THE PREVALENCE OF PLASMODIUM FALCIPARUM POINT MUTATIONS ASSOCIATED WITH RESISTANCE TO CHLOROQUINE AND ARTEMISININ IN LUSAKA URBAN DISTRICT

By

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A dissertation submitted to the University of Zambia in partial fulfillment of the requirements of the degree of Master of Science in Medical Parasitology.

University of Zambia
June, 2008
Declaration

I Enesia Banda Chaponda declare that this dissertation represents my own work and that it has not previously been submitted for a degree, diploma or other qualification at this or another university.

Signature........................................ Date..................
This dissertation of Enesia Banda Chaponda has been approved as fulfilling the partial requirement for the award of Master of Science in Medical Parasitology at the University of Zambia.

Examiner's signature

Date

27-06-2008
ABSTRACT

Malaria is the leading cause of morbidity and mortality in Zambia where 95 percent of the cases are caused by Plasmodium falciparum. Chloroquine, the former drug of choice for uncomplicated malaria treatment became less effective as a result of parasite resistance. Therefore in 2002, the Malaria Treatment Policy was revised to replace chloroquine with Coartem® as first line treatment for uncomplicated malaria except in children below 10 kilograms of body weight and pregnant women for which the drug is not registered.

This decision was based on in vivo efficacy studies conducted in various sentinel sites around the country between 1995 and 2000. These studies showed treatment failure rates higher than 25 percent prompting the Government of the Republic of Zambia to change the Malaria Treatment Policy as recommended by the World Health Organization. Early and late treatment failure figures were summed up to obtain resistance rates. In 1996, the resistance figure for Lusaka Province represented by Chongwe district was determined at 43.4 percent. However the molecular epidemiology of the chloroquine resistance marker was not established. Therefore, there is still a need for base line information on the prevalence of the chloroquine resistance marker in Zambia.

A point mutation at amino acid codon 76 of P. falciparum chloroquine resistance transporter (Pfcrt) gene is a molecular marker of resistance to chloroquine (Djimde et al. 2001). While a mutation in the sarcoplasmic reticulum calcium dependant Plasmodium falciparum adenosine triphosphatase6 (SERCA-PfATPase6) gene at nucleotide 2307 is the putative molecular marker of resistance to artemisinin (Jambou et al., 2005).
The general objective of this study was to determine the prevalence of the *P. falciparum* point mutations associated with chloroquine and artemisinin resistance in Lusaka Urban district.

The prevalence of the chloroquine and the artemisinin resistance markers was measured in a cross-sectional survey in Lusaka urban district. A total of 161 blood samples were collected from patients onto filter paper and air-dried. Parasite DNA was extracted by the chelex extraction method from air-dried filter papers. Nested polymerase chain reaction (PCR) followed by restriction enzyme digestion was carried out. The digestion products were analyzed by electrophoresis on ethidium bromide stained 2 percent agarose gel and visualized under ultra-violet (UV) transillumination.

Of 119 individuals interviewed in Lusaka Urban district 3 (2.5 percent) had used chloroquine for malaria after the withdrawal of chloroquine from health centres in Lusaka Urban District. The number of respondents that had taken Coartem® at the time of the study since its introduction in Zambia was 38 (31.9 percent). The *Pfert* K76T mutation was detected in 53.8 percent of the field samples assayed. The point mutation in the SERCA-ATPase6 associated with *P. falciparum* resistance to artemisinin was not detected in any of the samples.

Coartem® is available both in health centres and drug stores in Lusaka Urban District. Chloroquine is no longer in stock in government health centres but is still in stock in some drug stores in Lusaka Urban District. The chloroquine-resistant K76T marker is still prevalent in Lusaka urban district five years after the malaria treatment policy was changed from the use of chloroquine to the use of Coartem® as first line treatment for uncomplicated malaria. The findings indicate that if the persistence of chloroquine-resistant *P. falciparum* malaria is to be avoided, the reintroduction of chloroquine, either as a monotherapy or in
combination with other drugs, should not be considered at present. The SERCA-PfATPase6 mutation reported to be associated with *P. falciparum* resistance to artemisinin (Jambou et al., 2005) was not observed in this study. The absence of the artemisinin resistance mutation seems to suggest total *P. falciparum* sensitivity to artemisinin, unless a different resistance mechanism occurs in the area. This can therefore serve as a base-line for future monitoring of artemisinin sensitivity.
To my husband Joseph
Acknowledgement

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<th>Description</th>
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<tbody>
<tr>
<td>ACT</td>
<td>Artemisinin combination therapy</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>CBoH</td>
<td>Central Board of Health</td>
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<tr>
<td>CDC</td>
<td>Center of Disease Control and Prevention</td>
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<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>CSO</td>
<td>Central Statistical Office</td>
</tr>
<tr>
<td>LUDHMT</td>
<td>Lusaka Urban District Health Management Team</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>n.d</td>
<td>No date</td>
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<tr>
<td>NMCC</td>
<td>National Malaria Control Centre</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pfmdrl</td>
<td><em>Plasmodium falciparum</em> multi-drug resistance 1</td>
</tr>
<tr>
<td>Pfcrt</td>
<td><em>Plasmodium falciparum</em> chloroquine resistance transporter</td>
</tr>
<tr>
<td>SERCA-PfATPase6</td>
<td>Sarco/endoplasmic reticulum calcium dependent <em>Plasmodium falciparum</em> adenosine triphosphatase6</td>
</tr>
<tr>
<td>SP</td>
<td>Sulphadoxine-Pyrimethamine</td>
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TBE  Tris-Borate-EDTA
UV    Ultra- Violet
WHO   World Health Organization
µl    Microlitre
1.0 INTRODUCTION.

Malaria persists as a leading cause of morbidity and mortality in Zambia, claiming more than 4 million clinical cases and 50,000 deaths annually on the country’s population of 10.6 million people, (Central Board of Health, 2003; Central Statistical Office, 2000).

In Zambia *Plasmodium falciparum* accounts for approximately 95 percent of malaria cases, with *P. malariae* contributing 3 percent and *P. ovale* 2 percent. *P vivax* is rare in Zambia. Chloroquine (CQ), the former drug of choice for treatment of uncomplicated malaria for the last four decades, became less effective as a result of parasite resistance (Central board of Health, n.d).

Schnitzer and Gruberg (1957), who were faced with the necessity of selecting a definition for resistance that could be applied to any micro-organism, suggested the following: “Drug resistance manifests itself after exposure of the organism to an antimicrobial agent either in vivo or in vitro. It can be defined as the temporary or permanent loss of the initial sensitivity of the micro-organisms to the effect of the active substance.”

A different approach was taken by Covelle *et al.* (1955), who defined drug-resistant malaria from a clinical point of view. The important feature of their definition is their recognition that the limit of resistance in clinical practice is the maximum dose of a drug which the host will tolerate. According to Covelle *et al.* (1955) drug resistance is the ability of the malaria parasite species to withstand the action of drugs administered
to the vertebrate host in adequate and safe doses, that normally destroy or contribute to
the destruction of the parasites at some stage or other in their life-cycle.

In 1963 a special committee of the World Health Organisation (WHO) set out to select
a more precise definition of the term “resistance” and they differentiated “resistance”
from “treatment failure”.

They defined drug resistance as “the ability of a parasite strain to multiply or survive
in the presence of concentrations of a drug that normally destroy parasites of the same
species or prevent their multiplication”. They defined treatment failure as the absence
or insufficiency of drug action after administration of a normally effective dose.

According to Peters (1970), it is important to discriminate between such causes of drug
failure as insufficient absorption, unusual rate of degradation or excretion of the drug,
and parasite resistance. Therefore, according to these definitions, parasite resistance is
only one cause of treatment failure.

When some of the difficulties that the earlier definitions entailed were realised, the
WHO Scientific Group in their report on resistance of malaria parasites to drugs
(WHO, 1965), modified the definition of parasite resistance that was in the 1963 report
as “the ability of a parasite strain to survive and/or multiply despite the administration
and absorption of a chemotherapeutic drug in doses equal to or higher than those
usually recommended but within the limits of tolerance of the subject”.

*In vivo* efficacy studies that were conducted in different parts of Zambia between 1995
and 2000 demonstrated levels of CQ resistance ranging from 24% to 52% (CBoH and
NMCC, 2000). In an effort to reduce the malaria disease burden, the Ministry of
Health adopted the use of anti-malarial combination therapy strategy as a replacement
for the monotherapy options in management of uncomplicated malaria. Artemether-lumefantrine, under the trade name Coartem®, an artemisinin combination therapy (ACT) was introduced as the first line drug for the management of uncomplicated malaria except in children below 10kg of weight and pregnant women. During the transition period, sulphadoxine-pyrimethamine (SP) was to be used as first line drug and quinine as the second line drug for treatment of malaria (Central Board of Health, 2002). Artemether-lumefantrine has a broad stage activity on the parasite ranging from very young ring forms to mature schizonts. It is well tolerated and has a shorter parasite clearance time than CQ (Duraisingham et al., 2000).

The presence of parasites in a malaria patient after treatment can either be as a result of re-infection or as a result of parasite resistance to the anti-malarial drug. Parasite resistance can only be concluded if the same genotype of the parasite is found before and after treatment by means of molecular methods (Viriyakosol et al., 1995).

In their study, carried out in Malawi, Kublin et al. (2003) showed that the Plasmodium falciparum chloroquine resistance transporter (PfCRT) gene mutation persisted eight years after CQ had been replaced with sulphadoxine-pyrimethamine nationwide but the prevalence of the chloroquine-resistant PfCRT genotype decreased from 85 percent in 1992 to 13 percent in 2000. Therefore it is assumed that if the CQ resistance marker was prevalent in Zambia when CQ was in use it is unlikely that it has disappeared three years after the withdrawal of CQ in the country.

Artemisinin derivatives are an essential component of treatment against multidrug-resistant falciparum malaria. In their study Jambou et al. (2005), aimed at investigating in-vitro resistance to artemisinin in field isolates from Senegal, French Guiana and Cambodia and this study supports the sarco/endoplasmic reticulum calcium-dependant
*Plasmodium falciparum* adenosine triphosphatase6 (*SERCA-PfATPase6*) as the target for artemisinins. The resistant isolates came mostly from areas with uncontrolled use of artemisinin derivatives, and according to Jambou *et al* (2005) this rise in resistance indicates the need for increased vigilance and a coordinated and rapid deployment of drug combinations.

**2.0 STATEMENT OF THE PROBLEM**

Based on results from *in vivo* efficacy studies (Central Board of Health, 2002), the National Malaria Treatment Policy of Zambia 2002 was revised to replace CQ with artemether-lumefantrine as the first line malaria treatment drug. The decision was based on treatment failure rates higher than 25 percent as standard threshold for changing first line drugs (WHO, 1993). This creates a need for base-line information on the molecular epidemiology of chloroquine-resistant *P. falciparum* which can be useful in the monitoring of chloroquine resistant falciparum malaria.

**3.0 STUDY JUSTIFICATION.**

This study is important in that it generated baseline information on the frequency of the chloroquine and the putative artemisinin resistance markers in Lusaka urban district. This study provides evidence to support the need for the establishment of the procedure for monitoring drug resistance in Zambia. Data obtained from this study can be used as a reference point for future monitoring of resistance markers for chloroquine and artemisinin in Lusaka Urban District.
4.0 LITERATURE REVIEW

The World Health Organization recommends that any country with a total treatment failure rate of 25 percent or more should change its first line drug (WHO, 1993). By 1999 in vivo efficacy studies in which early and late treatment failure rates were combined to obtain Zambian CQ resistance figures showed resistance rates as high as 52 percent in some areas. For example Choma and Isoka district reported CQ resistance rates of 52 percent and 54 percent respectively (NMCC and CBoH, 2000).

Chloroquine resistance has been linked to a number of mutations in the P. falciparum genes. Earlier studies suggested an association between CQ resistance and the P. falciparum multidrug resistance (Pfmdr1) gene located on chromosome 5 and encoding the P-glycoprotein 1 (Pgh-1) (Wilson et al., 1989; Cox et al., 1995; Barnes et al., 1992). Resistance of P. falciparum to chloroquine was thought to be linked to point mutations, such as the asparagine-to-tyrosine substitution at position 86 (Foote et al., 1990). Other probably compensatory point mutations at position 184, 1034 1042 and 1246 were also thought to be linked to resistance to chloroquine (Reed et al., 2000).

However, the results from field studies of the association of these mutations with in vivo and in vitro chloroquine resistance were not consistent. Subsequent studies identified other mutations in different genes of chromosome 7 (Wellem et al., 1990). Polymorphisms in one gene, a single candidate gene (cg2) were highly associated with chloroquine resistance (Su et al., 1997; Adagu et al., 1999) but allelic modification experiments ruled out the role of this gene in chloroquine resistance (Fidock et al., 2000a). However the observed association was later disapproved and it was concluded that the association was probably due to the close proximity of cg2 to the Plasmodium falciparum chloroquine resistance transporter (Pfcrt) gene encoding the P. falciparum
CQ resistance–related transporter protein on chromosome 7 (Fidock et al., 2000b). The involvement in the modulation of resistance of other mutations in the Pfcrtr gene at positions 72 to 78, 97, 220, 271, 326, 356, and 371, as well as mutations in Pfmdrl was suggested in a study by Reed et al. (2000).

Duraisingh et al. (1997) found an association between Pfmdrl mutations and chloroquine resistance. However, CQ resistance was shown to segregate independently of the Pfmdrl gene, following a genetic cross between a CQ-sensitive parasite, P. falciparum HB3, and a CQ-resistant one, DD2; in addition the absence of a clear association between Pfmdrl and CQ responses in natural parasite populations strongly suggested the involvement of other gene(s) mutations. It was shown that CQ resistance was not linked to mdr-like genes in a P. falciparum cross (Wellem et al., 1990) and that alleles of Pfmdrl gene were not associated with CQ resistance in India (Bhattacharya et al., 1997).

Later evidence strongly suggested that the mechanism of P. falciparum resistance to chloroquine is linked to the Pfcrtr gene, especially the substitution of threonine for lysine at position 76 (Djimdé et al., 2001). Djimdé et al. (2001) concluded that chloroquine resistance involves a progressive accumulation of mutations in the Pfcrtr gene, and the mutation at position 76 seemed to be the last one in the long process leading to chloroquine clinical failure. It was also concluded that the Pfcrtr T76 mutation can be used as a marker in surveillance of chloroquine resistance. This marker was successfully used in Malawi for CQ resistance surveillance (Kublin et al., 2003).

Widespread multi-drug-resistant P. falciparum malaria led WHO to recommend combination therapy as first line treatment, with formulations containing an
artemisinin compounds as policy standard. Artemisinin and its derivatives are the most potent and rapidly acting antimalarials. However, artemisinin resistance has been reported in murine models of malaria (Ferrer et al., 2004). Therefore diligent surveillance is needed to monitor continued susceptibility to artemisinin derivatives in endemic areas.

*In vitro* susceptibility of *P. falciparum* isolates from Cambodia, French Guiana and Senegal, countries with different artemisinin use, was assessed and DNA sequencing in a sub-sample of 60 isolates lends support to sarcoplasmic reticulum calcium-dependent *P. falciparum* adenosine triphosphatase6 (SERCA) *PfATPase6* as the target for artemisinins (Jambou et al., 2005).

Reduced susceptibility is not synonymous with diminished therapeutic effectiveness, but it is the probable first step of an alarming cascade and definitely pleads for increased vigilance and a coordinated and rapid deployment of drug combinations (Jambou et al, 2005).

5.0 HYPOTHESIS

The hypotheses for this study were:

(a) The CQ resistance marker K76T is not present in *P. falciparum* infections in Lusaka Urban district.

(b) The putative artemisinin resistance marker is not present in *P. falciparum* infections in Lusaka Urban District
6.0 GENERAL OBJECTIVE

The general objective of this study was to determine the prevalence of *P. falciparum* point mutations associated to with resistance to chloroquine and artemisinin in Lusaka Urban District.

6.1 Specific Objectives

The specific objectives of the study were to:

6.1.1 Determine the extent to which CQ is still in use by individuals and health facilities in Lusaka Urban District.

6.1.2 Identify falciparum malaria infections using microscopy and PCR.

6.1.3 Determine the prevalence of *Pfcrt* 76T molecular marker for CQ resistant falciparum malaria in Lusaka Urban District.

6.1.4 Determine the prevalence of the putative artemisinin resistance marker in Lusaka Urban District.

7.0 METHODOLOGY

7.1 Study design

The study was a cross sectional and prospective evaluation of the prevalence of the CQ and the putative artemisinin resistance markers. The study was carried out from September 2006 - November 2007.

7.2 Study site and population.

The study sites for this survey were 10 government urban health centres in various Lusaka district townships and the target was the Lusaka district population.
7.3 Inclusion and exclusion criteria

The study included consenting patients with a positive malaria test by microscopy. The patients were required to sign a consent form (Appendix 1). There was no age restriction. Only Lusaka residents were included in the study. A resident was defined as a person who has lived in Lusaka for more than three months. Non-Lusaka residents, non-consenting patients consenting patients with a negative malaria test were excluded from the study.

7.4 Sampling and Sample size

7.4.1 Sampling method

The sampling method aimed at coming up with a representative sample of the Lusaka population. A total of 10 health centres were randomly selected using the lottery method.

7.4.2 Sample Size calculation

The classical statistical method for determining sample size based on an unknown proportion of markers was used at 95 percent confidence level and 10 percent precision. Since there is no known figure for the prevalence of Pfcrf in Lusaka, a prevalence rate of 50 percent was used to calculate the sample size using the formula below (Mendenhall et al, 1981).

\[ n = \frac{Z^2 PQ}{d^2} \]

Where \( n \) = Sample size

\( P \) = Prevalence rate

\( Z = 1.96 \) at \( \alpha = 0.05 \) (\( \alpha \) = desired confidence level)
\[ d = \text{desired width of confidence (precision)}\]
\[ Q = 100 - P \]

Therefore the sample size \((n)\) was determined as:
\[ n = \frac{1.96^2 \times 50 \times 50}{10^2} \]
\[ n = 96.04 \approx 96 \]

However, a total of 104 PCR positive samples were collected. The numbers of samples collected from the health centres were based on proportions calculated from the diagnoses compiled by the Lusaka Urban District Health Management Team for the year 2005.

**7.5 Administration of questionnaires**

Questionnaires were administered to Lusaka residents visiting the selected health facilities regardless of the reason they were visiting the health centre. The number of respondents at each health centre was in proportion to the number of people attended to at each centre in the previous year, 2005. The administrators of the selected health centres and/or health workers who had worked in the respective clinics at both the time of policy change and chloroquine withdrawal were interviewed. Workers/owners of randomly selected drug stores near each clinic and at Lusaka town centre were also interviewed on chloroquine and Coartem® stocks and sales (Appendix 2).
7.6 Sample collection

Consent was sought from the patients visiting the laboratory and had a malaria test request from their physician. Blood was collected from each consenting patient by means of the ring finger ball puncture using sterile lancets. Thick smears were prepared and blood was collected on Whatman® 3MM filter paper that had been previously serrated for blood sample collection according to Kublin et al. (2005). The filter paper was cut into rectangles, 2cm by 5cm, and folded in half. One half was cut into four strips of approximately 0.5cm by 2cm and the other half was used to enter the patient’s details. Finger stick blood was blotted onto the tips of the four strips. Approximately 25µl of blood was collected on each of the strips. Therefore, approximately 100µl of blood was collected from each patient. The filter papers blotted with blood were air-dried by placing them on a clean surface with the strips pointing upwards and individually packed in envelopes, transported and stored at room temperature pending DNA extraction.

The smears were air dried or dried on a heating block. Dried blood smears were stained using 10 percent Giemsa solution prepared using 10ml Giemsa stock solution and 90ml buffer. The slides were submerged in Giemsa stain and left to stand for 25 minutes. Each slide was gently rinsed with clean water and after air drying the stained smears were examined to determine the presence of the malaria parasite using microscopy. Parasite densities were estimated by the plus sign method (CBoH, 2004). Approximately 19,800 patients were included in this exercise.
7.7 Molecular Methods

7.7.1 Chelex method for parasite DNA extraction

Extraction of parasite DNA was carried out using the chelex method (Kain and Lanar, 1991). One of the four strips of filter paper was used for each patient to extract DNA and rest of the dried blood was preserved at room temperature. The dried sample strips where inserted into the mouth of sterile pre-labelled 1.5ml Eppendorf tubes by making a right angular fold at the blood front base. Using a clean pair of scissors the blood-free section of the sample strip was cut to avoid contamination of the scissors and the tip of the strip with the dried blood was dropped into the Eppendorf tube. The pair of scissors was wiped with a fresh cotton wool swab of 70 percent ethanol and flamed on a Bunsen burner between cuttings to preclude cross contamination of the samples. Negative controls were included by using filter paper strips without blood. Each set of 10 samples had a negative control as the tenth sample. After loading the sample strips into the Eppendorf tubes, one ml of autoclaved 1X phosphate buffered saline (PBS) 1% (W/V) Saponin solution was added to each tube and left at room temperature for 20 minutes.

The sample tubes were spun at top speed (14 000 revolutions per minute (rpm) in a microcentrifuge for 2 minutes. The supernatant was aspirated and discarded while the sample strips were retained in the tubes. I ml of 1X PBS was added to the sample tube and the 2 minute centrifugation was repeated. The supernatant was discarded again and 150 µl of autoclaved double distilled water and 50 µl of 20 % w/v chelex resin suspension in autoclaved double distilled water were added to each of the tubes. The sample tubes were closed and a fine hole pierced in the lid of each of the tubes using a hot 23G hypodermic needle. The needle was flamed between sample tubes to avoid
sample contamination. The sample strips were then boiled in the chelex resin for 8 minutes by floating them on boiling water. The fine hole introduced in the lids of the sample tubes prevented them from popping open during the boiling step. After boiling the tubes were spun at maximum speed in a microcentrifuge for 1 minute. The supernatant from this spin was transferred in approximately 100 µl duplicate aliquots to storage vials and were kept at 4 °C pending use in PCR assays.

7.7.2 DNA Amplification and Digestion

7.7.2.1 *Plasmodium falciparum* chloroquine resistant transporter (Pfcrt),

codon 76.

After DNA had been extracted from dried filter papers, nested PCR was carried out as described by Djimde et al (2001). A primary round of PCR amplification was performed using the primers CRTP1 and CRTP2. The PCR was carried out using 2µL template, 10µM primer, 25mM magnesium ions, 10mM dNTPs, 10X chloride ions and 5U/ µL Taq in 25 µL PCR reactions. A second 25 µL nested PCR was performed with internal primers CRTD1 and CRTD2 flanking the K76T mutation. The PCR primers (Appendix 3) and parameters (Appendix 4) that were employed were according to Djimdé et al. (2001). Detailed information on methods for molecular analysis is available on the Internet at http://medschool.umaryland.edu/CVD/appendix1.asp. A volume of 5 µL of the secondary PCR amplicon was loaded into wells on a 2 % agarose gel made in 1 X TBE buffer stained with ethidium bromide. One middle lane was loaded with 1.5 µL of a 100 base pair (bp) ladder (New England Biolabs) and the DNA bands were visualised under ultra-violet trans-illumination after electrophoresis. The 145bp amplicon from the secondary PCR was digested using 4 µL of substrate
and 1.0 units of the restriction enzyme Apo I in the presence of 1.0 X New England Buffer 3 (NEB 3) and 1.00 X BSA in a 30μL digestion reaction at 50°C for 18 hours. The digests (15 μL per lane) were analysed by electrophoresis on 2% ethidium bromide stained agarose gels. Apo I digests the sensitive allele, resulting in a size reduction of 34 base pairs but does not digest the resistant allele. A single 111bp band was observed in the cases where cleavage had occurred. In the case of mutated genotype 145bp band was observed. Positive and negative controls were included in all the steps described above. To rule out the possibility of cross contamination negative controls were prepared by exposing strips of Whatman® 3MM filter paper without blood to the same conditions of the extraction process and amplification PCR as the samples. Each set of ten samples include a negative control right from beginning of the extraction process. Every batch of PCR amplification included a known malaria positive sample as a positive control. For the Apo I digests, the chloroquine-resistant (DD2) and the chloroquine-sensitive (3D7) laboratory standard clones were used as the negative and positive controls respectively.

7.7.2.2 Sarco/endoplasmic reticulum calcium dependent adenosine triphosphatase6 (SERCA) PfATPase6.

The same DNA extract for each sample was used as DNA template for primary round PCR amplification of the region containing nucleotide codon 2307of the PfATPase6 gene. The same PCR composition as for Pfcrtn described above was used with primers 2307FW and 2307RV. A secondary 25 μL PCR amplification was performed with engineered (EN) internal primers 2307FW-EN and 2307RV-EN. The latter primers
were engineered to create 2 Csp6 I restriction sites (Mharakurwa, personal communication).

In both the primary and the secondary rounds the following were used: 2 μL of template, 0.3μM primer, 25mM magnesium ions, 200μM dNTPs, 1X chloride ions and 1.0 unit Taq in 25 μL PCR reactions. The PCR programme that was employed was adopted from Macha laboratory PCR protocol and was as shown in Appendix 4. A volume of 5 μL of the secondary PCR product was analysed for the presence of the 432bp secondary PCR amplicon and 4 μL of the secondary PCR amplicon was digested with 1.5 units of the restriction enzyme Csp6 I in the presence of 1X buffer B in 30 μL reaction during 12 hours of incubation at 37 °C. The digestion reaction was then analysed by electrophoresis on a 2% agarose gel stained with ethidium bromide. The sensitive allele reduced in size by 42 base pairs. The resistant allele was expected to reduce by 10 base pairs. As earlier mentioned the SERCA-PfATPase6 secondary primers (2307 FW_EN and 2307RV_EN) used were engineered to create 2 restriction sites, bold lower case g and t (Appendix 3). The one site which will always be present will serve as an internal control for complete digestion by the restriction enzyme, cleaving off 10bp from the PCR product. The second restriction site cleavage will only occur if the infection is wild type at codon 2307 of PfATPase6 gene, causing a further 32bp to be cleaved off by the enzyme. The latter site is therefore used to detect the artemisinin resistance-associated mutation when no cleavage occurs. 1F11, a standard laboratory clone was used as a positive control. A negative control will in this case be a P. falciparum clone carrying the mutation associated with resistance to artemisinin. However this was not available.
8.0 DATA ANALYSIS

Base-line frequencies of the prevalence of *P. falciparum* point mutations associated with resistance to chloroquine and artemisinin in Lusaka Urban District were calculated using Microsoft Excel®.

9.0 ETHICAL AND ADMINISTRATIVE CONSIDERATIONS

Clearance of the study was obtained from the University of Zambia School of Medicine Ethics Committee. Permission to carry out the study was obtained from the Ministry of Health (copies of the clearance letters from the ethics committee and Ministry of Health are attached as appendices 5-7). Informed consent was obtained from the participants, parents or guardians (Appendix 1). Treatment of the participants was not affected by the study in anyway.

10.0 RESULTS

10.1 Anti-malarial drug use

Of the 119 individuals interviewed in Lusaka Urban District, 3 (2.5 percent) had used chloroquine for malaria treatment after the withdrawal of the drug from the health centres in Lusaka urban district (since April 2004). Twenty (16.8 percent) knew at least one person who had taken chloroquine for malaria treatment since its withdrawal. The number of respondents that had taken artemether-lumefantrine, (Coartem®) since its introduction in Zambia to the time the study was conducted was 38 (31.9 percent) and 83 (69.7 percent) knew someone, on first hand basis, who had used Coartem® for malaria treatment. The rest of the respondents had taken SP and other anti-malarial drugs, 34 (28.6 percent) and 15 (12.6 percent) respectively. Examples of the other
antimalarials that the respondents used were Halfan® (Halofantrine), Quinine® and Co-Arinate®.

Of the 20 drug store owners/workers who were interviewed at their drug stores 5 had chloroquine in stock. However, all of the stores rarely got people requesting for chloroquine. All the drug stores with chloroquine in stock reported that they dispensed CQ on request once in one or two months. Requests were ranging from none to two times per month. The number of drug stores that had Coartem® in stock was 15 and 5 of these also had chloroquine. Hence, all the drug stores that had chloroquine also stocked Coartem® and 5 had neither chloroquine nor Coartem® in stock. Chloroquine stocks were withdrawn and replaced with Coartem® from all the government owned health centres in Lusaka urban district following a workshop for health workers in March 2004 (LUDHMT, Personal communication).

10.2 Falciparum malaria infections

Approximately 19,800 patients from 10 Lusaka urban district health centres were screened for malaria between September 2006 and April 2007. Of these 161 (0.8 percent) were positive for malaria by microscopy of Giemsa stained thick smears. After subjecting the 161 positive samples to PCR, 104 (64 percent) of these were falciparum malaria positive. Table 1 shows the health centres and the number of malaria positives by both microscopy and PCR from each health centre. The numbers of malaria positives by microscopy were higher than the number of positives by PCR and this pattern was also observed in all age groups as shown in Figure 1. The largest age group infected with malaria was the under five year group followed by the 5-9 age group. The 20-24 years age group was the third largest group. Of the 161 candidates
recruited on this study, 85 (52.8 percent) were female and of these 45 (53.9 percent) were between the age of 15 and 44 years. Of the 45 women in 15-44 year age group 12 (26.7 percent) were pregnant and 5 of these fell in the 20-25 age group. The age range between the youngest and the oldest pregnant woman was between 17 and 40 years.

Table 1: *Plasmodium falciparum* infections detected by microscopy and PCR, on samples collected from health centres around Lusaka urban district from September 2006 to April 2007.

<table>
<thead>
<tr>
<th>Study site (Clinic)</th>
<th>No. of falciparum malaria positives by microscopy</th>
<th>No. of positives by PCR</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chawama</td>
<td>29</td>
<td>18</td>
<td>62</td>
</tr>
<tr>
<td>Chilenje</td>
<td>9</td>
<td>6</td>
<td>66</td>
</tr>
<tr>
<td>Chipata</td>
<td>19</td>
<td>12</td>
<td>63</td>
</tr>
<tr>
<td>George</td>
<td>11</td>
<td>7</td>
<td>64</td>
</tr>
<tr>
<td>Kabwata</td>
<td>8</td>
<td>5</td>
<td>63</td>
</tr>
<tr>
<td>Kalingalinga</td>
<td>7</td>
<td>5</td>
<td>71</td>
</tr>
<tr>
<td>Kamwala</td>
<td>23</td>
<td>16</td>
<td>69</td>
</tr>
<tr>
<td>Kanyama</td>
<td>26</td>
<td>18</td>
<td>69</td>
</tr>
<tr>
<td>Matero Main</td>
<td>8</td>
<td>5</td>
<td>63</td>
</tr>
<tr>
<td>Matero Reference</td>
<td>21</td>
<td>12</td>
<td>57</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>161</strong></td>
<td><strong>104</strong></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 1.** Proportions of malaria positive samples by microscopy of Giemsa stained thick smears and PCR in various age groups. Bracketed numbers are n values.

10.3 The Prevalence of the chloroquine resistance marker (Pfcr 76 mutation)

Amplicons from PCR positive samples were digested by Apo I restriction enzyme to determine the presence of Pfcr 76 mutation. Digest fragments were resolved by agarose gel electrophoresis and visualised under UV transillumination. Figure 2 shows an example of ethidium bromide stained 2 percent agarose gel for the detection of the chloroquine-resistant marker (76T).
Figure 2. Apo I digest to detect the Pfcrt K76T mutation among Lusaka Urban District field samples. Lanes S1, S4 and S5 show samples carrying the wild type residue (111 bp). Lane S2 shows a field sample carrying mixed mutated and wild type. Field samples with the mutation (145 bp) appear in lanes S3, S6, and S7. L is the New England 100 bp DNA ladder. DD2 is the laboratory standard clone carrying mutated (negative Control) and 3D7 is the laboratory standard clone carrying wild type K76 (positive control). Numbers below bands are the band sizes.
Figure 3 shows the proportion of the mutated K76T among Lusaka field samples collected from each health centre. The *Pf* **er**t 76T allele was detected in samples from all health centres occurring as monoinfections or mixed with the wild type. George Clinic had the smallest proportion (1/7) of the chloroquine resistance mutation occurring in a mixed infection with the wild type. The health centre with the highest proportion of the 76T mutation was Kabwata where 4/5 infections carried the chloroquine resistance mutation. The 76T mutation was detected in all the samples from Kalingalinga Clinic both as mixed and single infections. The wild type K76 was observed in all the health centres. However, the wild type among samples from Kalingalinga clinic was only detected in mixed infections. Of the 104 *P. falciparum* positive samples collected from the 10 health centres, 7 (6.7 percent) mixed infections were detected in samples from 4 health centres namely George, Chilenje, Kalingalinga and Kamwala clinics.
Figure 3  Distribution of Pfcr-76 allele among 10 health centres in Lusaka Urban District. Bracketed numbers are n values.
Figure 4 shows the proportion of the \textit{P}fcrt K76T mutation on falciparum malaria infections in children under 5 and compared to the age groups 5 years and above. The chloroquine-resistant mutant was more prevalent among infections in children under the age of 5 years than the older age group (Figure 5), although this was not statistically significant (OR [95percent CI]: 2.1 [0.90-4.86], p=0.084, n=104).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Proportions of the \textit{P}fcrt K76T mutation on \textit{P.falciparum} infections in children under the age of 5 years, compared with participants 5 years and above (bracketed numbers are n-values)}
\end{figure}
The prevalence of the *P. falciparum* chloroquine resistance mutation among 104 Lusaka urban district field samples was as shown in Table 2. The table shows proportions of infections with the mutated (76T) chloroquine-resistant strain (47.1 percent) and the wild type (K76) chloroquine-sensitive strain (46.2 percent) and mixed infections with the wild type and mutated strains, (6.7 percent). Hence the overall prevalence of the T76 mutation was 53.8 percent (95 percent confidence interval, 44.2 percent to 63.4 percent).

*Table 2:* Prevalence of the chloroquine resistance marker (*P.fcr*t 76 mutation) in Lusaka Urban District.

<table>
<thead>
<tr>
<th>PfCRT codon 76</th>
<th>Frequency</th>
<th>(%)</th>
<th>95% confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>48</td>
<td>46.2</td>
<td>36.3 - 56.2</td>
</tr>
<tr>
<td>Mixed</td>
<td>7</td>
<td>6.7</td>
<td>2.7 - 3.4</td>
</tr>
<tr>
<td>Mutated</td>
<td>49</td>
<td>47.1</td>
<td>37.2 - 57.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>104</strong></td>
<td><strong>100</strong></td>
<td></td>
</tr>
</tbody>
</table>
10.4 The Prevalence of the SERCA-PfATPase6 mutation associated with *P. falciparum* resistance to artemisinin

The same DNA extracts of the 104 falciparum malaria positive samples as in section 6.7.1 were used to amplify a fragment containing codon 2307 of the *PfATPase6* gene. The secondary PCR amplicons were digested with Csp6 I enzyme to detect the mutation associated with *in vitro* artemisinin resistance. The digests were also analysed by 2 percent agarose gel electrophoresis with ethidium bromide staining and UV transillumination (Figure 5).
Figure 5  Agarose gel showing Csp6 I digests of PfATPase6 codon 2307 in P. falciparum from Lusaka Urban District. All infections carried the wild type (artemisinin sensitive) allele Ser-2307, appearing as a 390bp band (lanes S1-S7). Lane 8 shows undigested secondary amplicon (432bp). L is the New England, 100bp ladder. Numbers below bands show band sizes.
The proportions of the \textit{SERCA} type \textit{PfATPase6} genotypes were as shown in Table 3. All the 104 (100 percent) infections carried the artemisinin sensitive wild type allele, Ser-2307.

\textbf{Table 3:} Prevalence of \textit{P. falciparum} point mutation linked to artemisinin resistance in Lusaka Urban district.

<table>
<thead>
<tr>
<th>\textit{SERCA-PfATPase-6} codon 2307</th>
<th>Frequency</th>
<th>%</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>104</td>
<td>100</td>
<td>100 - 100</td>
</tr>
<tr>
<td>Mutated</td>
<td>0</td>
<td>0</td>
<td>0 - 0</td>
</tr>
<tr>
<td>Total</td>
<td>104</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
11.0 DISCUSSION

11.1 Anti-malarial drug use

This study shows that low level use of Chloroquine by individuals (2.5 percent) persisted in Lusaka Urban District 3 years after the withdrawal of chloroquine from Lusaka Urban District health facilities. Chloroquine stocks in Lusaka were withdrawn from all the Government health centres and replaced with Coartem® by Medical Stores Limited (MSL) following a workshop for health centre administrators in March 2004 (LUDHMT, unpublished). The number of respondents that had taken Coartem® since its introduction was higher (31.9 percent) than those who had taken sulphadoxine-pyrimethamine (28.6 percent) and other drugs combined (12.6 percent). The number of respondents that knew someone who has used Coartem® since its introduction was higher (83/119 or 69.7 percent) than those that knew someone who had taken CQ since its withdrawal (20/119 or 16.8 percent). In addition to this, 15 of 20 drug stores surveyed had Coartem® in stock. This indicates that a good proportion of Lusaka urban district residents had access to Coartem® the recommended first line treatment drug for uncomplicated malaria. Some participants expressed fear of side effects of Coartem® they either experienced or witnessed from someone else and others complained about the high cost of Coartem® in drug stores when it is not available at the health centres.

11.2 Falciparum malaria infections

The falciparum malaria positives by microscopy were not all positive by PCR in all the health centres. PCR based diagnostic methods for malaria surpass microscopic methods with respect to sensitivity and specificity (Farcas et al. 2004). Therefore it
would be expected that a malaria sample positive by microscopy would also be positive by PCR. The observation above may be attributable to a number of factors including human error on the part of the principle investigator and the microscopists who helped with screening of the samples. On the other hand, if the amount of blood collected on filter paper strips was too little, much less than 25μl coupled with low parasitaemia a negative PCR result may be the outcome.

The numbers of malaria cases in different age groups among the Lusaka urban district field samples showed that children under the age of 5 years were the largest group infected with falciparum malaria. Studies have shown that children under 5 years of age are among the most vulnerable groups, which include pregnant women and travelers or migrants who move from areas with little or no transmission to high endemic areas (Lusungu et al., 2004; CDC, 2004). This also explains why the 20-24 year age group was the third largest group after the 5-9 year age group because the highest proportion of the pregnant women fell in the 20-24 years age group.

11.3 Prevalence of *P. falciparum* point mutations associated with resistance to chloroquine and artemisinin resistance.

The findings show that the *Pfcrt* mutation associated with resistance to chloroquine is prevalent 3 years after the withdrawal of chloroquine as first line treatment for uncomplicated malaria. The policy was changed from the use of chloroquine to the use of Coartem® as first line treatment for uncomplicated malaria in 2002 and chloroquine was withdrawn from health centres in Lusaka Urban District in March 2004 (CBoH 2002: LUDHMT, unpublished). The malaria treatment policy in Zambia was changed based on the WHO guidelines which recommend that any country with a total treatment failure of 25 percent or more should change its first line drug (WHO, 1993).
By 2000, Zambian CQ treatment failure rates were determined to be as high as 52 percent in some sites. For instance, chloroquine treatment failure rates for Choma and Isoka districts were determined at 52 and 54 percent respectively (CBoH and NMCC, 2000). The therapeutic failure rate of chloroquine in Chongwe, which was a representative site for all the districts in Lusaka Province and where in vivo efficacy studies (using a 14 day follow up period) were conducted in 1996, was determined at 43.4 percent (CBoH and NMCC, 2000). Treatment outcomes are affected in part by factors other than parasite resistance, including insufficient absorption, unusual rate of degradation and excretion of a drug by the host (Peters, 1970) and host’s immune response (Wellem and Plowe, 2001). In vivo efficacy studies based on standard methods for measuring antimalarial drug efficacy using a 14-day follow up period provide information only on short term efficacy. If a drug has a moderately impaired ability to eradicate parasites, it may still have high rates of therapeutic efficacy at 14 days but have more late recrudescences associated with higher rate of recurrent episodes of symptomatic malaria requiring treatment (Plowe, 2003).

The treatment failure rates determined at 43.4 percent in 1996 would correspond to similar prevalence of the Pfcr76 at that time. It would be expected that the prevalence of the chloroquine resistance marker was higher at the time of policy change which came about five years after the in vivo efficacy studies were conducted (1996-2002) and during this five year period chloroquine was still the first line treatment drug for uncomplicated malaria. However, there are no baseline data available on the prevalence of the K76T mutation at the time the policy was changed or at the time of chloroquine withdrawal from health facilities. Depending on the prevalence of the K76T mutation at the time chloroquine was withdrawn from health centres in Lusaka
Urban District, the prevalence of the chloroquine resistant marker may have decreased since chloroquine withdrawal or the prevalence may still be around the same rate. It must be noted that withdrawal of antimicrobial drug pressure does not always compromise the fitness of resistant micro-organisms and result in selection of drug sensitive phenotypes (Levin et al. 2000). Incomplete withdrawal of the drug, cross-resistance to agents still in use, and compensatory mutation that reduce the burden of resistance without jeopardising resistance itself (Sherman et al. 1996) may allow the resistant organisms to persist. Anti-malarial drugs that are widely used in Zambia, Coartem®, quinine and sulphadoxine-pyrimethamine have different mechanisms of resistance and they should be adhered to for a number of years, if the persistence of chloroquine resistant *P. falciparum* is to be avoided.

The proportion of Lusaka field samples found with K76T mutation in the *Pfcr* gene, which encodes a transporter protein of the *P. falciparum* digestive vacuole, was lower by 42.2 percent than what was observed by Thuma in 2003 (unpublished data) around Macha where the prevalence of the *Pfcr* K76T was determined at 96 percent. This may not necessarily imply that the prevalence of the K76T mutation in Lusaka in 2003 was as high as Macha as the two areas are expected to have different transmission patterns. Studies show that lower altitude and accompanying temperatures are associated with a higher rate of transmission and resistance (Bødker et al., 2003; Mharakurwa et al., 2004; Mharakurwa, 2004). Macha lies at 1006m above sea level while Lusaka is higher at 1260m above sea level (Ministry of Lands, 1950). In addition, Macha unlike Lusaka Urban is a rural setting and people build their homes near water sources where they are exposed to high vector densities and infection challenge. Thus the prevalence of the *Pfcr* K76T mutation might well have been
higher in Macha than Lusaka. Nevertheless the observed difference was too high to be explained by these factors and it does seem likely that Lusaka has experienced a reduction in *Pfcr* K76 prevalence over time.

There is a possibility that chloroquine use may return to Zambia in an event that Coartem®, the current first line treatment for uncomplicated malaria, fails or becomes unaffordable and if *P. falciparum* total sensitivity to chloroquine re-emerges. However chloroquine would have to be used in combination with other drugs because if used as a monotherapy its useful therapeutic life span may be short-lived.

The re-emergence of chloroquine sensitive *P. falciparum* malaria has been recorded in Malawi. In their study, Kublin *et al.* (2001), noted that the prevalence of the Chloroquine-resistant *Pfcr* genotype decreased from 85 percent in 1993 to 13 percent in 2000 and in 2001 chloroquine cleared 100 percent of 63 asymptomatic *P. falciparum* infections. However, Kublin (2001) emphasized that Malawi made a concerted and sustained effort to withdraw chloroquine from use and that less rigorous efforts to reduce chloroquine drug pressure may not result in the same re-emergence of chloroquine sensitivity. A decline in the measure of chloroquine resistance after a reduction in chloroquine use has also been reported in Gabon (Schwenke *et al.*, 2001).

However countries in South America, such as Columbia and Venezuela, were reported to have continued sustaining the mutant *Pfcr 76T*- harbouring parasites despite having minimised the use of chloroquine (Cortese *et al.*, 2002).

Each health centre used in this study had at least one sample in which the K76T mutation was detected indicating an even distribution of the chloroquine resistance marker in Lusaka urban district. The prevalence of the chloroquine resistance
mutation at individual health centres could not be determined because the numbers of samples from the individual health centres were too small, less than 30 in all cases.

The proportion of Pfcrt K76T mutation in malaria infections in children under the age of 5 was higher than in the older age group. However this was not statistically significant. Presumably the sample size did not give sufficient study power to detect the difference between age groups since n was only calculated to measure the proportion of Pfcrt K76T mutation with 10 percent precision. Djimdé et al. (2003) found that the ability to clear chloroquine resistant parasites improves with age. Other studies have also shown in vivo clearance of chloroquine-resistant parasites with the K76T mutation (Basco et al., 2001; Mayor et al., 2001). According to Djimdé et al. (2003) residents of malaria endemic areas sometimes spontaneously clear P. falciparum infection without drug treatment, implying an important role for host factors such as immunity in the clearance of chloroquine resistant parasites. Immunity against malaria develops over years of repeated P. falciparum infections (March, 1992). In populations of northern Nigeria not receiving drug treatment, age related immunity was evident in the increase of daily spontaneous clearance rates 0.2 percent in children less than 5 years old to 0.5 percent in children 9 years old and above (Molineaux et al., 1980).

This is the first study conducted on prevalence of chloroquine resistance markers in Lusaka Urban district. Therefore it serves as base line data and supports, although in retrospect, the decision by the Zambian government to revise the National Malaria Policy from the use of chloroquine to the use of Coartem® as first line treatment for uncomplicated malaria.
The *PfATPase* mutation associated with resistance to artemisinin does not appear to have arisen in Lusaka urban district. None of the 104 samples that were analyzed in this study bore the point mutation at locus 2307 in the *PfATPase* gene that is thought to be associated with *P. falciparum in vitro* resistance to artemisinin. According to Jambou *et al.* (2005), field isolates that exhibited *in vitro* resistance to artemisinin all came from areas with uncontrolled use of artemisinin derivatives. The observation on the artemisinin resistance putative marker from this study can be due to the pristine conditions under which Coartem® was used having not been in use for long enough to give rise to resistance in Lusaka Urban District.

Despite the fact that *P. falciparum* resistance to artemisinin was not observed in this study, there is need for increased vigilance so that policy markers can make evidence based decisions on which antimalarials to introduce. The absence of the artemisinin resistance mutation in *P. falciparum* would seem to suggest total artemisinin sensitivity at the time of the study, unless a different mechanism of resistance occurs in the study area. Continued regulated use of ACT’s as first line treatment for uncomplicated malaria is indicated.

**12.0 CONCLUSIONS**

Chloroquine is no longer in stock in government health centres but is still in stock in some of the private drug stores and there has been low level use of the drug after its withdrawal in Lusaka Urban District. Coartem® is available both in health centres and drug stores around Lusaka Urban District. The chloroquine resistance K76T mutation is prevalent (53.8 percent) in Lusaka Urban District three years after the withdrawal of chloroquine from health facilities and five years after the Malaria Treatment Policy
was changed from the use chloroquine to the use of Coartem as first line treatment for uncomplicated malaria. The \textit{SERCA-PfATPase6} mutation thought to be associated to \textit{P. falciparum} resistance to artemisinin was not observed in this study.

\textbf{13.0 RECOMMENDATIONS}

The findings from this study show that the reintroduction of CQ either in combination with other drugs or as a monotherapy should not be considered at present if effective treatment is to be a strategy for malaria control. In an event that a drug containing chloroquine is available and its introduction considered it might be necessary to carry out this kind of research to establish the prevalence of the chloroquine resistance marker before the administration of such a drug. Treatment failures presumably indicate malabsorption, although continuous monitoring of efficacy and molecular resistance markers is recommended. Controlled use of Coartem® as first line treatment for uncomplicated malaria is also recommended.

\textbf{14.0 LIMITATIONS}

There were various problems that were faced during the research period. Firstly time (3 months) was lost due to the period of time that it took to get clearance from the University of Zambia Ethics Committee. Secondly the funds that were available at the beginning of the research were limited. Therefore recruitment of assistants was not possible which made sample collection from 10 health centres very difficult. Sample collection commenced around the low transmission period (September 2006) and this resulted in prolonged sample collection period. This was the time everything that was required for the study was in place.
15.0 LIST OF REFERENCES


Cox S. J., B. Singh, A. Alias, and M. S. Abdullah, 1995. Assessment of the association between three pfmdr1 point mutations and chloroquine resistance


16.0 APPENDICES

Appendix 1: Consent form

I Enesia Banda Chaponda will be conducting a research study on the prevalence of chloroquine and the putative artemisinin resistance marker genes in Lusaka urban district. I am a Master of Science student at the University of Zambia in the School of medicine.

The study will benefit the Zambian community in that it will generate baseline data for future monitoring of the trends in the prevalence of the artemisinin and chloroquine resistant genes and will also enhance the establishment of procedure for monitoring drug resistance in the country.

Blood will be collected on filter paper as you/ your child test(s) for malaria at your health center and this will not affect your treatment. There are no risks of any kind in being involved in this study and you will not be recalled or followed up. If your blood sample is found to be malaria positive, it will be subjected to further molecular procedures.

Be assured that your malaria test results will be treated in the strictest confidence and that it will only be used for purposes stated above. I would be very grateful if you agree to take part in this study but understand that you are at liberty to either participate or not.

If you are willing to take part in this study please sign below:

41
I....................................................... Understands the information given above and I would like to participate in the study. I also understand that the study will benefit the Zambian community as a whole.

Sign............................................ (Participant)  Date..................

For questions and clarifications contact the principal investigator at 096-807318 or 099-518088 or E-mail: enesiachaponda@yahoo.com, University of Zambia Main Campus, Biological Sciences Department, P.O Box 32379, Lusaka. You can also contact University of Zambia Research Ethics Committee Tel No: 256067, E-mail: unzarec@zamtel.zm, P.O Box 50110, Lusaka.
Appendix 2: Questionnaire

PREVALENCE OF THE CHLOROQUINE AND THE PUTATIVE ARTEMETHER RESISTANCE MARKER GENES IN LUSAKA DISTRICT

Dear respondent,

I am a Master of Science student in the school of Medicine conducting a study on the distribution of chloroquine resistant marker genes in Lusaka district.

The study is aimed at generating baseline data for future reference and enhancing the establishment of procedure for future monitoring of drugs resistance in the country. This questionnaire will help me to establish whether chloroquine is at all used by individuals and exactly when some individuals stopped using chloroquine for malaria treatment. It will also shed light on when Coartem was first in use

Be assured that your responses to all the questions in this questionnaire will be treated in the strictest confidence and that they will only be used for purposes stated above. I would be grateful if you would take a bit of your time to answer the questions that follow

Thanking you in anticipation for your cooperation

Section A: Personal information. (Tick where appropriate)

1. Sex: □ Male □ Female

2. Age

3. Area of residence

4. Study site

5. Have you had malaria in the since April 2004?
   Yes □ No □

6. If your answer to question 4 is yes, what treatment did you receive?
☐ Co-artem®  ☐ Chloroquine  ☐ Fancidar  ☐ Other (specify..........................)

7. Have you ever used chloroquine before?
   Yes ☐ No ☐

8. If yes, when was the last time you used chloroquine for malaria treatment?

9. Do you know anyone who was on chloroquine for malaria treatment since April 2004 to date?
   Yes ☐ No ☐

10. Have you ever used Coartem® for Malaria treatment before?
    Yes ☐ No ☐

11. If yes, when was the first time you used Coartem® for malaria treatment?
    2002 ☐ 2003 ☐  2004 ☐  2005 ☐  2006 ☐  2007 ☐

12. Do you know anyone who has used Coartem® for malaria treatment?
    Yes ☐ No ☐

Section B- For health worker

1. Do you still give chloroquine for malaria treatment?
   Yes ☐ No ☐

2. Did this health center stop the use of chloroquine as the first line treatment drug for uncomplicated malaria immediately after the change of policy (2002) from the use of chloroquine to Co-artem? ☐ Yes ☐ No ☐

3. When was the last time this health centre stocked chloroquine.........(MM-YY)

4. Do you have Coartem® in stock? Yes ☐ No ☐
5. When was the first time this health centre stocked Coartem®? ........(MM-YY).

Section C- For drug store owners/workers

A. Do you have chloroquine in stock?  
   Yes □ No □

B. If yes, how often do people buy chloroquine from your chemist?  
   (Give range).............

C. If your answer to A is no, when was the last time you had chloroquine in stock?  

D. Do you have Coartem® in stock?  
   Yes □ No □

E. If yes, how often do people buy Coartem® from your drug store?  
   (Give range)..................
**Appendix 3:** Primer names, sequences and product sizes.

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE</th>
<th>PRODUCT bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>2307FW</td>
<td>5'-TGA GCA TGG CAC AAG TTT T-3'</td>
<td>486bp</td>
</tr>
<tr>
<td>2307RV</td>
<td>5'-TCA ATA ATA CCT AAT CCA CCT AAA TA-3'</td>
<td>(primary AMP)</td>
</tr>
<tr>
<td>2307FW.EN</td>
<td>5'TGA GCA TGG tAC AAG TT T-3'</td>
<td>432bp</td>
</tr>
<tr>
<td>2307RV.EN</td>
<td>5'-TCA TCT GTA TTC TTA ATA TTT AAA TCT gTA CTA-3'</td>
<td>(Secondary AMP)</td>
</tr>
<tr>
<td>CRTP1</td>
<td>5'-CCGT TA ATA ATA AAT ACA CGC AG-3'</td>
<td>537bp</td>
</tr>
<tr>
<td>CRTP2</td>
<td>5'-CGG ATG TTACAA AAC TAT AGT TAC C-</td>
<td>(Primary AMP)</td>
</tr>
<tr>
<td>Pre-digestion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRTD1</td>
<td>5'-TGT GCT CAT GTG TTT AAA CTT-3'</td>
<td>145bp</td>
</tr>
<tr>
<td>CRTD2</td>
<td>5'-CAA AAC TAT AGT TAC CAATTT TG-3'</td>
<td>(secondary AMP)</td>
</tr>
</tbody>
</table>

**Apo 1**

Undigested: 145bp  
Wild type (lys-76): 111bp, 34bp  
Mutated (Thr-76): 145bp  
(Djimde et al., 2001)

**Csp61**

Undigested: 432bp  
Wild type (ser-2307): 390bp, 32bp, 10bp  
Mutated (Asn-2307): 422 10bp  
(Mharakurwa, 2007, Unpublished data)
Appendix 4: PCR parameters employed for analysis of *Pfcr* and SERCA-PfATPase6.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Reaction</th>
<th>Initial Denaturation °C, Time</th>
<th>Denaturation °C, Time</th>
<th>Annealing °C, Time</th>
<th>Extension °C, Time</th>
<th>Final extension °C, Time</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERCAPfATPase6</td>
<td>Primary &amp; secondary</td>
<td>94, 2 min</td>
<td>94, 45 sec</td>
<td>46, 45 sec</td>
<td>65, 1 min</td>
<td>65, 2 min</td>
<td>25</td>
</tr>
<tr>
<td><em>Pfcr</em> K76T</td>
<td>Primary</td>
<td>94, 3 min</td>
<td>94, 30 sec</td>
<td>56, 30 sec</td>
<td>60, 1 min</td>
<td>60, 3 min</td>
<td>45</td>
</tr>
<tr>
<td><em>Pfcr</em> K76T</td>
<td>Secondary</td>
<td>95, 5 min</td>
<td>92, 30 sec</td>
<td>48, 30 sec</td>
<td>65, 30 sec</td>
<td>65, 3 min</td>
<td>25</td>
</tr>
</tbody>
</table>

PCR parameters employed for *Pfcr* K76T available on the internet at [http://medschool.umaryland.edu/CVD/appendix1.asp](http://medschool.umaryland.edu/CVD/appendix1.asp)

SERCA-PfATPase6 PCR parameters were as described by Mharakurwa (2007, Personal communication).
RESEARCH ETHICS COMMITTEE

Assurance No. FWA00000338
IRB00001131 of IORG0000774

28 August, 2006
Ref.: 025-06-06

Ms Enesia Banda Chaponda
Department of Biomedical Sciences
School of Medicine
University of Zambia
P.O. Box 50110
LUSAKA

Dear Ms Chaponda,

RE: RESEARCH PROPOSAL ENTITLED: “PREVALENCE OF THE CHLOROQUINE RESISTANCE MARKER GENE IN LUSAKA URBAN DISTRICT”

The above research proposal was presented to the Research Ethics Committee meeting on 26 July, 2006 where changes were recommended. We would like to acknowledge receipt of the corrected version with clarifications. The proposal has now been approved. Congratulations!

CONDITIONS:

• This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.

• If you have need for further clarification please consult this office. Please note that it is mandatory that you submit copy of your final report at the end of the study.

• Any serious adverse events must be reported at once to this Committee.

Yours sincerely,

Prof. J. T. Karashani, MB, ChB, PhD
CHAIRMAN

Date of approval: 28 August, 2006
Date of expiry: 27 August, 2007
19 February, 2007
Our Ref: 025-06-06

Ms Enesia Banda Chaponda
Department of Biomedical Sciences
School of Medicine
University of Zambia
P.O. Box 50110
LUSAKA

Dear Ms Chaponda,

RE: APPLICATION FOR RESEARCH PROPOSAL AMENDMENT

We acknowledge receipt of your letter dated 12 February, 2007.

Please be informed that this Committee has approved the change on the title of your research topic: “Prevalence of the Artemether and the Chloquine resistance marker genes in Lusaka Urban District”. We also take note that this amendment will not affect the participants in anyway as the samples that have been collected will be used to check the prevalence of both the artemether and the Chloquine resistance marker genes.

Yours sincerely,

[Signature]

Dr E. M. Munalula-Nkandu
SECRETARY

cc Dr C. Shinondo, Head, Department of Biomedical Sciences, School of Medicine, UNZA, Lusaka
20th October, 2006

Ms. Chanda Enesia Banda
University of Zambia
Box 50110
Lusaka

RE: RESEARCH PROJECT - YOURSELF

Reference is made to your letter dated 18th October, 2006.

I wish to inform you that permission has been granted for you to do carry out the above mentioned activity.

However this should be done at no cost to DHMT.

By copy of this letter the health Centre In-Charges are informed forthwith.

Yours faithfully,

[Signature]

DR. MAKUNGU KABASO
CLINICAL CARE MANAGER
FOR/DISTRICT DIRECTOR OF HEALTH