemics 75% of field isolates from the epidemic sites still harbor Y86 mutation in *Pfmdr*1 while 25% have the wild type allele N86 compared to the 91.6% and 8.4% prevalence observed in an epidemic area in 1997 (χ^2=1.585, P=0.208, 95% CI=0.701-19.176). From the study there is a significant change in the proportions of the resistant genotypes in the endemic areas while in the epidemics, there was also a noticeable shift though not significant. This therefore indicates a slow but steady re-emergence of *P. falciparum* CQ sensitive strains in the country. Though does not warrant the reintroduction of CQ for malaria treatment.

Key words: *Plasmodium falciparum*; chloroquine (CQ); resistance; endemic; epidemic.

INTRODUCTION

Despite considerable efforts during the last century to eradicate or control *Plasmodium falciparum* malaria, it remains the most prevalent and devastating parasitic disease in the tropics. Worldwide, it causes 300-500 million clinical cases with estimates putting the yearly malaria burden at around 1 million deaths, 90% in sub-Saharan Africa (WHO, 2003). In the last decades, malaria control and treatment has been complicated by the rapid emergence of resistance to widely used anti-malarial drugs such as chloroquine (CQ). This is particularly dramatic for Africa as few alternative drugs that are cheap and safe as CQ are available. Drug pressure has been identified as one of the key factors in the emergence of resistance to anti-malarial drugs.

Selection of resistant strains can occur when a drug is misused or used alone extensively (Bloland and Ettling, 1999). CQ has been in use since the 1940s and it proved to be the most effective drug that was cheap and recorded little contraindications. Together with DDT, it became the most promising tool for total eradication of malaria. This yielded fruit in North America and Europe where malaria was eradicated whereas in Africa, no such efforts were mounted. These were impeded by DDT and CQ resistance in the *Anopheles* mosquito and malaria parasites respectively including DDT's adverse effects to the environment (Chapin and Wasserstrom,1981). Resistance of *P. falciparum* to CQ was first noted in the late 1950s from Colombia and Thailand (Pettersson *et al.*, 1981). Similar resistance patterns quickly followed from other countries in South America and South East Asia. The first well-documented case of CQ-resistant *P. falciparum* from Africa was reported from Kenya in 1979 (Pettersson *et al*, 1981). Wide spread resistance to CQ led to change of drug policy and sulfadoxine-pyrimethmine (SP) drugs replaced it as the first line drug (Shretta *et al*., 2000). However, resistance to SP drugs was reported almost spontaneously and has increased over the years. Currently drug combinations are thought to be the only alternative in the management of malaria cases so as to delay development of resistance. Resistance is known to arise due to mutations (White, 2004). Where known, these mutations can be used as molecular markers for monitoring drug resistance in *P. falciparum* useful either in predicting therapeutic outcome of a drug or target treatment in regions where *in vivo* studies are not possible (Djimdé *et al.*, 2004). Point mutations in a 13-exon gene, *crt*, in CQ-resistant-*P. falciparum* lines have implicated its product, *Pfcrt*, as the primary culprit behind CQ resistance (Djimdé *et al.*, 2001; Djimdé *et al.*, 2004; Mackinnon *et al*., 1998; Mutero *et al*., 1998). Other studies have shown a correlation of *in vitro* CQ resistance with the substitution of lysine for threonine at position 76 in field isolates and laboratory strains. This suggests that *Pfcrt* polymorphism is a useful tool for surveillance of CQ resistance. Other point mutations in the *Pfcrt* gene at positions 72 to 78, 97, 220, 323, 356 and 371 as well as mutations in other genes might be involved in the modulation of CQ resistance (Fidock *et al.*, 2000; Lim *et al.*, 2003; Tran and Saier, 2004). After the withdrawal of CQ for several years, there have been reports indicating reversion of *P. falciparum* to CQ sensitivity. In a study conducted in Malawi demonstrated a rapid decline of the resistant strain (Kublin *et al*., 2003; Laufer *et al*., 2006). However, in Kenya the decline has been slow; from 90%-60% for *Pfcrt*-76 mutant while there has been no significant decline for *Pfmdr*1-86 (Omar *et al*., 2007; Mwai *et al*., 2009).

The present study was aimed at establishing the CQ resistance status in the country, 10 years after its withdrawal as first line treatment drug, by looking at high malaria transmission zone, Mbita, a malaria endemic area and some malaria epidemic areas of the Kenyan highlands. Both restriction fragment length polymorphism and dot blot hybridization were used to determine the prevalence of molecular markers for CQ resistance. Although the resistance to CQ has long been established and its use as a first line of treatment stopped by the Kenyan government, inline with what has been observed in other countries, the drug still stands a chance of reuse. There was therefore a need to carry out a molecular study that would assess the current sensitivity level. The study hypothesized that point mutations at position 76 of the *Pfcrt* gene has not declined following withdrawal of CQ in Kenya. An association between *P. falciparum Pfmdr*1, which encodes a trans-membrane glycoprotein (Pgh1, for P-glycoprotein homologue 1) and the multi drug-resistant (MDR) phenotype was first reported in 1989 (Foote *et al*., 1989). Point mutations in *Pfmdr*1, most notably at amino acid 86, have been associated with decreased CQ sensitivity (Price *et al*., 1999; Agbonlahor *et al*., 2008). Furthermore, in a study involving genetic cross, CQ resistance was found to segregate with cg2 (located on chromosome 7) rather than with *Pfmdr*1 (located on chromosome 11) (Su *et al*., 1997). Amplification of the *Pfmdr*1 gene copy number is associated with resistance to mefloquine and halofantrine, both in laboratory and field isolates (Wilson *et al*., 1993). In *Plasmodium, mdr* homologues encoding P-glycoprotein–like molecules have been proposed as determinants of malaria drug resistance and associations have been reported between CQ resistance and

amplification or mutation of the mdr-like gene *Pfmdr*1, which encodes Pgh1 (Reed *et al*., 2000). The mutations is at amino acid 86 resulting in the substitution of tyrosine for asparagine (N86T) in the *P. falciparum* MDR (Foote *et al*., 1989). Thus the need to establish the presence of L76T mutation in the *P. falciparum* CQ resistance transporter (*Pfcrt*) gene and N86T in the *mdr* gene, a decade after withdrawal of CQ as first line treatment in high malaria transmission areas.

MATERIALS AND METHODS

Study site (geographical)

This present study was conducted from March to July in 2007 in Mbita, a rural village on the shores of Lake Victoria in Suba District, western Kenya. The main malaria vectors in the area are *An. gambiae*, *An. funestus* and *An. arabiensis*. Malaria transmission is high and perennial, (entomologic inoculation rate approximately six infectious bites per person per month) (Mutero *et al*., 1998) with *P. falciparum* as predominant parasite species accounting for more than 95% of the clinical malaria cases (Gouagna *et al*., 2003). With parasite prevalence in the human population ranging from 24.4% to 99.0% (Mutero *et al*., 1998). Generally, the rainfall pattern is bimodal, with a long rainy season between March and May and a short rainy season between October and December.

Several other low transmission or non transmission were included in the study sites list for control purposes (Lugari, Uasin gishu, Mosoriot, Embu, Nandi South, Nandi North, Narok, West pokot and Kericho in the Kenyan Higlands and some Kenyan arid areas).

Study population

Patients with *P. falciparum* mono-infection were enrolled for this study as part of a larger drug sensitivity study. The study protocol (SSC 948) was approved by the Scientific Steering Committee and the Ethical Review Committee of the Kenya Medical Research Institute. Written, informed consent was obtained from a parent or guardian of the participating children. Children aged 6 months to 12 years (144 months) visiting the outpatient clinic with signs of uncomplicated malaria and residing in the research area were recruited for the study. Inclusion criteria included patients aged between 6 months and 12 years, residents of research area, temperature > 37.5˚C and < 39.5˚C, or a history of fever in the previous 24 hours, understanding of the procedures of the study by parent or guardian and willing to participate (informed consent signed). Additional inclusion criteria for participation in the drug study included; diagnosed with uncomplicated malaria, *P. falciparum* only, parasitemia 1,000-100,000/µL (Giemsa-stained blood smears counted against 200 WBC, negative result if 100 parasite negative microscopic fields). The subject were excluded if they had; general danger signs of severe malaria or Hb count < 5 g/dL, severe malnutrition, diseases other than malaria causing febrile conditions, inability to take drugs orally, known hypersensitivity to any of the drugs given, reported treatment with antimalarial chemotherapy in the past 2 weeks, evidence of chronic disease or acute infection other than malaria, and domicile outside the study area and if they were unwilling to participate and sign informed consent forms.

Enrolment and blood sampling

A fingerprick blood sample for parasite detection was taken from children presenting at the outpatient clinic with symptoms indicating uncomplicated malaria. Name of the child, father and mother, age, weight and clinical symptoms including fever were recorded on a case record form (CRF) and/or the patient card distributed by the health centre. The blood sample was used to prepare thick and thin blood smears, to make dry blood spots for molecular analysis and to measure haemoglobin level by using HemoCue (HemoCue AB).

Blood smears were Giemsa-stained and parasites counted against 200 WBC, with parasite negative results based on screening of 100 microscopic fields. Parasitological data was added to the patient card. Children diagnosed with uncomplicated *P. falciparum* malaria and meeting all inclusion/exclusion criteria will be enrolled in this study and the main drug study after explaining the purpose and procedures of the study and obtaining informed consent from the parent(s) or guardian(s). All children not included in the study were referred back to the clinician with their patient cards for further diagnosis and treatment. They were treated as any other outpatient and received treatment as required.

DNA extraction and amplification of *Pfcrt* gene

DNA extraction was done on dried blood spots as described (Warhurst *et al*., 1991). The amplification of the *Pfcrt* gene was done on MJ Thermocycler™ PCR machine. PCR amplification (Djimdé *et al*., 2001) was done with modifications. The outer PCR: each reaction tube consisted x10 PCR buffer (Roche) to a final concentration of x1, 25 mM $MgCl_2$ to a final concentration of 1.5 mM, 20 mM dNTP mix to a final concentration of 2 mM, 10 µM each of CRTP1 (5'-CCG TTA ATA ATA AAT ACA CGC AC-3') (MWG Biotech) and CRTP2 (5'-CGG ATG TTA CAA AAC TAT AGT TAC-3') (MWG Biotech) to a final concentration of 100 nM, 5 U/µL *Taq* polymerase (Roche) to a final concentration of 1 U/reaction tube. The mixture was topped up to a volume of 25 or 30 µL with double distilled water. PCR programme run was set at 94°C for 3 minutes, 94°C for 30 seconds, 56°C for 30 seconds, 60°C for 1 minute, 45 cycles, final extension at 60°C for 3 minutes then held at 4°C. Nested PCR: each reaction tube consisted of 5 µL outer PCR product thawed on ice, x10 PCR buffer (Roche) to a final concentration of x1, 25 mM $MgCl_2$ to a final concentration of 1.5 mM, 20 mM dNTP mix to a final concentration of 2 mM, 10 µM CRTD1 (5'-TGT GCT CAT GTG TTT AAA CTT-3') (MWG Biotech) and CRTD2 (5'-CAA AAC TAT AGT TAC CAA TTT TG-3') (MWG Biotech) forward and reverse primers respectively each to a final concentration of 100 nM, 5 U/µL *Taq* polymerase (Roche) to a final concentration of 1 U/reaction tube. The mixture was topped up to a volume of 25 or 30 µL with double distilled water. PCR programme run was set at 94°C for 3 minutes, 94°C for 30 seconds, 48°C for 30 seconds, 64°C for 1 minute, 35 cycles, final extension at 64°C for 5 to 10 minutes then halted at 4°C. RFLP (Ranford-Cartwright *et al*., 2002) done using restriction endonuclease *Apo* I (New England Biolabs). Incubation was done for 12 to 14 hours. Each sample was mixed with 2 µL of ABgeneTM x6 type II DNA electrophoresis gel loading dye. Samples were then loaded on a 2% agarose gel in TAE buffer. (The dye consists of: 15% (w/v) Ficoll R400, 0.06% (w/v) Xylene cyanol FF 30 mM EDTA). The gel was run for 35 minutes at a voltage of 80 volts on gel electrophoresis tank from Biorad and then viewed on the gel/photo-documentation system.

Analysis of *Pfcrt* gene by dot blot hybridization

Nested PCR products were denatured using an alkaline denaturation reagent prepared to a final concentration of 10 mM EDTA and 0.4 M NaOH. Each reaction tube thus consisted of 20 µL nest II PCR product, 3 µL of 0.1 M ETDA, 3 µL of 4 M NaOH and 4 µL of distilled water. The mixture was boiled at 100°C for 10 minutes to ensure complete denaturation. Thereafter, the tubes were spanned briefly on a microfuge and the reaction was neutralized using 30 µL of 2 M ammonium acetate at pH 7. Thirty µL of each denatured sample were loaded onto two Gene screen membranes that had been cut and pre-wetted in TE buffer, layered onto the dot blot apparatus (BIORAD) carefully avoiding bubbles. Vacuum had been applied as the screws were tightened and the membrane rehydrated by adding 200 µL of TE buffer into each spot. The loaded samples were allowed to remain on the membrane for 30 minutes before releasing the vacuum. The membrane was then removed and neutralized by washing in x2 SSC for 60 seconds, then washed in 0.4 M NaOH for 60 seconds to ensure complete denaturation of immobilized DNA and rinsed in neutralizing solution (1 M Tris-HCl, 1.5 M

NaCl, pH8.0) for 30 seconds. The membrane was placed onto a glass plate and exposed to UV for 5 minutes in a UV cross linker to fix the DNA onto the membrane. The membrane was then air dried and wrapped in cling film, then stored at -20^{o}C.

Oligonucleotide probes, MNK, MNT, MEK, MET (MWG Biotech) were reconstituted according to the manufacturer's instruction to a final concentration of 100 pmol/µL. To label each probe, 25 µL of distilled water was first added to Ready-To-Go T4 PNK (Amersham Biosciences, UK) and incubated at room temperature for 5 minutes. One µL of 10 µM probe was then added, followed by 23 µL of water to make a total volume of 49 µL. One µl of [^{32}P]-γ-ATP was then added. The contents were then spanned briefly and incubated at 37^{o}C for 7 minutes. The reaction was then stopped by addition of 5 µL of 250 mM EDTA. Unincorporated [^{32}P]-γ-ATP was removed by spinning in resuspended microspin G-25 resin columns for two minutes at 3,000 rpm and the purified sample was collected at the bottom of the support tube. Each membrane was hybridized in 20 mL hybridization buffer (every 100 ml contained 25 mL of x20 SSPE, 10 ml of x50 Denhardt's reagent, 5 mL of 10% SDS, 59.9 mL of DNase-free water and 0.1 mL (100 µL) of 10 mg/mL salmon sperm (Salmon sperm DNA), sonicated) (Gibco BRL®) in a hybridization bottle. The membranes were stringently washed with agitation in 20 ml of x2 SSC for 15 minutes, then in 20 mL of x1 SSC/0.1% SDS for 10 minutes. The membranes were loaded into cassettes and Kodak X-ray films in the darkroom. The X-ray films were exposed to -70^{o}C for 12 to 24 hours and developed thereafter. The labeled probes were stripped off by incubating the membrane in excess 0.1 M NaOH with agitation, at room temperature for 15 minutes twice followed by a brief wash with x5 SSC. These were then stored at -20^{o}C or reprobed again.

Analysis *of Pfmdr*1 gene.

The amplification of the *Pfmdr*1 gene was done using MDR/A1 (5'-TGT TGA AAG ATG GGT AAA GAG CAG AAA GAG-3') (MWG Biotech) and MDR/A3 (5'-TAC TTT CTT ATT ACA TAT GAC ACC ACA AAC-3') (MWG Biotech) as forward and reverse primers respectively. The samples were then loaded onto the thermocycler set at 94^{o}C for initial denaturation for 3 minutes. This was followed by denaturation at 94^{o}C for 1 minute, annealing temperature of 45^{o}C for 30 seconds, 72^{o}C extension. These conditions were repeated for 40 cycles followed by a final extension for 3 minutes at 72^{o}C, then halted at 4^{o}C.

Nested PCR was done using MDR/A2 (5'-GTC AAA CGT GCA TTT TTT ATT AAT GAC CAT TTA-3') and MDR/A4 (5'-AAA GAT GGT AAC CTC AGT ATC AAA GAA GAG-3') forward and reverse primers respectively. Nest 1 PCR products were thawed on ice and 5 µL from each tube was transferred into labeled sterilized PCR tube. In addition, each reaction tube consisted x10 PCR buffer to a final concentration of x1, 25 mM magnesium chloride to a final concentration of 1.5 mM, 20 mM dNTPs to a final concentration of 200 µM, 10 µM each MDR/A2 and MDR/A4 to a final concentration of 100 nM, 5 U/µL *Taq* polymerase (Roche) to a final concentration of 1 U/reaction tube. The mixture was topped up to a volume of 25 or 30 µL with double distilled water. PCR was then run with the initial denaturation being set at 94^{o}C. This was followed by a denaturation temperature of 94^{o}C for 30 seconds, annealing temperature of 45^{o}C for 1 minute and extension at 72^{o}C for 1 minute. These steps were repeated for 40 cycles and then followed by a final extension at 72^{o}C for 3 minutes before halting the reaction at 4^{o}C.

The nested PCR products (MDR/A2 and MDR/A4) were digested using *Apo* I (New England Biolabs) following the procedure described above. The digest products were then electrophoresed on 1.5% agarose gel at 80 volts for 30 minutes.

Dot blot hybridization of nested *Pfmdr*1 products was done as per procedure described above. Probes, *Pfmdr*1-86Asn (N) and *Pfmdr*1-86Tyr (Y) (MWG Biotech) were reconstituted according to the manufacturer's instruction and used in hybridization procedure.

RESULTS

SITE / YEAR	*Pfcrt*							
	IEK	IET	XD	MEK	MET	XD	BLANK	MNK
EPIDEMICS /2007	-	15	-	3	11	1	23	4
OYUGIS /1998	-	32	-	4	22	8	1	15
MBITA /2007	-	-	-	7	32	21	4	33

XD- mixed

*Pfmdr*1 and *Pfcrt* score summary

A total of 137 samples were analyzed by dot-blot hybridization for polymorphisms in the CQ resistance molecular markers, *Pfcrt* and *Pfmdr*1 genes. These comprised of 38 samples from epidemic sites (collected in 2007), 64 samples from Mbita (collected in 2007) an endemic site and 35 samples from Oyugis (collected in 1998) an endemic site. None of the samples from both Oyugis and epidemic zones hybridized with IEK probe. In Mbita IEK and IET were not done. However, 15 (39.50%) and 32 (91.42%) samples from epidemic sites and Oyugis, respectfully, hybridized to the IET probe.

When hybridized with the MEK probe, only 3 samples (7.9%) from epidemic zones, 4 samples (11.42%) from Oyugis and 7 samples (10.9%) from Mbita hybridized. Eleven (28.94%), 22 (62.86%) and 32 (50.00%) samples from epidemic sites, Oyugis and Mbita respectfully hybridized to the MET probe. One (2%) sample from sites, 8 (22.36%) samples from Oyugis and 21 (32.81%) samples from Mbita hybridized to both MEK and MET.

Four (10.53%), 15 (42.86%) and 33 (51.56%) samples from epidemic sites, Oyugis and Mbita respectively hybridized to the MNK probe. Three, 4 and 5 samples from epidemic sites, Oyugis and Mbita respectively hybridized to both MEK and MNK. Two samples each from epidemic sites and Oyugis showed IET, MEK and MNK polymorphism. Eight samples from Oyugis and one sample from epidemic sites showed IET, MEK, MET and MNK polymorphisms while 3 samples in Oyugis had IET, MET and MNK.

SITE / YEAR	*Pfmdr*1			
	TYROSINE	ASPARAGINE	XD	BLANK
EPIDEMICS / 2007	10	8	14	6
OYUGIS / 1998	9	2	10	14
MBITA / 2007	20	18	17	9

Dot-blot analysis of samples from epidemic sites for the *Pfmdr*1 gene gave 10 (26.32%) tyrosine mutant polymorphism, 8 (21.05%) had asparagine and 14 (36.84%) samples had both tyrosine and asparagine. Six (15.78%) samples however did not hybridize to any of the probes.

Analysis of samples from Oyugis showed that 9 (25.71%) samples had tyrosine only, 2 (5.71%) carried asparagine alone while 10 (28.57%) had both tyrosine and asparagine. Fourteen (40%) samples did not hybridize with either of the probes.

Analysis of samples from Mbita had no samples with tyrosine only, 18 out of 56 (32.14%) carried

asparagine alone while 37 out of 56 (66.07%) had both tyrosine and asparagine. One out of 56 (1.79%) samples did not hybridize with either of the probes.

Sites/Year	Chi test	P- Value	95% CI	OR
Mwea 2004 vs Mbita 2007	10.08	0.00015	2.09 - 27.86	7.62
Mbita 2007 vs Busia 1998	13.40	0.0003	0.09 - 0.46	0.20
Epidemics 2007 vs 1997	1.59	0.208	0.70 - 19.18	3.67
Mbita vs Epidemics 2007	0.27	0.6056	0.26 - 1.82	0.69

Comparing the incidence of the resistant genotypes over the different time periods and transmission zones gave the values in the table above. Between Mwea 2004 and Mbita 2007 both of which are malaria endemic areas, the OR was 7.62 (P=0.00015) indicating a significant decline in the resistant genotype in 2007. The same trend was observed after comparing this with Busia 1998 giving an OR of 0.2 (P=0.0003). In the epidemics, comparison over the 10 years time period gave an OR of 3.667 (P=0.208) while comparing these with the endemics in the same year (2007) the OR was 0.6852 (P=0.6056).

DISCUSSION

Samples collected from epidemic sites and endemic site in 2007 showed that mutation in amino acid 76 of the *Pfcrt* genes suggesting parasites from these sites are likely to be resistant to CQ. The *Pfcrt* 76T mutation has been found in both CQ sensitive and CQ resistant strains (Omar, 2002). Since it has been perfectly associated with CQ resistance in *in vitro* studies, it is possible that the pharmacokinetics and pharmacodynamics of the drug in an individual, and their immune status would determine the ultimate treatment outcome. This is mainly applies to areas of high transmission where individuals are likely to have acquired immunity to asexual stages of the parasite. Studies in Mali have shown that presence of *Pfcrt* 76T can be used as a predicator of treatment outcome in areas of low transmission. The regions lack semi-immune asymptomatic individuals and transmission is only from symptomatic patients (Djimde *et al*., 2001). Moreover the results show that there is co-existence of more than one polymorphism in amino acids 74, 75 and 76 in both endemic and epidemic sites. That mutant forms still exist 10 years after cessation of CQ use in the endemic sites in Kenya suggesting that mutation at position 76 may have become stable or alternatively there could be compensatory mutations in the *Pfcrt* gene that have maintained the functional integrity of the Pfcrt1 protein. This would negate the need for reverse mutations since there hasn't been any loss of fitness of fit. If this were the case, then there hasn't been any need to select against mutant parasites hence their continued survival.

The result from epidemic sites also suggests that there is co-existence of IET, MEK, MET and MNK polymorphism. The fact that some samples hybridized with all these probes indicate mixed clones of the parasite population within the samples.

Studies on Cambodian isolates using PCR followed by sequencing have revealed that there are more polymorphisms in this region of the *Pfcrt* gene than what was initially thought. The initial assumption was that all field isolates of *P. falciparum* from this country where CQ resistant phenotypes was first observed had IET or IEK polymorphisms. The study by Lim *et al.* (2003) identified six distinct *Pfcrt* haplotypes at amino acids 72 to 78 and 218 to 220. Four of these, CVTNTIF//ISS (methionine is replaced with threonine at position 74), CVIDTIF//ISS (aspartic acid replaced asparagine at amino acid 75), CVMNTIF//ISA and CVIETTF//ISS, had never been observed before, indicating that the extent of *Pfcrt* polymorphism is larger than reported so far. The

fact that this Cambodian study used samples from close regions implies that even a single locus can carry *P. falciparum* parasites with different genotypes. This study was conducted 20 years after withdrawal of CQ as first line treatment in Cambodia (Lim *et al.*, 2003).

It has been shown that mutations in the *Pfcrt* 75 and 76 amino acids are key to defining the CQ resistant phenotype. Thus the presence of this mutations in the Mwea samples suggest that CQ treatment is likely to fail, 10 years after cessation of use of the drug as first line of treatment. The good news is that there is possibility of reversal since these are revertant mutations. The reversal can only possibly occur if the compensatory mutations fail to maintain fitness of the mutant genotypes in the absence of drug pressure. This means that the cost of fitness of fit is raised in the mutant genes so that individual parasites carrying these mutations fail to survive or get disadvantaged when drug pressure is removed. The long-term maintenance of *P. falciparum* CQ resistance markers in Kenya just as in Cambodia, more likely results from lack of loss of fitness associated to the CQ resistance phenotype rather than from continued drug pressure. Findings from Malawian studies suggest that CQ resistance mutations in *Pfcrt* gene come at a cost and are less likely to be selected in absence of drug pressure. The high prevalence of the mutant allele may also be due to slow reversal rate.

*Pfmdr*1 Y86 mutations together with *Pfcrt* 76T have been shown to have a stronger association with both CQ treatment and in *in vitro* sensitivity assay outcomes. Thus a high prevalence of the two mutations in field isolates from the same population is more likely to predict *in vivo* treatment outcomes in the field. These mutations have a higher predictive value when present together.

The present study has revealed that the high prevalence of *Pfcrt* 76T and *Pfmdr*1 86Y mutations imply that CQ resistance is still high in all the sites studied. Comparing the incidence of the resistant genotypes over the different time periods and transmission zones gave the values which indicated a significant decline in the resistant genotype in 2007. Comparison over the 10 years time period in the epidemics and in the endemics showed a similar trend. Hence, our study reveals the slow reversal to the wild type allele and hence the CQ sensitivity in these region. This is in line with what was previously reported of the Kenyan coastal endemic area where it was demonstrated that CQ resistance had been on decline and this decline will take 13 more years for the clinical efficacy of CQ to be restored (Mwai *et al.,* 2009). As indicated, it appears that the CQ ban in Kenya was both too late and was ineffectively applied, so that it gave rise to the use of other drugs such as AQ, as well as continued use of CQ, thereby maintaining selection for CQ resistance. Thus the effectiveness of drug policy implementation can have important and far-reaching effects on the useful life of life-saving drugs. The newer policies of using drugs in combinations in order to prolong the resistance-free period is a good (Mackinnon and Hastings, 1998; White, 2004).

ACKNOWLEDGEMENTS

We owe profound gratitude to the members of staff and students in the Malaria lab, P3 lab and Radioisotope lab for all the technical assistance they offered. The Director KEMRI for supporting this work and making it's publication a success. The International Atomic Energy Agency (IAEA) for funding the project and sending the supplies on time. JKUAT for financial support.

REFERENCES

Agbonlahor, D.E., Mirabeau, T.Y., Faith, O., Inoigbe, O.P., Samuel, T.K. and Clarence, Y.S. 2008. High prevalence of *Plasmodium falciparu*m Pfmdr1 86Y mutant gene in sickle cell disease in Nigeria. J. Microbiol. Immunol. Infect. 41: 393-396.

Bloland, P.B. and Ettling, M. 1999. Making malaria-treatment policy in the face of drug resistance. Ann. Trop. Med. Parasitol. 93**:** 5-23.

Chapin, G. and Wasserstrom, R. 1981. Agricultural production and malaria resurgence in Central America and India. Nature. 293: 181–185.

Djimdé, A., Doumbo, O.K., Cortese, J.F., Kayentao, K., Doumbo, S., Diourté, Y., Dicko, A., Su, X.Z., Nomura, T., Fidock, D.A., Wellems, T.E., Plowe, C.V. and Coulibaly, D. 2001. A molecular marker for chloroquine-resistant *falciparum* malaria. N. Engl. J. Med. 334: 257-263.

Djimdé, A.A., Dolo, A., Ouattara, A., Diakité, S., Plowe, C.V. and Doumbo, O.K. 2004. Molecular diagnosis of resistance to antimalarial drugs during epidemics and in war zones. J. Infect. Dis. 190: 853-855.

Fidock, D.A, Nomura, T., Talley, A.K., Cooper, R.A., Dzekunov, S.M., Ferdig, M.T., Ursos, L.M., Sidhu, A.B., Naudé, B., Deitsch, K.W., Su, X.Z., Wootton, J.C., Roepe, P.D. and Wellems, T.E. 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein *PfCRT* and evidence for their role in chloroquine resistance. Mol. Cell. 6: 861-871.

Foote, S.J., Thompson, J.K., Cowman, A.F. and Kemp, D.J. 1989. Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. Cell. 57: 921-930.

Gouagna, L.C., Okech, B.A., Kabiru, E.W., Killeen, G.F., Obare, P., Ombonya, S., Bier, J.C., Knols, B.G., Githure, J.I. and Yan, G. 2003. Infectivity of *Plasmodium falciparum* gametocytes in patients attending rural health centres in western Kenya. East Afr. Med. J. 80: 627-634.

Kublin, J.G., Cortese, J.F., Njunju, E.M., Mukadam, R.A., Wirima, J.J., Kazembe, P.N., Djimdé, A.A., Kouriba, B., Taylor, T.E. and Plowe, C.V. 2003. Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. J. Infect. Dis. 187: 1870-1875.

Laufer, M.K., Thesing, P.C., Eddington, N.D., Masonga, R., Dzinjalamala, F.K., Takala, S.L., Taylor, T.E. and Plowe, C.V. 2006. Return of chloroquine antimalarial efficacy in Malawi. N. Engl. J. Med. 355: 1959-1966.

Lim, P., Chy, S., Ariey, F., Incardona, S., Chim, P., Sem, R., Denis, M.B., Hewitt, S., Hoyer, S., Socheat, D., Merecreau-Puijalon, O. and Fandeur, T. 2003. polymorphism and chloroquine resistance in *Plasmodium falciparum* strains isolated in Cambodia. Antimicrob. Agents Chemother. 47: 87-94.

Mackinnon, M.J. and Hastings, I.M. 1998. The evolution of multiple drug resistance in malaria parasites. Trans. R. Soc. Trop. Med. Hyg. 92: 188-195.

Mutero, C.M., Ouma, J.H., Agak, B.K., Wanderi, J.A. and Copeland, R.S. 1998. Malaria prevalence and use of self-protection measures against mosquitoes in Suba District, Kenya. East Afr. Med. J. 75: 11-15.

Mwai, L., Ochong, E., Abdirahman, A., Kiara, S.M., Ward, S., Kokwaro, G., Sasi, P., Marsh, K., Borrmann, S. Mackinnon, M and Nzila, A. 2009. Chloroquine resistance before and after its withdrawal in Kenya. Malar. J. 8:106.

Omar S.A. Drug Susceptibility and Molecular Characterization of Epidemic and Endemic Malaria in Kenya." Ph.D Thesis; University of London. 2002: 176-182.

Omar S.A, W. M Francis, A. M Fat'hia, F. T Kimani and G. Magoma 2007. Prevalence of *Plasmodium falciparum* chloroquine resistant gene markers, Pfcrt-76 and pfmdr1-86, eight years after cessation of chloroquine use in Mwea, Kenya. The Journal of Infectious Diseases in developing countries 1: 195-201.

Pettersson, T., Kyrönseppä, H. and Pitkänen, T. 1981. Chloroquine-resistant *falciparum* malaria from East Africa. Trans R Soc Trop Med Hyg. 75: 112-113.

Price, R.N., Cassar, C., Brockman, A., Duraisingh, M., van Vugt, M., White, N.J., Nosten, F. and Krishna, S. 1999. The *pfmdr1* gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. Antimicrob Agents Chemother. 43: 2943-2949.

Ranford-Cartwright, L.C., Johnston, K.L., Abdel-Muhsin, A.M., Khan, B.K. and Babiker, H.A. 2002. Critical

comparison of molecular genotyping methods for detection of drug-resistant *Plasmodium falciparum*. Trans. R. Soc. Trop. Med. Hyg. 96: 568-572.

Reed, M.B., Saliba, K.J., Caruana, S.R., Kirk, K. and Cowman, A.F. 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature. 403: 906-909.

Shretta, R., Omumbo, J., Rapuoda, B. and Snow, R.W. 2000. Using evidence to change antimalarial drug policy in Kenya. Trop. Med. Int. Health. 5: 755-764.

Su, X., Kirkman, L.A., Fujioka, H. and Wellems, T.E. 1997. Complex polymorphisms in an approximately 330 kDa protein are linked to chloroquine-resistant *P. falciparum* in Southeast Asia and Africa. Cell. 91: 593-603.

Tran, C.V. and Saier, M.H. 2004. The principal of chloroquine resistance protein of *Plasmodium falciparum* is a member of the drug/metabolite transporter super-family. Microbiology. 150: 1-3.

Warhurst, D.C., Awad el Kariem, F.M. and Miles, M.A. 1991. Simplified preparation of malarial blood samples for polymerase chain reaction. Lancet. 337: 303-304.

White, N.J. 2004. Antimalarial drug resistance. J. Clin. Invest. 113**:** 1084-1092.

Wilson, C.M., Volkman, S.K., Thaithong, S., Martin, R.K., Kyle, D.E., Milhous, W.K. and Wirth, D.F. 1993. Amplification of *pfmdr1* associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand. Mol. Biochem. Parasitol. 57: 151-160.

World Health Organization. 2003. Africa Malaria report.