IMPACT OF CHLOROQUINE DRUG WITHDRAWAL ON RESISTANCE OF LOCAL *Plasmodium falciparum* MALARIA PARASITES TO THE DRUG IN TIWI AND MBITA TOWNS OF KENYA

Clarence Maikuri Mang'era

A Thesis Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Award of Master of Science Degree in Biochemistry of Egerton University

EGERTON UNIVERSITY

JUNE 2011

DECLARATION AND RECOMMENDATION

DECLARATION

I hereby declare that this thesis is my original work and has not been submitted for award of a degree in any institution of learning to the best of my knowledge.

Clarence Maikuri Mang'era
SM14/2191/08
Signed:…….. Date:……..…….

RECOMMENDATION

We confirm that this thesis has our approval to be presented for examination as per the Egerton University regulations.

Dr. Paul Mireji,
Egerton University

Signed:……..... Date:…….....……

Dr. Sabah Ahmed Omar,
Kenya Medical Research Institute (KEMRI), Nairobi

Signed: …….....…… Date:..…….....…….....

Dr. Mbai Fiona
International Medical Corps, Nairobi, Kenya

Signed: .....…… Date:…….....……..…

COPYRIGHT

DEDICATION

I dedicate this thesis to my loving parents, Elkanah Mang'era and Esther Mang'era and my exceptional sister Lillian Moraa.

ACKNOWLEDGEMENTS

I give God all the glory, praise and honor for thus far he has brought me and He still has great and mighty plans in my life. The old adage that it takes a village to raise a child is also true for training of a graduate student. During the time of this research, I have learned so much from so many and without them, the completion of this thesis would not have been possible. I would first like to thank my research supervisors and mentors; Dr. Paul Mireji for spending countless hours guiding me through the trials and tribulations of biomedical research, Dr. Sabah Ahmed Omar for affording me a rare opportunity of carrying out the research in her laboratory, not to mention frequent consultations on the project that were of immeasurable magnitude, apt interventions that ensured lab work progressed well together with an overall proficient supervision and Dr. Mbai Fiona for patiently cultivating the research concept, always challenging me step out of my comfort zone and grow as a scientist. I appreciate the members of KEMRI-CBRD malaria laboratory; Kimani for teaching me various crucial molecular techniques that ensured my survival on the bench, Rahma for providing immense state-of-the-art scientific information, Too and Kaniaru for sharing their extensive knowledge on field work and sample collection and Makokha, whose previous work on similar area of research was very instrumental, Eric, Ibrahim, Nancy, Njeri, to name but a few. I thank the team of students and volunteers who have come and gone from the malaria lab for being great friends and making lab life more entertaining; Fatiah, Nathan, David, Betty, Jackie and Maureen. I would also like to recognize my fellow graduate students. It would not have been possible to work with a better group to help me along this journey. I also thank my parents, Esther and Elkanah Mang'era. You have loved me unconditionally and made me the person I am today. Without you, none of this would have been possible. Finally, I thank IAEA for funding part of my research work especially the radioisotope work for dot blot.

ABSTRACT

Chloroquine (CQ) drug was replaced in 1998 by Sulfadoxine - Pyrimethamine (SP) and later Artemisinin Based Combination therapy (ACT) as a first-line treatment of uncomplicated malaria in Kenya. This was due to development of resistance to CQ in *Plasmodium falciparum* Welch, 1897 malaria parasite. This study set out to determine the prevalence of the CQ resistance in malaria endemic Tiwi and Mbita towns of Kenya. Blood sample were collected from out-patient candidates with uncomplicated malaria using finger pricking techniques and spotted on filter paper *Plasmodium falciparum* were collected in blood samples drawn from naturally infected patients with uncomplicated malaria, visiting out-patient clinics in Tiwi (n=152) and Mbita (n=38) towns of Kenya in 1999 and 2008 or 2009. DNA extracted from individual spots in the paper were screened by nested PCR for presence of *P. falciparum* specific Chloroquine resistance markers using chloroquine resistance transporter (*Pfcrt)* and multi drug resistance (*Pfmdr1)* primers. Mutations and/or haplotypes in the *Pfcrt* and *Pfmdr1* markers in the samples were assessed using dot blot hybridization technique. Temporal changes in the prevalence of the resistance were determined using Chi Square. There was significant reduction in prevalence of *Pfmdr1-N86Y* (P = 0.0009) and *Pfcrt K76T* (P = 0.0001) mutations in Tiwi between 1999 and 2008. *Pfmdr1-N86Y* and *Pfcrt K76T* mutations reduced to 63.6 and 56.9% respectively. In Mbita, change in prevalence of *Pfmdr1-N86Y* mutations between 1999 and 2009 was not significant (P = 0.7826), reducing to 84.2%, while Mbita's prevalence of *Pfcrt K76T* mutation as at 2009 was 63.2%. There was a significant association in prevalence of *P. falciparum* between Tiwi 1999/Tiwi 2008 ($\chi^2_{df=1}$ = 10.958, P = 0.0009) and Mbita 2009/Tiwi 2008 ($\chi^2_{df=1}$ = 4.231, P = 0.00397). There was however no significant association in prevalence of *P. falciparum* between Tiwi 1999/Mbita 2009 ($\chi^2_{df=1}$ = 0.0715, P = 0.7891). Significant reduction in *Pfmdr1-N86Y* and *Pfcrt K76T* mutations in Tiwi, and not in Mbita suggest potential reduction in resistance to CQ in *P. falciparum* populations in Tiwi, but not Mbita. This indicates that CQ is still not a viable alternative to current antimalarial chemotherapy but can potentially be introduced as a combination therapy to treat and manage *P. falciparum* malaria in Tiwi, however, not in Mbita.

Key words: *Pfcrt*, *Pfmdr 1,* Chloroquine drug resistance, *Plasmodium falciparum*

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACT	Artemisinin based combination therapy
ATP	Adenosine triphosphate
Ca2+	Calcium ions
Ci	Curie
CQ	Chloroquine
DNA	Deoxyribonucleic acid
Kb	Kilo bases
kDa	Kilo dalton
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SP	Sulfadoxine-pyrimethamine
WHO	World health organization

APPENDICES

CHAPTER ONE

INTRODUCTION

1.1. Background information

World Health Organization estimates that malaria is responsible for about one million deaths annually, with *P. falciparum* as the leading causative agent (WHO, 2008). The *P. falciparum* is the most virulent and infectious to humans among malaria pathogens with widespread resistance to most drugs (Krogstad, 2000), including chloroquine (CQ), which has long since been withdrawn in many countries, including Kenya (Shretta *et al.,* 2000). Molecular markers associated with CQ resistance in the parasite have been identified and has been instrumental evaluation of prevalence of CQ in nature, which in turn has influenced anti-malarial policies in Kenya (Hastings and Smith 2008; Snow *et al.,* 2005; WHO, 2008).

Established markers for Chloroquine resistance in *P. falciparum* include *P. falciparum* chloroquine resistance transporter (*Pfcrt*) (Fidock *et al*., 2000) and multi-drug resistance gene 1 (*Pfmdr1*) (Babiker *et al.,* 2001; Reed *et al.,* 2000). The Pfcrt is a 10 transmembrane domain protein, localized to the food vacuole membrane of the parasite (Fidock *et al*., 2000). A threonine - lysine substitution mutation at residue 76 (K76T) of the gene confer resistance to CQ (Babiker *et al*., 2001; Djimde *et al.,* 2001; Fidock *et al*., 2000; Sidhu *et al.,* 2002). Similarly, the resistance to CQ in Pfmdr1 is conferred by asparagine - tyrosine substitution at residue 86 (N86Y) in the gene (Reed *et al.,* 2000; Wellems and Plowe, 2001). Continuous application of CQ in management of malaria can select the local *P. falciparum* for resistance to the drug (Schneider *et al.,* 2002) based on these substitutions in the markers. Although the resistance enhances fitness of the parasite in counteracting the CQ pressure, the mutations become costly to the fitness of the parasite with withdrawn the CQ pressure (Orr, 2000), coincident with replacement of CQ as a standard prescription against malaria. This can be attributed to a cost associated with maintaining the K76T or N86Ymutations segregating in the population (Zeyl *et al.,* 2001).

Unless the cost to fitness costs in *P. falciparum* is compensated for by secondary mutations (Levin *et al.,* 2000), frequency of resistant individuals is expected to decline in the absence of the selection pressure as populations of the parasite strain resistant to CQ is displaced by sensitive individuals having a greater reproductive success and superior rates of population

growth (Agnew *et al.,* 2004). This loss of CQ stress resistance is generally expected to be rapid, the rate and the magnitude of which should be proportional to the costs of resistance (Agnew *et al.,* 2004). A population or isolate of *P. falciparum* can be screened for presence of K76T or N86Y mutations to estimate the changes in CQ resistance in the parasites over time.

This study estimated putative changes in resistance to CQ in Tiwi and Mbita Kenya by screening local isolates collected in 1999 and 2008 (Mbita) or 2009 (Tiwi) for the K76T or N86Y mutations.

1.2. Statement of the problem

Plasmodium falciparum has rapidly developed resistance to novel drugs, including SP, adopted after discontinuation of CQ. However, the withdrawal of CQ can lead to reduction of the resistant strain from the local parasite populations, presenting a chance for re-introduction of the drug as a potential replacement for the currents drugs the parasite has developed resistance too.

1.3. Objectives

1.3.1. General objective

To determine putative changes in resistance to CQ in *P. falciparum* populations in endemic Tiwi and Mbita towns of Kenya based on *Pfcrt-K76T* and *Pfmdr1-N86Y* molecular markers.

1.3.2. Specific objectives

1. To determine changes in prevalence of *Pfcrt-K76T* and *Pfmdr1-N86Y* mutations in *P. falciparum* isolates collected between 1999 and 2009 from Tiwi and Mbita towns of Kenya.
2. To identify haplotypes in the *Pfcrt* gene at codon 74, 75 and 76 of *P. falciparum* isolates from Tiwi and Mbita towns of Kenya.

1.4. Hypotheses

1. Prevalence of *Pfcrt-K76T* and *Pfmdr1-N86Y* mutations in *P. falciparum* isolates between 1999 and 2009 from malaria endemic Tiwi and Mbita towns of Kenya has not reduced significantly.
2. There are no new haplotypes in *Pfcrt* gene at codon 74, 75 and 76 of *P. falciparum* isolates from malaria endemic Tiwi and Mbita towns of Kenya.

1.5. Justification

Chloroquine was considered a wonder drug for treatment of *P. falciparum* malaria, a major cause of human morbidity and mortality in Sub-Sahara Africa. However, almost exclusive use of this drug (intensive selection pressure on the parasite), coupled to abuse of the same through poor dosage compliance (exposure of the parasite to sub-lethal dosage of the drug) generated resistance genotype in the parasite to the drug. In the presence of the CQ selection pressure, parasites with the resistant alleles predominated the natural populations. However, with the discontinuations of the drug to counteract resistance to the drug, parasites with allele for susceptibility to CQ are expected to proliferate and replace those with alleles for resistance, since adaptations conferring resistance to the drug are expected to be costly to parasite in absence of CQ pressure. Assessment of status of this phenomenon in Tiwi and Mbita towns of Kenya can aid policy makers in determining whether or not CQ should be re-introduced in the malaria treatments in the towns to counteract development of resistance to current drugs in the towns.

CHAPTER TWO

LITERATURE REVIEW

2.1. Epidemiology of malaria

Malaria affects about half the world's population or 3.3 billion people with a 56% world-wide prevalence (Aregawi *et al.*, 2008). About 90% of all malaria deaths in the world occur in Sub-Saharan Africa, majority of which are caused by *P. falciparum* (WHO, 2008). Malaria burden and transmission patterns vary across Kenya (Division of Malaria Control, 2010), designated into four malaria transmission zones (Snow *et al.*, 2005). The first zone comprises regions with perennial high malaria transmission near Lake Victoria and South Coast, with the second zone consisting of regions of high malaria transmission with seasonal fluctuations, typically regions adjacent to the areas with perennial transmission. The first zone is also, especially along the coast, associated with chloroquine resistance in the *Plasmodium* parasite (Figure 1). The third zone is defined by presence of stable malaria transmission accompanied by seasonal peaks, common in most of the semi-arid and western highland regions of the country. Finally, the fourth zone is composed of low malaria transmission risk, normally in the arid and mountain regions of the country.

2.2. Biology of the malaria parasite

Malaria is caused by protozoa members of the genus *Plasmodium* with heteroxenous life cycle involving a vertebrate host and an arthropod vector. Species infecting humans in this genus include Plasmodium *falciparum*, *P. vivax*, *P. ovale* and *P. malariae* (Krogstad, 2000). The *P. falciparum* has 4 stages which mark the life cycle in the liver, blood and within the *Anopheline* mosquitoes (Paniker, 1989). Sporozoites are injected with the saliva during mosquito feeding and enter the circulatory system and invade a liver cell within 30-60 minutes before maturing into a merozoite (Yamauchi *et al.,* 2007). These merozoites then invade erythrocytes and mature into trophozoite which then undergoes erythrocytic schizogony (Alan and Brendan, 2006). Subsequent sexual stage is marked by micro - and macrogametocytes, pre-adaptive for infection of the *Anopheles* mosquito vector (Paniker, 1989). The gametes fuse into Zygote, which mature into ookinete (Ghosh *et al.*, 2000), which asexually reproduces and mature into oocyst which in

turn undergo sporogony to generate human infective mature sporozoites (Beier, 1993; Pimenta *et al*., 1994). The sporozoites are subsequently injected into the next human host as the mosquito takes a blood meal, reinitiating infection in vertebrate host (Kappe *et al.*, 2004).

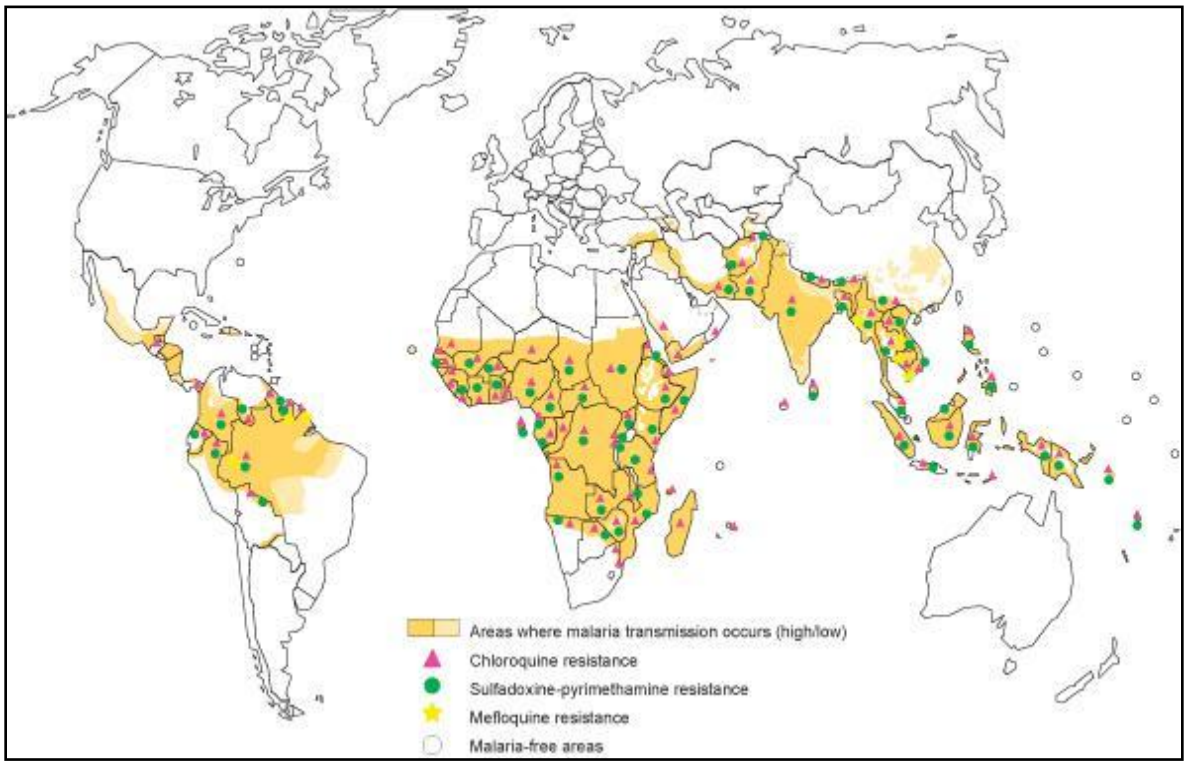


Figure 1: Worldwide distribution of drug resistance of *Plasmodium falciparum* (Reproduced in full World malaria report 2005)

2.3. Clinical manifestations of malaria and transmission

Malaria is naturally transmitted by *Anopheles* mosquitoes (Bjorkman and Bhattarai, 2005). Congenital transmission is relatively rare despite the heavy infection of the placenta (Greenwood, 2006). The pathology and clinical manifestations associated with malaria are almost exclusively due to the asexual erythrocytic stages of the parasite (Beier, 1993). The symptoms are described as 'flu-like' and include headache, slight fever, muscle pain, anorexia, nausea and lassitude (Paniker, 1989). The symptoms correlate with malarial paroxysms, which

exhibit periodicities of 48 hours for *P. falciparum* (Paniker, 1989), with blood-stage parasites within undergoing a synchronous schizogony (WHO, 2008).

2.4. Immunity against malaria

Persons living in malaria endemic areas develop a slow and short-lived immunity against malaria (al-Yaman *et al.,* 1997). Most of these people are asymptomatic (Males *et al.,* 2008) with low parasitemia due to premunition (Obi *et al.,* 2010), as defined by presence of antibodies that prevent merozoite invasion, or factors that eliminate infected erythrocytes (Hviid *et al.,* 1992), A very gradual long-term resistance to *P. falciparum* malaria develops years after the onset of disease among children three months and older in majority of African populations owing to trans-placental maternal IgG passed on to the children (Wahlgren and Qijun, 1999).

2.5. Chemotherapy as a malaria control method

Drugs used to combat malaria are classified according to their mode of action. Anti-folate combinations exert their therapeutic effect by interrupting the folate synthesis pathway in parasites (Hyde, 2005).

Artemisinin based combination therapies (ACTs), target sarcoplasmic and endoplasmic reticulum (ER), Ca^{2+} ATPase (SERCA)-type protein of the *P. falciparum* encoded by a gene denoted *PfATPase6* (Golsner *et al.,* 2006). Treatment with artemisinin drugs causes reduction of parasite burden below detectable levels without eliminating all parasites which results in a higher risk of recrudescence (Targett *et al.,* 2001; Bjorkman and Bhattarai, 2005). Artemisinins may also decrease malaria transmission because they act on the gametocytes (Pukrittayakamee *et al.,* 2004). Artesunate cannot be administered alone because if the duration of therapy is less than 5–7 days, there is a high risk of recrudescent infection (Targett *et al.,* 2001). The recurrent parasites are not resistant to artemisinins but evade their very short duration of action (Ittarat *et al.,* 2003). When combined with a longer-acting partner drug, the artemisinins can be administered as an ACT over 3 days (WHO, 2005).

The sulphadoxine-pyrimethamine (SP) combination was the most effective and relatively inexpensive therapy after the emergence of chloroquine (CQ) resistance (Nzila *et al.,* 2000). The

SP was subsequently replaced by ACTs. Chloroquine falls under 4-aminoquinolones and acts in the same way as amodiaquine (AQ) hence continued use of AQ can exert drug pressure the same way CQ does (Nsobya *et al.,* 2007).

Table 1: Main classes of drugs used in chemotherapy against malaria

Chemical Composition	Example of Drugs
Aryl amino alcohol	Quinine, quinidine (cinchona alkaloids), mefloquine, halofantrine
4-aminoquinolones	Chloroquine, amodiaquine
Folate synthesis inhibitors	Type 1 – Competitive inhibitors of dihydropteroate synthase – sulphones, sulphonamides Type 2 – Inhibit dihydrofolate reductase – biquanides like proguanil and chloroproguanil, diaminopyrimidine like pyrimethamine
8-aminoquinolones	Primaquine, WR238, 605
Antimicrobials	Tetracycline, doxycycline, clindamycin, azithromycin, fluoroquinolones
Peroxides	Artemisinin (Qinghasou) derivatives and analogues – artemether, arteether, artesunate, artelinic acid
Naphthoquinones	Atovaquone
Iron chelating agents	Desferrioxamine

2.5.1. The 4-Substituted Quinolines

Chloroquine and the other 4-substituted Quinolines such as Amodiaquine (AQ) kill malaria parasites by interfering with ferriprotoporphyrin IX (FP) detoxification process and causing it to accumulate to lethal levels (Fitch, 2004). Primary antimalarial action of chloroquine is to bind to and remove ferriprotoporphyrin IX from oxidized hemoglobin, producing toxic FP-chloroquine complexes and an excess of denatured globin (Fitch and Russell, 2006). Ferriprotoporphyrin IX is produced when the parasites denature or degrade hemoglobin (Fitch, 2004), which is detoxified by dimerization to β-hematin, promoted by unsaturated lipids (Fitch *et*

al., 2003). Chloroquine treatment interferes with detoxification by causing unsaturated lipids in parasitized erythrocytes to be unavailable for promotion of ferriprotoporphyrin IX dimerization (Fitch *et al.*, 2004).

Possible mechanisms for selective accumulation of chloroquine up to 1000-folds in food vacuole are: protonation and ion trapping of chloroquine due to low pH of food vacuole, active uptake of chloroquine by a parasite transporter(s); and/or binding of chloroquine to a specific receptor in the food vacuole (Martin *et al.,* 2009). Chloroquine exerts it toxic effect by interfering with conversion of free heme to hemozoin (Ouellette, 2001). Although chloroquine is no longer recommended for the treatment of *P. falciparum*, it is the drug of choice to treat *P. vivax* and *P. ovale*, less severe forms of malaria that can cause recurrent infections (Travassos and Laufer, 2009).

2.6. Resistance to anti-malarial drugs

WHO recommended ACTs as the first-line antimalarial treatment for *P. falciparum* malaria due to emergence of resistant of *P. falciparum* malaria (WHO, 2005). In addition, various molecular methods have been developed to detect point mutation of genes present in drug resistant malaria parasite strains (WHO, 2005), a process facilitated by availability of *P. falciparum* genome in 2002 (Gardner *et al.*, 2002; Roopam *et al.*, 2005).

2.6.1. Resistance to chloroquine in P. *falciparum*

Chloroquine resistance arises by mutation and is associated with a decrease in the amount of chloroquine that accumulates in the food vacuole, the site of action for chloroquine, due to an increase in drug efflux (Wellems, 2001). Parasitized red blood cells release drug at least 40 times faster than the sensitive strains (Ouellette, 2001). Verapamil partially restore accumulation of, and sensitivity to, chloroquine for chloroquine-resistant *P. falciparum* (Ravit and Theresa, 2005). The chloroquine-resistant gene that originated in Southeast Asia spread across the Asian continent and reached Africa in the late 1970s (Cooper *et al*., 2005). Over the course of a decade, chloroquine resistance became widespread in sub-Saharan Africa (Alifrangis *et al.,* 2006). Resistance did not emerge within infected individuals on a regular basis, as might be expected

based on the inherent mutation rate of the parasite and high rate of replication within the human host (Escalante *et al.*, 2009). Rather, resistant strains emerged a limited number of times and those ancestral strains spread in the permissive environment of drug pressure (Wellems and Plowe, 2001).

There has been one example of the effect of the removal of drug pressure on the prevalence of drug-resistant parasites. In 1993, Malawi was the first country to replace chloroquine with SP for the oral treatment of malaria, due to high rates of chloroquine resistance (Kublin *et al.*, 2003). A clinical trial in Malawi demonstrated that chloroquine is now highly effective in the treatment of malaria, after high rates of failure documented just 12 years prior (Lauffer *et al*., 2006). As chloroquine is withdrawn from use throughout the region (WHO, 1996), it is possible that chloroquine-susceptible parasites will return and chloroquine may once again play a role in the treatment or prevention of malaria.

2.6.2. *Plasmodium falciparum* chloroquine-resistance transporter

A gene with 13 exons, *Pfcrt* (*P. falciparum* chloroquine-resistance transporter) near *cg2* on chromosome 7, (Fidock *et al*., 2000) encodes PfCRT, a 45 kDa protein, containing 424 amino acids, with ten predicted transmembrane domains that immunolocalizes to the membrane of the digestive vacuole of the malaria parasites. The protein, a transporter, reduces chloroquine levels in the digestive vacuole by altering ion fluxes that change the acidity of the vacuole. This in turn reduces the accumulation of free heme and relieves the digestive vacuole of its cytotoxicity (Waller *et al.*, 2003).

Sets of point mutations in *pfcrt* have been associated with chloroquine resistance *in vitro* in laboratory lines of *P. falciparum* from Africa, South America, and Southeast Asia. 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 resistance to CQ (Fidock *et al*., 2000). Genes from resistant cells have at least five and up to eight mutations, all confined to ten positions that are clustered within or near transmembrane domains. One mutation, the substitution of threonine (T76) for lysine (K76) at position 76 (K76T), was present in all resistant isolates and absent from all sensitive isolates tested in vitro. When present in a mutated form, it is associated with decreased chloroquine accumulation. This mutation, K76T, now provides a valuable molecular marker in surveillance studies and a predictor of chloroquine efficacy (Djimdé *et al*., 2001; Lakshmann *et al*., 2005*)*.

2.6.3. *Plasmodium falciparum* multidrug-resistance 1 gene

There are associations between chloroquine resistance and mutations in a multidrug-resistance *mdr*-like gene, *P. falciparum* multidrug-resistance 1 (*Pfmdr 1*), on chromosome 5 that encodes a protein (Foote *et al*., 1990), Pgh1, located in the lysosomal membrane of the parasite (Babiker *et al*., 2001) The *Pfmdr1* gene is 4260 base pairs long coding 1419 amino acids (Reed *et al.,* 2000). The *Pfmdr1* gene is associated with CQ resistance, with a polymorphism resulting from the substitution of an asparagine for a tyrosine in amino acid 86 (N86Y) in *Plasmodium falciparum* (Babiker *et al*., 2001; Duraisingh *et al.,* 2000). Addition of mutant *Pfmdr1* to cells harboring mutant *Pfcrt* enhances chloroquine resistance, indicating that mutations in this gene

modulate the overall response to chloroquine (Reed *et al.,* 2000). Additionally, mutations in *Pfmdr1* are associated with resistance to Mefloquine, Quinine, and Halofantrine (Sidhu *et al*., 2002).

Of recent interest, *PfMDR* N86, the chloroquine susceptible- allele has been proposed as a molecular marker for lumefantrine resistance (Sisowath *et al.,* 2005). Artemether-lumefantrine treatment selects for N86 in recurrent infections (Sisowath *et al.,* 2005). Resistant lines originated in just a few discrete geographic locations from which they then spread (Wootton *et al*. 2002). Therefore, these mutations are not due to clonal expansion of a single genotype but most probably due to this gene being under strong selection by chloroquine (Escalante, 2009). Essential role of *Pfcrt* was firmly established by allelic exchange of the endogenous *Pfcrt* in chloroquine-sensitive cells for mutant alleles due to selective drug pressure, which effectively conferred a chloroquine-resistant phenotype (Sidhu *et al*., 2002). The documented reemergence of chloroquine-sensitive parasites when drug pressure is removed afford an opportunity to reintroduce chloroquine after years of nonuse (Kublin *et al*., 2003).

2.7. Dynamics of *Pfcrt* alleles

Substitutions in the wild type allele, encoding CVMNK, give rise to several resistant variants, of which the most common are CVIET in South-East Asia and Africa and SVMNT, which has been reported in South America (Fidock *et al.,* 2000) and Asia (Sutherland *et al.,* 2007), but rarely in Africa (Alifrangis *et al.,* 2006). When chloroquine pressure is increased, some *pfcrt* alleles associated with low fitness in chloroquine free environment will become associated with a high fitness in this chloroquine environment and could thus invade the African parasite population (Ariey *et al.,* 2006). If chloroquine pressure stops, and wild type allele is still present in the subpopulation, it will replace the mutant allele (Kublin *et al.,* 2003).

2.8. Methods used in drug resistance studies

There are four basic methods for testing malaria for drug resistance. In *in vivo* test, patients with clinical malaria are given a treatment dose of an antimalarial drug under observation and are monitored over time for either failure to clear parasites or for reappearance

of parasites (WHO, 2001). *In vitro* tests involve blood samples from malaria patients being obtained and the malaria parasites exposed to different concentrations of antimalarial drugs in the laboratory (WHO, 2001). Animal models are also used since *in vivo* tests conducted in non-human animal models are influenced by the same extrinsic factors as in the human body. They allow testing parasites, which cannot be adapted to *in vitro* environments, provided a suitable host is available since testing of experimental drugs is not yet approved for use in humans (Craig *et al.,* 1997).

For some drugs (chloroquine, antifolates, atovaquone), gene molecular markers have been identified that confer resistance (Djimde *et al.,* 2001; Omar *et al.,* 2001). Molecular techniques, such as PCR or gene sequencing can identify these markers in blood taken from malaria-infected patients (Duraisingh *et al.,* 2000; Warhust *et al.,* 1991). Molecular studies of resistance markers provide an early warning system and can inform therapeutic efficacy studies (Omar *et al.,* 2001). They can also be useful in monitoring the prevalence of molecular markers in places where a drug has been withdrawn or where a drug combination is in use (WHO, 2001).

2.8.1. Dot blot hybridization

Apart from increasing sensitivity of detection over ethidium bromide staining of DNA gels which can be laborious to perform, confirming the specificity of the amplicon, hybridization with specifically designed complementary probes, can be used to detect point mutations in its nucleotide sequence (Omar *et al.,* 2007). The radioisotope Phosphorus –32 is used to label the probes (Mayor *et al.,* 2001; Ranford-Cartwright *et al.,* 2002). The atom phosphorus –32 (^{32}P) is an unstable isotope of phosphorus-31 (^{31}P), with emissions that can be detected by methods such as autoradiography (Sinclair, 2000). The advantage of this method is that nylon membranes can be reused for dot blot hybridization. The probe can be stripped off the membrane after autoradiography, and re-probing carried out with other radio-labeled probes (Mayor *et al.,* 2001; Omar *et al.,* 2007). In addition the cost of radio isotopic hybridization is less expensive than non-radio isotopic probe based methods (Ranford-Cartwright *et al.,* 2002). Radioisotopes are simple, quick and convenient for labeling probes (Sinclair, 2000) and the short half-life of P-32, which is 14 days, is less problematic to dispose (Ranford-Cartwright *et al.,* 2002).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study sites

3.1.1. Tiwi

Tiwi (4° 14´ 0´´ South, 39° 35´ 0´´ East) is situated in a Kwale district, Coast province in Kenya. It is a small town 25 Km south of Mombasa by road (Fig 2c). Average temperatures in Tiwi range between 24°C and 30°, which favor breeding of *Anopheles gambiae* malaria vector with transmission peaking in May-July (Coetzee, 2000). Tiwi has continuous malaria transmission with children and pregnant women as most vulnerable group to the disease (Omar *et al*., 2001). In addition, the permanent inhabitants acquire a high degree of immunity to malaria due to re-infection many times in the year (Bull *et al.,* 1998). Malaria transmission in the area is endemic with varying transmission intensity (Division of Malaria Control, 2009).

3.1.2. Mbita

Mbita (0° 30´ 0´´ south, 34° 15´ 0´´ East) is a rural town situated along the shores of Lake Victoria in Suba District in Nyanza province of Kenya. The main malaria vectors in these areas are *A. gambiae*, *A. funestus*, and *A. arabiensis* (Division of Malaria Control, 2009). Malaria transmission is high with *P. falciparum* predominating as the principle *Plasmodium* parasite, accounting for more than 95% of malaria cases (Gouagna *et al.,* 2003). The parasite prevalence in the human population ranges from 24.4% to 99.0% (Mutero *et al.,* 1998).

3.2. Ethical considerations, inclusion and exclusion criteria

Archived dry blood spots on Whatman filter papers or Glass fiber membrane was used for this study. These samples were collected in the May–July malaria seasons in 1999 (Tiwi and Mbita), 2008 (Tiwi) and 2009 (Mbita) from patients visiting the outpatient clinics in the study sites during previous studies on antimalarial drug's efficacy and resistance monitoring at day zero. The Kenya Medical Research Institute (KEMRI) Nairobi, Scientific Steering Committee (SSC 791, 848, 948) and ethical review Committee of the Kenya Medical Research Institute issued approval to these studies.

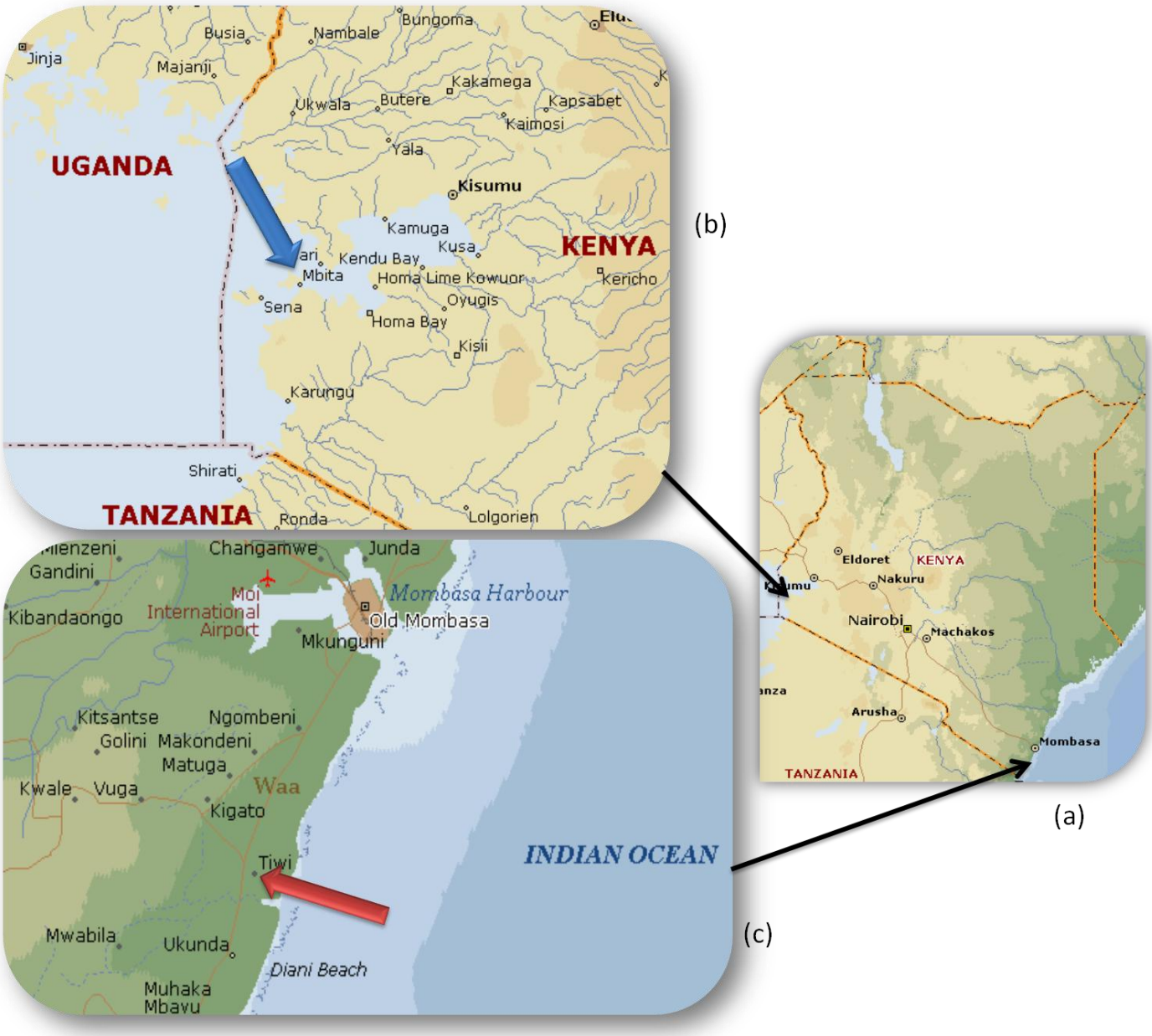


Figure 2: Study sample source sites; (a) Map of Kenya (b) Mbita (blue arrow) Suba District, and (c) Tiwi (Red arrow), Kwale District, Coast province in Kenya. Source: Microsoft®Encarta® 2009© 1993-2008 Microsoft corporation

Written informed consent was obtained from parent(s) or guardian(s) of the participating children. Patients visiting outpatient clinics were enrolled in these studies if they met the following criteria; 1) Approved informed consent, 2) mono-infection of *P. falciparum* with parasitemia between 1,000 and 200,000 parasites/ μL of blood, 3) auxiliary temperature ≥ 37.5 C or with a history of fever and 4) no history of anti-malarial drug intake during the previous week (confirmed by conventional drug blood level test). Patients were excluded from the study if 1) there was administration of any additional anti-malarial drugs, 2) emergence of any non-malarial febrile illness that would interfere with the classification of the treatment outcome, 3) patient relocation from the study site or 4) withdrawal from the study. The approved protocol for each study site was strictly adhered to in the treatment of the patients. Presence of *P. falciparum* parasites in the blood samples were determined by standard microscopy and conformed by Nested PCR.

3.3. Determination of prevalence of *Pfmdr1-N86Y* mutations in Tiwi and Mbita

3.3.1. DNA extraction

Blood spot samples and clones collected on 3mm Whatman filter paper (Whatman®) were excised from filter paper and DNA extracted using Chelex-100® Bio- Rad method as described by Warhust *et al* (1991). Briefly, scalpels, forceps and glass plate were sterilized with 5M HCl , 5M NaOH, and distilled water. Blood spots in filter papers were scalpel excised on glass and the paper was transferred into 1ml of 0.5% saponin in 1 x PBS in a sterile 1.5ml microfuge tube (eppendorf®). The tube was inverted several times to mix, and placed at 4^{o}C overnight. The contents were centrifuged and supernatant transferred into 10% Sodium Hypochlorite, and then into 1ml of PBS at 4^{o}C for 30 minutes. The solution was centrifuged and placed again in 10% Sodium Hypochlorite with. 50μl of 20% (w/v) chelex- 100® Bio-Rad solution and 100μl of DNase-free water (Sigma) were added to the remaining excised filter. The solutions were heated at 95^{o} – 100^{o} C for 10 minutes with vortexing at 2 minutes intervals (Genie vortexer®). The solutions (DNA containing) were centrifuged at 10,000 for 2 minutes and the supernatant transferred to a fresh tube, and further centrifuged for 2 minutes at 10,000 and transferred into another fresh tube, which was subsequently stored at -70^{o}C.

3.3.2. PCR amplification of the *Pfmdr1-86* gene

Oligonucleotides forward primers MDR/A1 (TGT TGA AAG ATG GGT AAA GAG CAG AAA GAG) and reverse MDR/A3 (TAC TTT CTT ATT ACA TAT GAC ACC ACA AAC) primers were used in single outer PCR reaction. Using the PCR products generated from the primary reaction as templates, a nested PCR reaction was conducted using forward MDR/A2 MDR/A2 (GTC AAA CGT GCA TTT TTT ATT AAT GAC CAT TTA) and reverse MDR/A4 (MDR/A4: AAA GAT GGT AAC CTC AGT ATC AAA GAA GAG) primers. Three *P. falciparum* clones, (3D7, 7G8 and Dd2) from WHO/IAEA, with *Pfcrt* and *Pfmdr1* alleles were used as standard positive controls in all the studies to optimize for specificity and sensitivity of the experiments.

A master mix for the outer PCR consisted of 15.16 µl nuclease-free water (Sigma), 3.0 µl 10 × PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl) (Roche), 3.0 µl 25mM magnesium chloride, 3.0 µl 2mM mixed dNTP's (dGTP, dATP, dTTP, dCTP) (Promega), 0.3 µl of each primers (10 µM) (Outer loop primers A1, forward, and A3, reverse) and 0.24 µl Taq polymerase enzyme (5 units/ µl). DNA (5 µl) of was added to 25 µl master mix to a total volume of 30 µl. Amplification was performed using MyCycler™ Thermal cycler PCR machine (BIORAD, USA). Initial denaturation was conducted for 3 min at 95^{o}C followed by a 40 cycles of denaturing for 1 min at 94^{o}C, annealing for 1 min at 46^{o}C and extension for 1 min at 72^{o}C. Final extension was carried out for 3 mins at 75^{o}C and the reaction brought to a hold at 4^{o}C. Second round of PCR reactions was conducted on the PCR products of the first round reactions targeting internal segment of the amplicons. Briefly, a master mix was prepared composed of 15.16 µl nuclease-free water (Sigma), 3.0 µl 10 × PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl) (Roche), 3.0 µl 25mM magnesium chloride, 3.0 µl 2mM mixed dNTP's (dGTP, dATP, dTTP, dCTP) (Promega), 0.3 µl of each primers (10 µM) (Outer loop primers A2, forward, and A4, reverse) and 0.24 µl Taq polymerase enzyme (5 units/ µl). DNA (5 µl) was added to 25 µl master mix to a total volume of 30 µl. Amplification was performed using a MyCycler™ Thermal cycler PCR machine (BIORAD, USA). Primary denaturation was conducted for 3 min at 95^{o}C followed by 40 cycles of denaturing for 30 sec at 94^{o}C, annealing for 1 min at 46^{o}C and extension for 1 min at 72^{o}C. Final extension was carried out for 3 mins at 75^{o}C and the reaction

brought to a hold at 4^{o}C. Five microlitres of nested PCR were mixed with a 2 µl of 10× loading dye and analyzed by agarose gel electrophoresis on 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) in Tris-acetate-EDTA buffer. The electrophoresis was run at 80 volts for 45 minutes. DNA was visualized by ultraviolet transillumination and the expected nested PCR product band size determined by comparison with a standard 100-base pair DNA ladder.

3.3.3. Preparation of dot blotting

Dot blots were prepared by methods of Abdel Muhsin (2002). Briefly, 20 µl each of PCR product were denatured in 10 mM EDTA and 0.4M NaOH at 100^{o}C for 10 minutes to a final volume of 30µl, and then neutralized in an equal volume of 2M-ammonium acetate, pH 7. Nitrocellulose membranes (Millipore) cut to exact size to fit manifolds were pre-wetted by soaking in 2× SSC for 10 minutes and fitted on to dot blotting apparatus (Bio Rad). The membranes were re-hydrated with TE (10mM Tris-HCl: 1mM EDTA) buffer and then dried using vacuum pump. Denature samples were individually loaded in duplicate into the manifold containing the blots, and were held for 30 min. The membranes were removed and neutralized in 2× SSC for 30-60 seconds and washed in 0.4M NaOH for 30-60 seconds to denature immobilized DNA. The membranes were rinsed in neutralizing solution (1M Tris-HCL, 1.5M NaCl, and pH 8) for 30 seconds and UV cross-linked at 0.120 joules for 5 minutes in to fix the PCR products onto the membrane. The membrane was then wrapped using cling film and stored at -20^{o}C until it was needed.

3.3.4. Labeling of oligonucleotide probes

Probes for *Pfmdr 1* Asn 86 - specific (5'-GAA ATA TTT TCG TGC AGT TAC-3') and *Pfmdr 1* Tyr 86 - specific (5'- GTA ATA TTT TCG TGC AGT TAC -3') were labeled as Asn and Tyr (MWG Biotech). Ten picomoles of each probe was labeled with 10µCi of [γ-^{32}P] dATP using 5 units of polynucleotide kinase. This was prepared by adding 1µl probe, 1µl T4 Polynucleotide kinase (5 units/µl) (USB, Cat 70031), 5µl T4 Polynucleotide kinase 10× buffer and 42µl nuclease free water to a tube. The contents of tube were then mixed by pippeting up and down briefly. From this stage all procedures were carried out in a radiation containment

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

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

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

3.3.6. Hybridization and stringent washes

The prepared blots were unwrapped and placed into a rotor bottle, making sure that there were no overlapping areas, into which 20 mls hybridization buffer were added (0.25-0.125ml per cm^2 of membrane). The hybridization buffer consisted of a final volume of 5× SSPE, 5x Denhardt's reagent, 0.5% SDS, 0.02mg/ml sonicated salmon sperm DNA (Gibco) in DNase-free Water. This was then pre-warmed at 41°C for Asn probe and 43°C for Tyr probe in a hybridization oven for 30 minutes with agitation making sure that the bottle was closed properly and the buffer does not leak. The blots were added into rotor bottles containing the pre-warmed

buffer for another 30 minutes. A volume of 20µl of the labeled oligonucleotide probe (1µl for every 1ml of the hybridization buffer) was then added into the bottle contents. Hybridization at the appropriate temperature for at least 5 hours with agitation followed. Overnight hybridization was preferred especially when the labeled probe was over 2 weeks old.

The hybridization solution was poured off and disposed of accordingly and stringent washes were carried out. An excess (at least 1ml/cm^2 blot) of wash buffer 1 (2 × SSC) was added at the corresponding temperature and incubated with agitation in the oven for 10 minutes at the same temperature. The washes were repeated twice using excess (at least 1ml/cm^2 blot) wash buffer 2 (1× SSC/0.1% SDS) at the same temperature and incubated with agitation for 10 minutes each. The washing solutions were poured off and disposed of accordingly then the blot sealed by wrapping in cling film without allowing the blot to dry out.

3.3.7 Autoradiography

The sealed blot was taped right side up (DNA-side up) into an autoradiography cassette (Kodak) with intensifying screens. To avoid problems with autorad orientation, the film was folded at bottom right corner and this allowed for accurate positioning of the autorad after developing. Blots were exposed on Kodak® (Rochester, NY) X-Omat film for 12-24 hours at –70ºC in the freezer (Revco®). The films were then removed from the cassette and developed to score the sample against the controls. If any of the controls showed non-specific hybridization, an extra stringent wash was carried out. The autorad was obtained by developing the image in a developer solution (Kodak) for 5 minutes followed by a brief rinse in clean water and finally fixing the developed image in a fixative solution (Kodak) for 5 minutes and the fixative rinsed off with clean tap water. These processes were done in a dark room as the films are light sensitive. The films were then air dried then scored.

3.3.8. Stripping the probe from the membrane

The membranes were stripped off the probe using excess of 0.1 M NaOH, for 15 minutes, at room temperature with agitation, repeating the process followed by a brief wash with 5× SSC. The blot was probed again or stored, after sealing the blot by wrapping in a cling film, at -20ºC

or dried and store at room temperature sandwiched and taped between two pieces of clean filter paper.

3.4. Identification of *Pfcrt* gene haplotypes at codon 74, 75 and 76 in Tiwi and Mbita

3.4.1. DNA extraction

DNA was extracted as described in section 3.4.1 using the chelex method. In addition, preparation of the DNA template from glass filter membrane was done as described by Warhurst *et al*. (1991). Briefly, blood spots on glass filter membrane were outlined with a pencil to clearly mark the spot. Each glass filter membrane was supported on a fresh 2.5 cm Whatman filter paper disc (Whatman®) and placed in a sintered glass vacuum filter then washed with 2-3 mls, sterile distilled water to lyse the blood cells. Blood proteins were removed by washing with 2-3 mls of sterile normal saline. The prepared glass filter membrane was then air dried, on a clean surface, at room temperature for 1-2 hours and later stored in fresh-labeled self-seal bags at 4° C with desiccant to maintain dryness.

3.4.2. PCR amplification of the *Pfcrt* gene

Oligonucleotides primers pairs P1 and P2 (P1: CCG TTA ATA ATA AAT ACA CGC AC; P2: CGG ATG TTA CAA AAC TAT AGT TAC) were used as forward and reverse primers respectively for outer PCR. Nested Primers D2 and D3 (D2: CAA AAC TAT AGT TAC CAA TTT TG; D3: AGG TTC TTG TCT TGG TAA ATT TGC) were included in a nested PCR reaction. Control amplification using DNA from *P. falciparum* parasites clones, from WHO/IAEA, known to contain the possible different haplotypes at position 74, 75 and 76 of *Pfcrt* gene were used to ensure specificity and sensitivity of the technique. The controls included; 3D7 for MNK, Dd2 for IET and 7G8 for MNT. There is no laboratory isolate known at present with the combination MEK, MET, INK or INT.

A master mix for the outer PCR was prepared composed of 12.3 µl nuclease-free water (Sigma), 2.5 µl of 10 × PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl) (Roche), 2.5 µl of 25mM magnesium chloride, 1.5 µl 2mM mixed dNTP's (dGTP, dATP, dTTP, dCTP) (Promega),

0.5 µl of each primers (10 µM) (Outer loop primers P1, forward, and P2, reverse) and 0.2 µl Taq polymerase enzyme (5 units/ µl) per PCR tube. DNA (5 µl) of was added to 20 µl master mix to a total volume of 25 µl. Amplification was performed using a MyCycler™ Thermal cycler PCR machine (BIORAD, USA). Primary denaturation was conducted for 3 min at 94^{o}C followed by a 35 cycles of, denaturing for 30 sec at 94^{o}C, annealing for 30 sec at 56^{o}C and extension for 1 min at 62^{o}C. Final extension was carried out for 5 min at 65^{o}C then followed by 15^{o}C for 5 min and the reaction brought to a hold at 4^{o}C.

Second round of PCR reactions was conducted on the PCR products of the first round reactions targeting internal segment of the amplicons. Briefly, a master mix was prepared composed of 14.3 µl nuclease-free water (Sigma), 2.5 µl of 10 × PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl) (Roche), 2.5 µl of 25mM magnesium chloride, 1.5 µl 2mM mixed dNTP's (dGTP, dATP, dTTP, dCTP) (Promega), 0.5 µl of each primers (10 µM) (Outer loop primers D2, forward, and D3, reverse) and 0.2 µl Taq polymerase enzyme (5 units/ µl) per PCR tube. DNA (3 µl) of was added to 22 µl master mix to a total volume of 25 µl. Amplification was performed using a MyCycler™ Thermal cycler PCR machine (BIORAD, USA). Primary denaturation was conducted for 3 min at 94^{o}C followed by a 30 cycles of, denaturing for 30 sec at 94^{o}C, annealing for 30 sec at 56^{o}C and extension for 1 min at 65^{o}C. Final extension was carried out for 5 min at 65^{o}C then followed by 15^{o}C for 5 min and the reaction brought to a hold at 4^{o}C.

Five microlitre of nested PCR were mixed with a 1-2 µl of 10× loading dye and analyzed by agarose gel electrophoresis on 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) in Tris-acetate-EDTA buffer. The electrophoresis was run at 80 volts for 50 minutes. DNA was visualized by ultraviolet transillumination and the expected nested PCR product band size, which is 164 base pairs, determined by comparison with a standard 100-base pair DNA ladder. Preparation of dot blot was done as with *Pfmdr1-86* gene in section 3.4.3.

3.4.3. Labeling of oligonucleotide probes

Probes for all the possible alleles at codons 74, 75 and 76 of the *Pfcrt* gene were labeled as MNK (5'-TAA TGA ATA AAA TTT TTG-3'), MNT (5'-TAA TGA ATA CAA TTT TTG-3'), IEK (5'-TAA TTG AAA AAA TTT TTG-3'), IET (5'-TAA TTG AAA CAA TTT TTG-3'),

MEK (5'-TAA TGG AAA AAA TTT TTG-3'), MET (5'-TAA TGG AAA CAA TTT TTG-3'), INK (5'-TAA TTA ATA AAA TTT TTG-3') and INT (5'-TAA TTA ATA CAA TTT TTG-3') (MWG Biotech). Ten picomoles of each probe were labeled with 10µCi of [γ-^{32}P] dATP using 5 units of polynucleotide kinase. This was prepared by adding 1µl probe, 1µl T4 Polynucleotide kinase (5 units/µl) (USB, Cat 70031), 5µl T4 Polynucleotide kinase 10× buffer and 42µl nuclease free water to a tube. The contents of tube were then mixed by pippeting up and down briefly. From this stage all procedures were carried out in a radiation containment room/area, using beta-shields such as 1cm acrylic for protection from the radiation, and wearing appropriate personal radiation monitors such as film badges. Solid and liquid wastes were disposed off according to the advice of the local Atomic Energy Agency/radiation protection advisors. To the reaction mix of 1µl of [γ-^{32}P] dATP (Amersham Biosciences, UK: Redivue [γ-^{32}P] ATP, 3000Ci/mmol: Cat No. AA00068) was added and mixed gently. These were then spun briefly in a microfuge to collect the contents at the bottom of the tube followed by incubation at 37ºC for 30 minutes in a programmable heating block (Eppendorf). Adding 5µl of 250mM EDTA, into the tube, stopped the reaction.

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

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

3.4.5. Hybridization and stringent washes

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

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

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

3.4.6. Autoradiography

The sealed blot was taped right side up (DNA-side up) into an autoradiography cassette (Kodak) with intensifying screens. To avoid problems with autorad orientation, the film was folded at bottom right corner and this allowed for accurate positioning of the autorad after

developing. Blots were exposed on Kodak® (Rochester, NY) X-Omat film for 12-24 hours at –70ºC in the freezer (Revco®).

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

3.4.7. Stripping the probe from the membrane

The membranes were then stripped off the probe using excess of 0.1 M NaOH, for 15 minutes, at room temperature with agitation, repeating the process followed by a brief wash with 5× SSC. The blot was probed again or stored, after sealing the blot by wrapping in a cling film, at -20ºC or dried and store at room temperature sandwiched and taped between two pieces of clean filter paper.

3.5. Determination of genetic profiles of *Pfcrt* and *Pfmdr1-86*

Genetic profiles were determined by statistical comparison of occurrence of both mutant and wild strains between the two sites. Presence of different haplotypes of the *Pfcrt* gene haplotypes at codon 74, 75 and 76 in the two study sites was also analyzed to determine any differences in gene profiles.

3.6 Data analysis

The prevalence of *Pfcrt* K76T was obtained by considering all haplotypes that had the mutation at position 76 (definitive mutation for chloroquine resistance). Chi square was used to compare proportional data. All statistical analyses were conducted using GraphPad Instat statistical software (version 2.04) and P values < 0.05 were considered significant.

CHAPTER FOUR

4.0 Results

4.1. Determination of prevalence of *Pfmdr1-N86Y* mutations in Tiwi and Mbita

4.1.1. Sample selection for samples with *P. falciparum*

Out of the total 257 (100%) samples from Tiwi collected in 2008, 72 (28%) had *P. falciparum* as revealed by PCR. Five (6.5%) of the samples negative by PCR but positive for parasite presence by microscopy were used for dot blot analysis. Seventy-one samples were positive for parasite presence by microscopy and nested PCR. Only one sample was positive after screening the remaining 180 samples that did not reveal presence of the parasite by microscopy but the reverse with PCR, giving a total of 72 PCR positive samples. Dot blot hybridization was able to detect parasite DNA in samples that could not be detected using PCR (CT35, CT47, CT48, CT54 and CT65). From Tiwi in 1999, 90 samples collected on glass fiber membrane (GFM's) had the parasite as revealed by microscopy examination, but PCR analysis of the same samples revealed that 15 (16.6%) of the samples did not have the parasites. All but one of the 38 samples from Mbita in 2009, screened for presence of parasite via PCR had the parasite. Both the negative and the positive by nested PCR were used in dot blot analysis.

4.1.2. Dot blot outcome after *Pfmdr 1-86* analysis

There was a significant reduction ($p = 0.05$) in the prevalence of *Pfmdr1-N86Y* mutation in both study sites ($\chi^2_{df=1} = 12.4$, P = .002). There was no significant ($\chi^2_{df=1} = 0.07615$, P = .7826) reduction in prevalence of *P. falciparum* in between 1999 and 2009 in Mbita. Tiwi had reduction in prevalence of *Pfmdr1-N86Y* from 88% in 1999 to 63% in 2008. However, Samples from Mbita 2009 had high levels (44.7%) of mixed infections than Tiwi in 2008 (7.8%), with 17/38 samples in Mbita 2009 having both mutant and wild type strains.

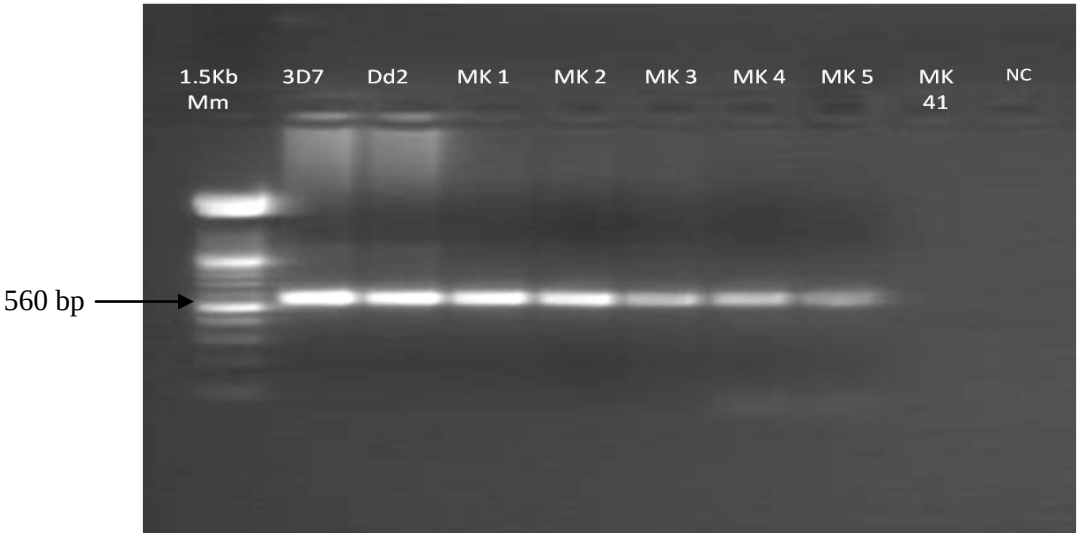


Figure 3: Amplification of the *Pfmdr* gene by nested PCR Mbita samples

The lanes denote the following DNA PCR products; Lane 1 = 1.5kb molecular marker, Lane 2 and 3 = *P. falciparum* positive controls, Lane 4 to 8 = Mbita samples positive for *Pfmdr 1* gene. Lane 9 = Mbita samples Negative for *Pfmdr 1* gene, Lane 10 = Negative control which was nuclease free water. Samples amplified by A1/A3 and A2/A4 primers. The PCR products were resolved in 1.5% agarose gel stained with ethidium bromide in Tris-acetate-EDTA buffer. The electrophoresis was run at 80 volts for 45 minutes. The expected nested PCR product band size, which is 560 base pairs was determined by comparison with a standard 100-base pair DNA ladder.

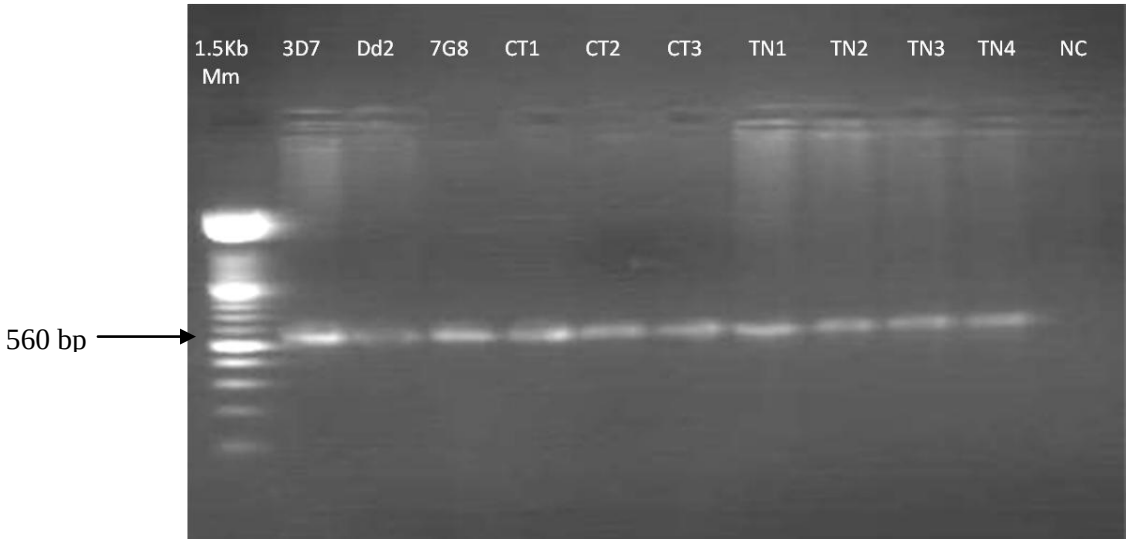
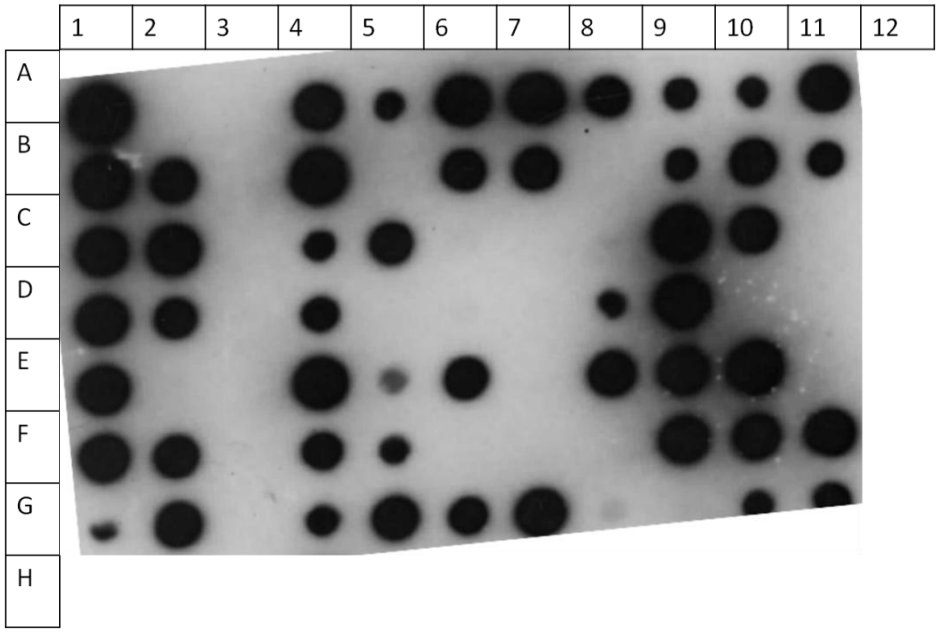


Figure 4: Amplification of the *Pfmdr1-N86Y* gene by nested PCR Tiwi samples

Lane 1 = 1.5kb molecular marker, Lane 2 to 4 = *P. falciparum* positive controls, Lane 5 to 7 = Tiwi 2008 samples positive for *Pfmdr 1* gene. Lane 8 to 11 = Tiwi 1999 samples positive for *Pfmdr 1* gene, Lane 12 = Negative control which was nuclease free water. Samples amplified by A1/A3 and A2/A4 primers. The PCR products were resolved in 1.5% agarose gel stained with ethidium bromide in Tris-acetate-EDTA. The molecular weight marker confirmed expected product size, 560 base pairs

Panel A



Panel B

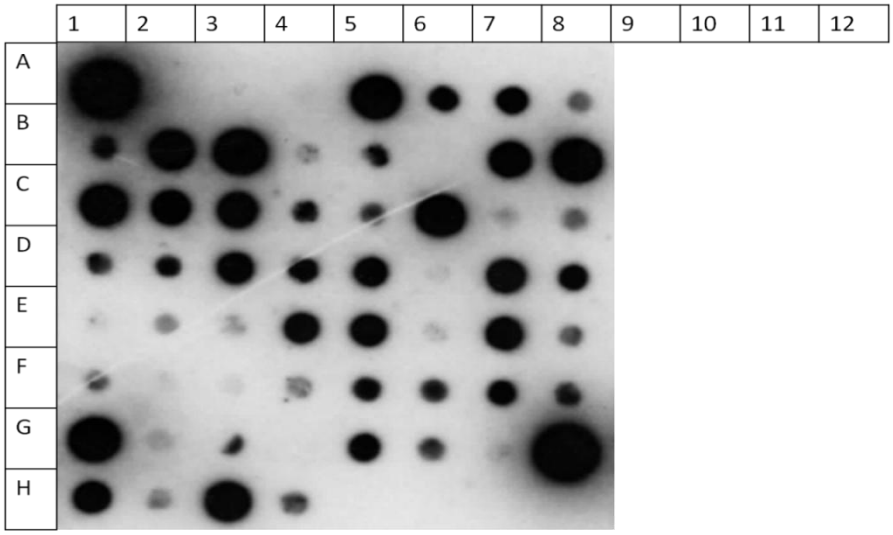


Figure 5: Prevalence of *Pfmdr* 1 N86Y by nested PCR Tiwi samples

The DNA amplified by A1/A3 and A2/A4 primers was blotted on a nitrocellulose membrane and hybridized with mutant (N86Y) and wild type (N86) probes for *Pfmdr 1* gene. Panel A represents a dot blot autoradiograph for 2008 samples from Tiwi (Tyrosine probe, mutant). A1 had Tyrosine mutant control, Dd2; A2 had the asparagine wild type control, 3D7 while A3 had PCR negative control which was nuclease free water. The mutant type control gave a strong signal hence samples that picked a similar signal had a single nucleotide polymorphism at codon 86. Panel B represents an autoradiograph for 2009 samples from Mbita (Tyrosine probe, mutant).

Table 1: Summary of the prevalence of the *Pfmdr 1* N86Y mutation in the Tiwi and Mbita towns as analyzed by dot blot technique ($\chi^2_{df=2} = 12.4$, P = .002)

	Pfmdr-1 86 Dot blot results			
	n	**Mutant**	**Wild**	**Mixed**
Tiwi 1999	75	88.0%	12.0%	17.3%
Tiwi 2008	77	63.6%	36.4%	7.8%
Mbita 2009	38	84.2%	15.8%	44.7%

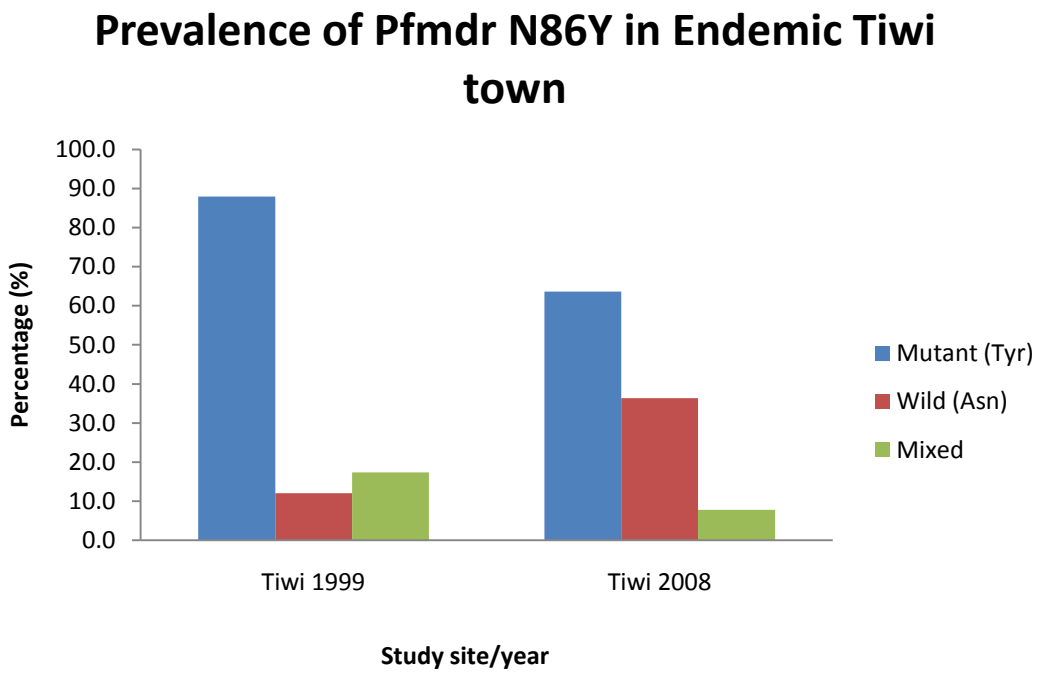


Figure 6: Graph showing the trends in prevalence of *Pfmdr1*-N86Y mutation between 1999 and 2008 in Tiwi ($\chi^2_{df=1}$ = 10.958, P = .0009).

All samples having both the mutant and wild type strains (mixed infections) are considered as mutant. There is a significant reduction in the prevalence of *Pfmdr1*-N86Y by 24.4% (p=0.05) between 1999 and 2008 in Tiwi and mixed infections were observed to decrease by 9.5%.

4.2. Identification of *Pfcrt* gene haplotypes at codon 74, 75 and 76 in Tiwi and Mbita

A total of 190 samples were analyzed by dot-blot hybridization for polymorphisms in the chloroquine resistance molecular markers, *Pfcrt* gene (Tiwi 1999 n = 75; Tiwi 2008 n = 77; Mbita 2009 n = 38). None of the samples from any of the sites hybridized to MNT, INK or INT probe. Of all the samples that were analyzed by dot blot hybridization, 15 (7.9%) did not pick signals, 10 (13.3%) from 1999 and 5 (6.5%) from 2008 samples from Tiwi. All samples from Mbita hybridized at least one probe.

When hybridized with the MEK probe, only 11 samples (29.0%) from Mbita hybridized. Seventeen (23.6%) and 18 (47.4%) samples from Tiwi 2008 and Mbita 2009 hybridized to the MET probe. There was no MET signal from 1999 Tiwi samples. Mbita samples did not hybridize to either IET or IEK probes. However, IET was present in 1999 (75.3%) and 2008 (22.2%) Tiwi samples while IEK was only present in 2008 (36.1%) of Tiwi samples. Out of the mixed infections in Mbita, 4 samples had both MNK and MEK, 5 samples had MNK and MET and one sample had MET, MEK and MNK haplotypes. In Tiwi samples collected in 2008, 5 of them had mixed IET and IEK while 3 contained both MNK and MET haplotypes. There was no mixed infection detected in 1999 Tiwi samples. All sites hybridized to the MNK probe, 16 (24.6%), 37 (51.3%) and 25 (65.8%) for 1999, 2008 Tiwi samples and 2009 Mbita samples respectively (Table 2).

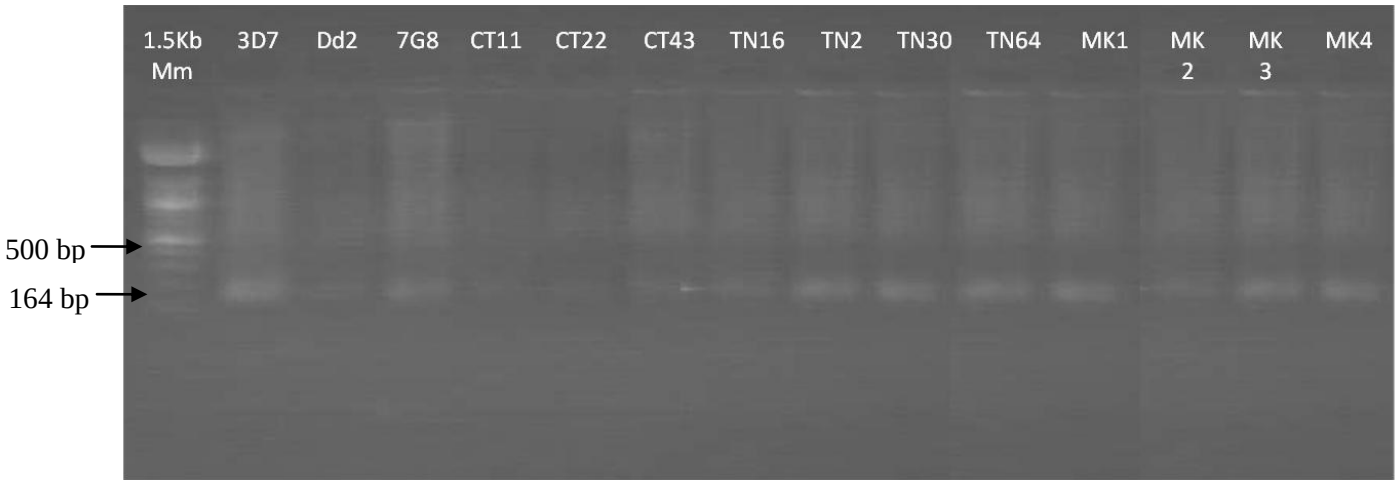
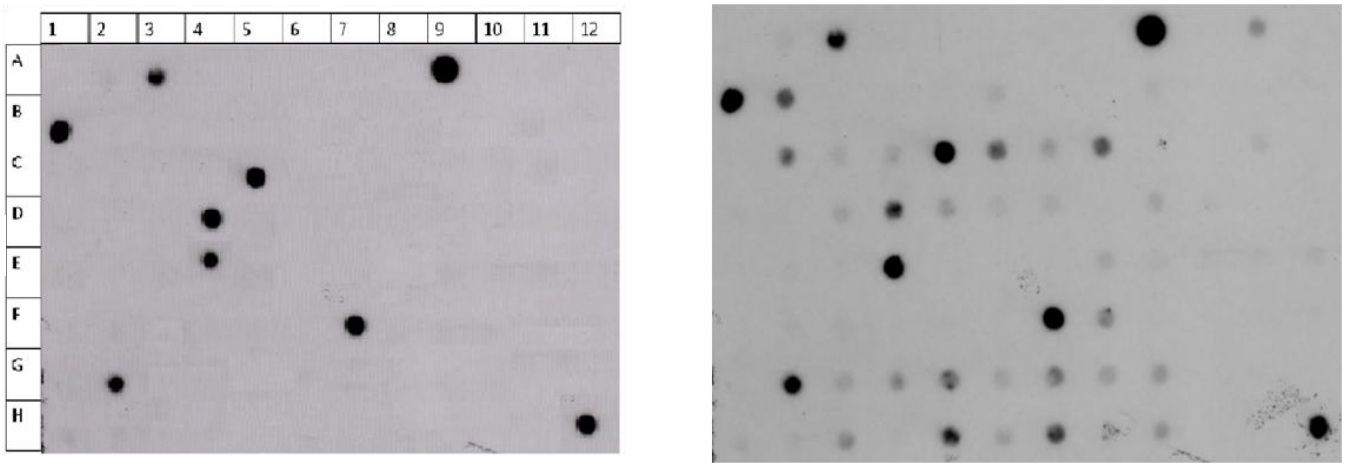


Figure 7: Amplification of the *Pfcrt* gene by nested PCR Tiwi and Mbita samples

The lanes denote the following DNA PCR products; Lane 1 = 100 base pair molecular marker, Lane 2 to 4 = *P. falciparum* positive controls, Lane 5 to 7 = Tiwi 2008 samples positive for *Pfcrt* gene. Lane 8 to 11 = Tiwi 1999 samples positive for *Pfcrt* gene, Lane 12 to 15 = Mbita 2009 samples positive for *Pfcrt.* DNA samples amplified by P1/P2 and D2/D3 primers. The PCR products were resolved in 1.5% agarose gel stained with ethidium bromide in Tris-acetate-EDTA. Electrophoresis was run at 80 volts for 30 minutes.The molecular weight marker confirmed expected product size, 164 base pairs

Panel A



(1) (2)

Panel B

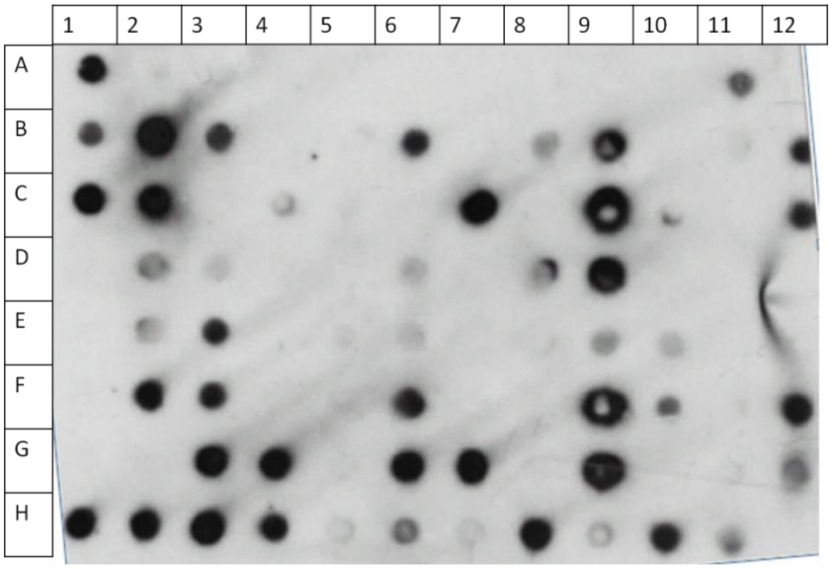


Figure 8: *Pfcrt* dot blot autoradiograph for Mbita and Tiwi.

DNA samples amplified by P1/P2 and D2/D3 primers was blotted on a nitrocellulose membrane and hybridized to *Pfcrt* haplotype probes for position 74, 75 and 76. Panel A represents an autoradiograph for 2008 samples from Tiwi (IET) after optimized stringent washes (2). A1 had 3D7 negative control (MNK); A2 had PCR negative control which was nuclease free water while A3 had Dd2, positive control (IET). IET is the complete mutation at position 74, 75 and 76 of the *Pfcrt* gene that is present in all strains resistant to chloroquine. Panel B represents a dot blot autoradiograph for 2009 samples from Mbita (MNK probe). MNK is the haplotype of *Pfcrt* at position 74, 75 and 76 present in all chloroquine sensitive strains of *P. falciparum.*

Table 2: Analysis for polymorphisms in *Pfcrt* gene at positions 74, 75 and 76 by dot-blot hybridization

	***PFCRT* HAPLOTYPES PROBES THAT HYBRIDIZED USING DOT BLOT**								
	MNK	**MNT**	**IEK**	**IET**	**MET**	**MEK**	**XD**	**BLANK**	**TOTAL**
TIWI 1999	16	-	-	49	-	-	-	10	75
TIWI 2008	20	-	26	16	17	-	8	5	77
MBITA 2009	17	-	-	-	18	11	10	-	38

XD stands for samples that contained a mixture of *P. falciparum* strains with different haplotypes of *Pfcrt* gene. Some samples were blank because they did not give any signals in any of the blots.

4.3 Genetic profiles of *Pfcrt* and *Pfmdr1-86* in *P. falciparum* isolates from malaria endemic Tiwi and Mbita towns of Kenya.

Mbita has more samples having more than one haplotype (mixed infection rate) 26.3% (10/38, 95% CI 13.0-43.0) which are higher than Tiwi 11.1% (8/72, 95% CI 5.0-21.0). It is also in Mbita where one sample harbored MET, MEK and MNK *Pfcrt* haplotypes. The mutant allele for *Pfcrt*, IET reduced in proportion from 75.3% to 11.1% while the wild type allele MNK increased from 24.6% to 51.4%. There was a significant association between Tiwi 1999/Tiwi 2008 ($\chi^2_{df=1}$ = 10.958, P = .0009) and Mbita 2009/Tiwi 2008 ($\chi^2_{df=1}$ = 4.231, *p* = .00397). There was however no significant association between Tiwi 1999/Mbita 2009 ($\chi^2_{df=1}$ = 0.0715, P = .7891). There were no samples collected from Mbita in 1999 or earlier. The odds ratio for all the comparisons are greater than 1 hence mutations are more likely to be reported in the sample populations than not.

Mbita has a higher frequency of sensitive MNK clones (65.8%, 95% CI 49.0-80.0) than Tiwi. In addition, Tiwi has the chloroquine resistant haplotype, IET (11.1, 95% CI 5.0-21.0) while samples from Mbita do not have IET. Samples collected in 2008 from Tiwi have a unique IEK (36.1%, 95% CI 25.0-48.0) allele which was not present in 1999. MET, which was not also present in 1999 from Tiwi, is present in 2008 samples from Tiwi (12.5%, 95% CI 6.0-22.0) and 2009 from Mbita (34.2%, 95% CI 20.0-51.0).

The prevalence of *Pfcrt* K76T was obtained by considering all haplotypes that had the mutation at position 76 (definitive mutation for chloroquine resistance). Tiwi had 56.9% (95% CI 45.0-69.0) in 2008 down from 75.4% (95% CI 63.0-85.0), a reduction of 18.5% ($\chi^2_{df=1}$ = 21.132, P = .0001). However 36% (95% CI 25.0-48.0) of samples from Tiwi in 2008 still had the IEK which may not have the definitive mutation, K76T, but has M74I and N75E mutations. Mbita had 63.2% (95% CI 60.0-89.0) of the samples bearing the K76T mutation.

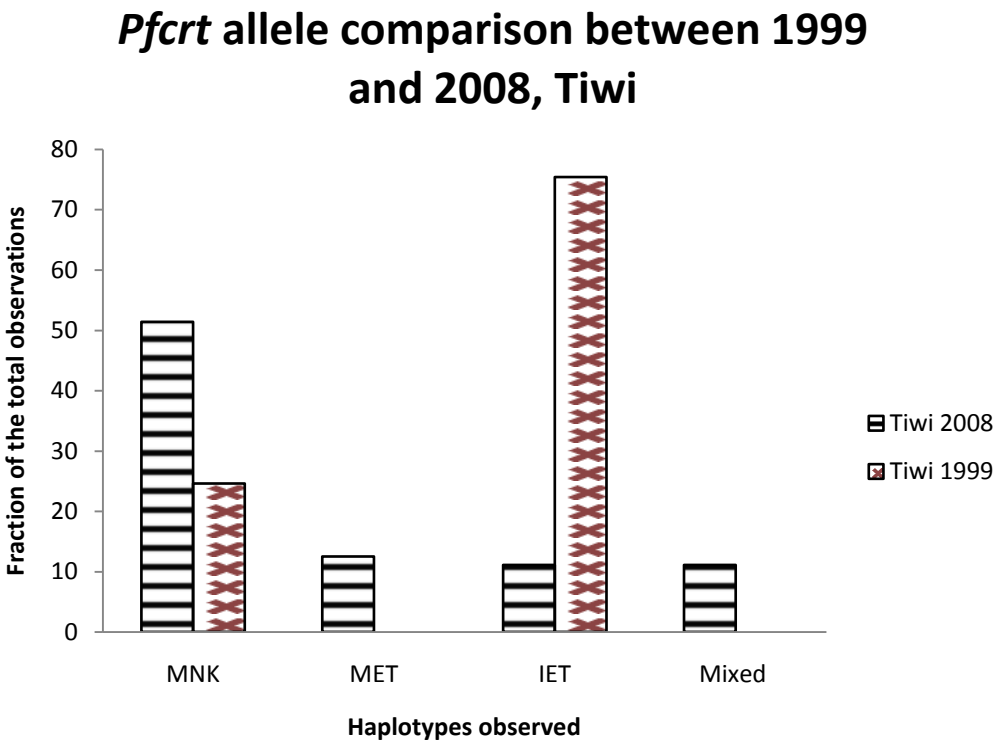


Figure 9: Trends of *Pfcrt* alleles over a decade in Tiwi

The mutant allele for Pfcrt, IET that is present in chloroquine resistant *P. falciparum* strains reduced in proportion from 75.3% in Tiwi in 1999 to 11.1% in 2008 while the wild type allele MNK that is present in all chloroquine sensitive *P. falciparum* strains increased from 24.6% in 1999 to 51.4% in Tiwi in 2008.

Table 4: Haplotype profiles for *Pfcrt* for Mbita

Mbita 2009						
	Pfcrt			Frequency		
Haplotype/Mutation	**M74I**	**N75E**	**K76T**	**n (38)**	**%**	**95% CI**
MNK	-	-	-	17	44.7	29.0-62.0
MNT	-	-	+	0	0	0
MET	-	+	+	18	47.4	31.0-64.0
MEK	-	+	-	11	29.0	15.0-46.0
IEK	+	+	-	0	0	0
IET	+	+	+	0	0	0
Mixed haplotypes				10	26.3	13.0-43.0
K76T	-	-	+	24	63.2	46.0-78.0

The '+' indicates presence of a mutation at each of the three respective codons while the '-' indicates absence of the mutation at codon positions indicated.

Table 5: Haplotype profiles for *Pfcrt* for Tiwi

Tiwi 2008						
	Pfcrt			Frequency		
Haplotype/Mutation	**M74I**	**N75E**	**K76T**	**n (72)**	**%**	**95% CI**
MNK	-	-	-	21	27.8	18.0-40.0
MNT	-	-	+	0	0	0
MET	-	+	+	17	23.6	14.0-35.0
MEK	-	+	-	0	0	0
IEK	+	+	-	26	36.1	25.0-48.0
IET	+	+	+	16	22.2	13.0-34.0
Mixed haplotype				8	11.1	5.0-21.0
K76T	-	-	+	41	56.9	45.0-69.0

Two new haplotypes were identified in Tiwi, IEK, 36.1%, which is a mutation whereby Methionine is replaced by Isoleucine at position 74 and Asparagine is replaced by Glutamate at position 75 and MET 23.6% which has Asparagine replaced by Glutamate at position 75 and Lysine replaced by Threonine at position 76.

CHAPTER FIVE

5.0 Discussion, conclusion and recommendation

5.1. Discussion

Prevalence of *Pfmdr1-N86Y* mutation in *P. falciparum* isolates between 1999 and 2009 from malaria endemic Tiwi has reduced significantly to 64% in 2008. The prevalence of *Pfcrt K76T* mutation in *P. falciparum* isolates reduced significantly to 56.9% in Tiwi 2008 while Mbita's prevalence of *Pfcrt K76T* mutation as at 2009 is 76.3%. This reduction in prevalence of mutations in the molecular markers for chloroquine, *Pfmdr1* and *Pfcrt* genes, is in agreement with several authors who have noted a slow reversal to the wild type in Mwea and Kilifi District (Omar *et al.,* 2007; Mwai *et al.,* 2009) in absence of drug pressure from chloroquine. Mutations in *Pfmdr1* may not entirely be sufficient for the malaria parasite to develop the chloroquine resistance phenotype; however the N86Y mutation can moderate drug response (Foote *et al.,* 1990; Reed *et al.,* 2000).

From the data on prevalence of mutation in *Pfcrt* and *Pfmdr 1* genes, we can infer that Mbita indicates relatively higher transmission rates than Tiwi, although both sites are high transmission zones and exhibit endemic malaria, since there is a more polyclonal population structure (high mixed infection rates in Mbita *Pfmdr* N86Y 44.7% compared to Tiwi 7.8%) owing to exclusive recombination among local *P. falciparum*, including other factors. Tiwi is less prone to exclusive recombination among local malaria parasite populations since it is in the coastal region where there is higher fluidity of host population, (Division of Malaria Control, 2009) due to close proximity to the coastline. Another reason may be due to the success in use of transmission barriers such as insecticide treated nets as compared to Mbita (Division of Malaria Control, 2010). Higher transmission rates are also evident from the presence of a sample having three different *Pfcrt* haplotypes strains of *Plasmodium falciparum* parasites from Mbita.

The probability of resistance parasite being exposed to drugs in Mbita is increased by higher rates of mixed infections, 26.3% for *Pfcrt* and 44.7% for *Pfmdr 1*, because the parasite population is more widely shared among the host population, and it is at the level of the host population that the degree of drug usage matters (Mackinnon and Hastings, 1998). In areas of high transmission intensity, such as Mbita, the host will have more polyclonal infections and,

consequently, will have a higher probability of transmitting gametocytes of multiple genetic backgrounds that will recombine in the mosquito (Mu *et al.*, 2005). This will directly reduce the number of independent gametes participating in reproduction and, consequently, the effective population size (Barton, 1995). Reduced effective population sizes will affect the ways selection acts on mutations associated with antimalarial drug resistance. Drug-resistant mutations, for example, could go to fixation by chance alone, even after drug pressure is eliminated, if they are already in high frequency (Escalante *et al.,* 2009). This explains the slowed reversal of prevalence of mutations in the chloroquine resistance molecular markers *Pfcrt* and *Pfmdr 1* in both study sites.

Haplotypes in *Pfcrt* gene at codon 74, 75 and 76 of *P. falciparum* isolates from Mbita and Tiwi exhibited diverse profiles. There were two new haplotypes reported in Tiwi, IEK (Methionine is replaced by Isoleucine at position 74 and Asparagine is replaced by Glutamate at position 75) and MET (Asparagine is replaced by Glutamate at position 75 and Lysine is replaced by Threonine at position 76). There is an indication of a wider gene pool in Tiwi because of the presence of the haplotype IET (Methionine is replaced by Isoleucine at position 74, Asparagine is replaced by Glutamate at position 75 and Lysine is replaced by Threonine at position 76) and IEK, which are both native to South-east Asia. However, there is a new MET haplotype which was not present in 1999 from Tiwi but present in 2008 samples. There might have been a probable introduction of this parasite strain from Western and Central malaria endemic regions of Kenya, such as Oyugis, Mbita and Mwea, through host population interactions since MET has been discovered in these regions (Omar *et al.*, 2007; Wangai, 2009). Mbita has however maintained a unique MNK, MEK (Asparagine is replaced by Glutamate at position 75) and MET profile. MET and MNK, which has increased in frequency over the decade, is present in both study sites.

The most likely reason why chloroquine took long to develop resistance since its introduction is that under CQ pressure, malaria parasites acquire *Pfcrt* mutations sequentially, with each mutation providing an incremental increase in chloroquine tolerance until parasites become refractory to chloroquine at therapeutic doses (Hastings *et al.,* 2002). However there are no intermediate alleles with a partial set of resistance mutations. This is probably because, the

major mutation (K76T) is acquired first before the other mutations, which may be compensatory, follow. Majority of the samples of 2008 from Tiwi that had the IET haplotypes also had IEK. This may be due to continued slow reversal of the resistant genotype at position 76 to the sensitive type. The chloroquine resistant mutant haplotypes (IET) observed in Tiwi ten years ago seems to be slowly reverting to its sensitive type (MNK). However the codon positions are not reverting at the same rate.

If other resistance mutations in *Pfcrt* are compensatory in support of K76T, they may in fact, confer a disadvantage on the chloroquine sensitive parasites, possibly explaining the lack of known single mutation revertants in Mbita and Tiwi where chloroquine is no longer used. The mode of selection of resistant strains may be due to clonal expansion of sensitive strains that are genetically fit to survive under absence of drug pressure. The cost of survival of the mutant haplotypes such as IET is increasingly high and hence there is clonal selection against such as these. In absence of drug pressure, chloroquine resistant *Pfcrt* mutations in the South American genetic background appear fit and stable due to a combination of low parasite transmission rates and the possibility that there are no longer chloroquine sensitive alleles in the population of *P. falciparum* to re-emerge (Wooton *et al.,* 2002). However, in our study sites, we have the sensitive MNK haplotype that is re-emerging in both sites, with MNK increasing in Tiwi by 26.7% over a decade.

The notable absence of MNT, INK and INT haplotypes in both sites may indicate high fitness cost for the strains harboring this genotype. Absence of MNT may be explained by geographical distribution because it is unique to South America (Equador, Brazil and Guyana) and is not native to East Africa (Cooper *et al.,* 2005).

Apart from reduced drug pressure, various mechanisms work naturally against spread of drug resistance such as force of recombination. The number of parasites with resistance combinations that are broken down by recombination exceeds the numbers that are built up. This recombination breakdown acts as a counter-balance to drug selection for combinations of resistance alleles. However, the force of recombination is weak when drug selection is low such as in the study sites after chloroquine withdrawal. Degrading force of recombination is only sufficient if the two alleles are rare, drug pressure is low and resistance alleles act in a strong

epistatic way (Hastings and Mackinnon, 1998). Recombination breakdown is therefore higher in high transmission areas and this would slow the spread of resistance. Host population is generally more immune in high transmission areas; drug usage might be lower, thus reducing the rate of spread of resistance.

5.2 Conclusion

This study has characterized *P. falciparum* isolates from two geographically distant endemic sites in Kenya. Using established molecular markers for chloroquine resistance, *Pfcrt* and *Pfmdr1,* the results indicated that there was a significant reduction in prevalence of mutations in *Pfcrt* and *Pfmdr 1* over a decade in both sites. This observation however, remains as a prediction of chloroquine drug failure and does not have a direct correlation to treatment outcome since several factors such as transmission rates, forces of recombination and in-host dynamics come into play.

The research presented further identified two new haplotypes for the *Pfcrt* gene at codon 74, 75 and 76 in Tiwi which are IEK and MET which were not previously present a decade ago (1999) in Tiwi. There was however no new haplotypes identified in Mbita. This observation, together with other findings discussed above, established that the gene profiles of *Pfcrt* and *Pfmdr 1* in these malaria endemic sites are not similar.

5.3. Recommendations

1. The effect of differences in gene profiles on efficacy of artemisinin based drugs in malaria endemic regions such as Tiwi and Mbita should be carried out.
2. Further investigations should be done on the role of *Pfcrt* mutations other than position 76 in chloroquine resistance. The focus should shift to the basic biology of the PfCRT protein, its regulation, expression, and network of gene partners in cellular processes. In addition, one should be able to know whether other genes other than *Pfcrt* are required to produce chloroquine resistance. Much will be revealed about the evolution of resistance to drugs with complex mechanisms of action such as the frontline artemisinin based antimalarial drugs.

REFERENCES

Abdel-Muhsin, A. A., Ranford-Cartwright, L. C., Abdel-Rahman, M., Ahmed, S., Suleiman, S., Khan, B., et al. (2002). Detection of mutations in *Plasmodium falciparum* drug resistance gene by dot-blot hybridization. *American Journal of Tropical Medicine and Hygiene, 67*, 24-27

Agnew, P., Berticat, C, Bedhomme S., Sidobre C., & Michalakis Y. (2004). Parasitism increases and decreases the costs of insecticide resistance in mosquitoes. *Evolution, 58*, 579–586.

al-Yaman, F., Genton, B., Reeder, J. C., Anders, R. F., Smith, T., & Alpers, M. P. (1997). Reduced risk of clinical malaria in children infected with multiple clones of *Plasmodium falciparum* in a highly endemic area: a prospective community study. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 91*(5), 602-605.

Alan, F. C., & Brendan, S. C. (2006). Invasion of Red Blood Cells by Malaria Parasites. *Molecular Cell, 124*, 755–766.

Alifrangis, M., Dalgaard, M. B., Lusingu, J. P., Vestergaard, L. S., Staalsoe, T., Jensen, A. T., et al. (2006). Occurrence of the Southeast Asian/South American SVMNT haplotype of the chloroquine-resistance transporter gene in *Plasmodium falciparum* in Tanzania. *Journal of Infectious Diseases, 193*, 1738-1741.

Aregawi, M., Cibulskis, R., Otten, M., Williams, R., & Dye, C. (2008). *World Malaria Report*, WHO Press.

Ariey F., Fandeur, T., Durand, R., Randrianarivelojosia, M., Jambou, R., Legrand E., et al. (2006). Invasion of Africa by a single *pfcrt* allele of South East Asian type. *Malaria Journal, 5*, 34.

Babiker, H. A., Pringle, S. J., Abdel-Muhsin, A., Mackinnon. M., Hunt, P., & Walliker. D. (2001). High level chloroquine resistance in Sudanese isolates of *Plasmodium falciparum* is associated with mutations in the chloroquine resistance transporter gene *Pfcrt* and the multidrug resistance gene *pfmdr1*. *The Journal of Infectious Diseases, 183*, 1543-1548.

Barton, N.H. (1995) Linkage and the limits to natural selection. *Genetics, 140*, 821–841.

Beier, J. C. (1993). Malaria sporozoites: survival, transmission and disease control. *Parasitology Today, 9*, 210–215.

Bjorkman, A., & Bhattarai, A. (2005). Public health impact of drug resistant *Plasmodium falciparum* malaria. *Acta Tropica, 94*, 163–169.

Bull, P. C., Lowe, B. S., Kortok, M., Molyneux, C. S., Newbold, C. I., & Marsh, K. **(**1998). Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. *Nat. Med.* 4:358–360.

Coetzee, M., Craig, M., & le Sueur, D. (2000). Distribution of African malaria mosquitoes belonging to the *Anopheles gambiae* complex. *Parasitology Today, 16,* 74-77.

Cooper, R. A., Hartwig, C. L., & Ferdig, M. T. (2005). *Pfcrt* is more than the *Plasmodium falciparum* chloroquine resistance gene: a functional and evolutionary perspective. *Acta Tropica, 94*, 170–180.

Division of malaria control, (2009). *Kenya malaria monitoring and evaluation Plan.* Retrieved November 19, 2010, Available from http://www.nmcp.or.ke

Division of malaria control, (2010). *Kenya malaria fact sheet.* Retrieved November 19, 2010, from http://www.nmcp.or.ke

Djimdé, A., Ogobara, O. K., Cortese, J. F., Kayentao, K., Doumbo, S. & Diorté Y., et al. (2001). A molecular marker for chloroquine-resistant falciparum malaria. *New England Journal of Medicine, 344*, 257-263.

Duraisingh, M. T., Jones, P., Sambou, I., von Seidlein, L., Pinder, M & Warhurst, D. C. (2000) The tyrosine-86 allele of *pfmdr1* gene of *plasmodium falciparum* in associated with increased sensitivity to the antimalarials mefloquine and artemisinin. *Molecular and Biochemical Parasitology, 108,* 13-23.

Escalante, A., Smith, D., & Kim, Y. (2009). The dynamics of mutations associated with anti-malarial drug resistance in *Plasmodium falciparum. Trends in Parasitology*, *25*(12), 557-563

Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekuov, S. M., & Ferdig, T., *et al.* (2000). Mutations in the *Plasmodium falciparum* digestive vacuoles transmembrane protein *Pfcrt* and evidence for their role in chloroquine resistance. *Molecular Cell, 6*, 861-871.

Fitch, C. D., Chen, Y. F., & Cai, G. Z. (2003). Chloroquine-induced masking of a lipid that promotes ferriprotoporphyrin IX dimerization in malaria. J. Biol. Chem. **278:**22596–22599.

Fitch, C. D. (2004). Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs. *Life Science, 74*, 1957–1972.

Fitch, C. D., & Russell, N. V. (2006). Accelerated denaturation of hemoglobin and the antimalarial action of Chloroquine. *Antimicrobial agents and chemotherapy, 50*, 2415–2419.

Foote, S. J., Kyle, D. E., Martin, R. K., Oduola, A. M., Fornsyth, K., & Kemp D. J., et al. (1990). Several alleles of the multi-drug resistance gene are closely linked with chloroquine resistance in *Plasmodium falciparum. Nature, 345*, 255-258.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., et al. (2002). Genome sequence of human malaria parasite *P. falciparum*. *Nature, 419*, 498–511.

Ghosh, A., Edwards, M. J., & Jacobs-Lorena, M. (2000). The Journey of the Malaria Parasite in the Mosquito: Hopes for the New Century. *Parasitology Today, 16*, 196-201.

Golsner, J., Waknine, J. H., Krugliak, M., Hunt, N. H., & Grau, G. E. (2006). Current perspectives on the mechanism of action of artemisinins. *International Journal of Parasitology, 36*, 1427-1441.

Gouagna, L. C., Okech, B. A., Kabiru, E. W., Killeen, G. F., Obare, P., Ombonya, S., et al. (2003). Infectivity of *Plasmodium falciparum* gametocytes in patients attending rural health centres in western Kenya. *East African Medical Journal, 80*, 627–634.

Greenwood, B. (2006). Review: Intermittent preventive treatment: A new approach to the prevention of malaria in children in areas with seasonal malaria transmission. *Tropical Medicine and International Health,11*(7), 983-91.

Hastings, I. M., & Mackinnon, M. J. (1998). The emergence of drug resistant malaria. *Parasitology, 117*, 411-417.

Hastings, I. M., Bray, P. G., & Ward, S. A. (2002). Parasitology. A requiem for chloroquine. *Science, 298*, 74-75.

Hastings, I. M., & Smith, T. A. (2008). MalHaploFreq: A computer program for estimating malaria haplotype frequencies from blood samples. *Malaria Journal, 7*, 130.

Hviid, L., Jakobsen, P. H., Abu-Zeid, J. A., & Theander, T. G. (1992). T-cell responses in malaria. *Acta Pathologica, Microbiologica et Immunologica Scandinavica, 100*, 85-106.

Hyde, J. E. (2005). Exploring the folate pathway in *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene, 94*, 191-206.

Ittarat, W., Pickard, A. L., Rattanasinganchan, P., Wilairatana, P., Looareesuwan, S., Emery, K.,et al. (2003). Recrudescence in artesunate treated patients with falciparum malaria is dependent on parasite burden not on parasite factors. *American Journal of Tropical Medicine and Hygiene,* 68, 147–152.

Kappe, H. I., Buscaglia, C. A., & Nussenzweig, V. (2004). *Plasmodium* Sporozoite molecular Cell Biology. *Annual Review of Cell Development Biology, 20*, 29–59.

Kaur, M., Zhang, Y., Liu, W. H., Tetradis, S., Price, B. D., & Makrigiorgos, G. M. (2002). Ligation of a primer at a mutation: a method to detect low level mutations in DNA. *Mutagenesis, 17*, 365–374.

Kublin, J. G., Cortese, J. F., Njunju, E. M., Mukadam, R. A., G, Wirima, J. J., Kazembe, P. N., et al. (2003). Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *The Journal of Infectious Diseases, 187*, 1870-1875.

Laufer, M. K., Thesing, P. C., Eddington, N. D., Masonga, R., Dzinjalamala, F. K., Takala, S. L., *et al.,* (2006). Return of chloroquine antimalarial efficacy in Malawi. *New England Journal of Medicine, 355*, 1959–1966.

Levin, B. R., Perrot, V., & Walker, N. (2000). Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics, 154*, 985–997.

Makrigiorgos, G. M. (2004). PCR-Based detection of minority Point Mutations. *Human Mutation, 23,* 406-412.

Males, S., Gaye, O., & Garcia, A. (2008). Long-Term Asymptomatic Carriage of *Plasmodium falciparum* Protects from Malaria Attacks: A Prospective Study among Senegalese Children. *Clinical Infectious Disease, 46*, 516-522.

Martin, R. E., Marchetti, R. V., Cowan, A. I., Howitt, S. M., Bröer, S., & Kirk, K. (2009). Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science, 325,* 1680-1682

Mayor, A. G., Gómez-Olivé, X., Aponte, J. J., Casimiro, S., Mabunda, S., Dgedge, M., *et al.*, (2001). Prevalence of the K76T mutation in the putative *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) gene and its relation to chloroquine resistance in Mozambique. *Journal of Infectious Diseases, 183,* 1413-1416.

Mu J., Awadalla, P., Duan J., McGee K. M., Deidre A. J., McVean G. A. T., *et al.* (2005). Recombination Hotspots and Population Structure in *Plasmodium falciparum*. PLoS Biol 3(10): e335. doi:10.1371/journal.pbio.0030335.

Mutero, C. M., Ouma, J. H., Agak, B. K., Wanderi, J. A., & Copeland, R. S. (1998). Malaria prevalence and use of self-protection measures against mosquitoes in Suba District, Kenya. *East African Medical Journal, 75,* 11–15.

Mwai, L., Ochong, E., Abdirahman, A., Kiara, S., Ward, S., Kokwaro, G. *et al.* (2009). Chloroquine resistance before and after its withdrawal in Kenya. *Malaria Journal, 8,* 106.

Nzila, A. M., Nduati, E., Mberu, E. K., Sibley, C. H., Monks, S. A., *et al.* (2000). Molecular evidence of greater selective pressure for drug resistance exerted by the long-acting antifolate pyrimethamine/ sulfadoxine compared with shorter acting Chlorproguanil/ Dapsone on Kenyan *Plasmodium falciparum*. *The Journal of Infectious Diseases, 181*, 2023-2028.

Nsobya, S. L., Dokomajilar, C., Joloba, M., Dorsey, G., & Rosenthal, P. J. (2007). Resistance-mediating *Plasmodium falciparum pfcrt* and *pfmdr1* alleles after treatment with artesunate-amodiaquine in Uganda. *Antimicrobial agents and chemotherapy, 51*, 3023-3025.

Obi, R. K., Okangba, C. C., Nwanebu F. C., Ndubuisi, U. U., & Orji, N. M. (2010). Premunition in *Plasmodium falciparum* malaria. *African Journal of Biotechnology, 9(10),* 1397-1401.

Omar, S. A., Adagu, I. S., & Warhurst, D. C. (2001). Can pretreatment screening for *dhps* and *dhfr* point mutations in *Plasmodium falciparum* infections be used to predict sulfadoxine-pyrimethamine treatment failure? *Transactions of the Royal Society of Tropical Medicine and Hygiene, 95*, 315-319.

Omar, S. A., Makokha F. W., Fat'hia, A. M., Kimani, F. T., & Magoma, G. (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*(2), 195-201.

Orr, H. A. (2000). Adaptation and the cost of complexity. *Evolution, 54,* 13–20.

Ouellette, M. (2001). Biochemical and molecular mechanisms of drug resistance in parasites. *Tropical Medicine and International Health, 6,* 874.

Padmanabhan, G., & Rangarajan, P. N. (2000). Heme metabolism of Plasmodium is a major antimalarial target. *Biochemical and Biophysical Research Communications, 268,* 665–668.

Paniker, C. K. (1989). *A textbook of Medical Parasitology,* 2nd edition. Medical Publishers. New Delhi. India. p. 58-83.

Pimenta, P. F., Touray, M., & Miller, L. (1994). The journey of malaria sporozoites in the mosquito salivary gland. *The Journal of Eukaryotic Microbiology, 41,* 608–624.

Pukrittayakamee, S., Chotivanich, K., Chantra, A., Clemens, R., Looareesuwan, S., & White, N. J. (2004) Activities of artesunate and primaquine against asexual- and sexual-stage parasites in falciparum malaria. *Antimicrobial agents and chemotherapy, 48,* 1329–1334.

Ranford-Cartwright, L. C., Johnson, K. L., Abdel-Muhsin, A. A., Khan, B., & Babiker, H. A. (2002). Critical comparison of molecular genotyping methods for the detection of drug resistant *Plasmodium falciparum*. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 96,* 568-572

Ravit, A. B., & Theresa, A. S. (2005). Molecular mechanisms of resistance in antimalarial chemotherapy: The unmet challenge. *The Annual Review of Pharmacology and Toxicology, 45,* 565–585.

Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K., & Cowman, A. F. (2000). Pgh 1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature, 24* 403*(6772),* 906-909.

Schneider, A. G., Premji, Z., Felger, I., Smith, T., Abdulla, S., Beck, H., et al. (2002). Point mutation in codon 76 of *pfcrt* of *P. falciparum* is positively selected for by chloroquine treatment in Tanzania. *Journal of Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases, 1,* 183-189.

Seesod, N., Nopparat, P., Hedrum, A., Holder, A., Sodsri, T., Uhlen, M., et al. (1997). An integrated system using immune-magnetic separation, polymerase-chain reaction and colorimetric detection for diagnosis of *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene, 56*, 322–328.

Shretta, R., Omumbo, J., Rapuoda, B., & Snow, R. W. (2000). Using evidence to change antimalarial drug policy in Kenya. *Tropical Medicine & International Health, 5*, 755–764.

Sidhu, A, B., Verdier-Pinard, D., & Fidock, D. A. (2002). Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. *Science, 298*, 210–213.

Sinclair, B. (2000). Isotopic labels still have their place in DNA hybridization**.** *The Scientist*, 14, 22.

Sisowath, C., Stromberg, J., Martensson, A., Msellem, M., Obondo, C., & Bjorkman, A., et al. (2005). *In vivo* selection of *Plasmodium falciparum pfmdr1 86N* coding alleles by artemether-lumefantrine (Coartem). *Journal of Infectious Diseases, 191*, 1014–1017.

Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. Y., & Hay, S. I. (2005). The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature, 434* (7030), 214-217.

Sutherland, C. J., Haustein, T., Gadalla, N., Armstrong, M., Doherty, J. F., & Chiodini, P. L (2007). Chloroquine-resistant *Plasmodium falciparum* infections among UK travellers returning with malaria after chloroquine prophylaxis. *Antimicrobial agents and chemotherapy*, *59*, 1197-1199.

Targett, G., Drakeley, C., Jawara, M., von Seidlein, L., Coleman, R., Deen, J., et al. (2001). Artesunate reduces but does not prevent post-treatment transmission of *Plasmodium falciparum* to *Anopheles gambiae*. *Journal of Infectious Diseases, 183*, 1254–1259.

Travassos, M. A., & Laufer, M. K. (2009) Resistance to antimalarial drugs: molecular, pharmacologic, and clinical considerations *Pediatric Research 65*, 64R-70R

Trouiller, P., Olliaro, P., Torreele, E., Orbinski, J., Laing, R., & Ford, N. (2002). Drug development for neglected diseases: a deficient market and a public-health policy failure. *Lancet, 359*, 2188-2194.

Wahlgren, M., & Qijun, C. (1999). Waves of malarial variations. *Molecular Cell 96*, 603-605.

Waller, K. L., Muhle, R. A., Ursos, L. M., Horrocks, P., & Verdier-Pinard, D. (2003). Chloroquine resistance modulated in vitro by expression levels of the *Plasmodium falciparum* chloroquine resistance transporter. *Journal of Biological Chemistry, 278*, (33) 593–600.

Wangai, L. (2009). Characterization of Malaria parasites from endemic and epidemic regions of Kenya. (Doctoral dissertation, Institute of tropical medicine, Jomo Kenyatta University of Agriculture and Technology).

Warhurst, D. C. (2001). A molecular marker for chloroquine-resistant falciparum malaria. *New England Journal of Medicine, 344*, 299-302.

Warhust, D. C., Kariem, F. M. A., & Miles, M. (1991). Simplified preparation of malarial blood samples for polymerase chain reaction. *Lancet, 337*, 303-304.

Wellems, T. E., & Plowe, C. V. (2001). Chloroquine-resistant malaria. *The Journal of Infectious Diseases, 184*, 770.

Wootton, J. C., Feng, X., Ferdig, M. T., Cooper, R. A., & Mu, J. (2002). Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. *Nature, 418*, 320– 323.

World Health Organization (1996). *Assessment of therapeutic efficacy of antimalarial drugs for uncomplicated falciparum malaria in areas with intense transmission:* Report WHO/MAL/96 1077, Geneva.

World Health Organization. (2001). *Monitoring Antimalarial Drug Resistance*. Retrieved on 1st October 2009 from www.who.int/emc

World Health Organization. (2002). *The World Health Report 2002: reducing risks, promoting healthy life.* Geneva.

World Health Organization. (2005). *Strategic orientation paper on prevention and control of malaria* Retrieved October 1st 2009 from ww.who.int/malaria/docs/trainingcourses/NPOreport.pdf

World Health Organization. (2008). *World malaria report.* Geneva

Yamauchi, L. M., Coppi, A., Snounou, G., & Sinnis, P. (2007). *Plasmodium* sporozoites trickle out of the injection site. *Cell Micorbiology, 9*(5), 1215-1222

Zeyl, C., Mizesko, M., & de Visser, M. (2001). Mutational meltdown in laboratory yeast populations. *Evolution. 55*, 909–917.

APPENDICES

APPENDIX I

Calculations of buffer preparations

Calculating concentrations and volumes $C_1V_1 = C_2V_2$

C_1 = starting concentration

V_1 = starting volume

C_2 = final concentration

V_2 = final volume

Use same units for concentration (mM, 1X) and volume (l, ml, µl) on both sides of the equation.

$$V_1 = \frac{C_2 \text{ X } V_2}{C_1}$$

For example, to make 200ml of TE buffer (10mM TrisHCl:1mM EDTA)

Beginning with 1M TrisHCl to 10 mM TrisHCl,

$C_1V_1 = C_2V_2$

$1000 \text{ mM} \times V_1 = 10\text{mM} \times 200\mu\text{l}$

$$V_1 = \frac{10\text{mM} \times 200\mu\text{l}}{1000 \text{ mM}} = 2\text{ml of 1M TrisHCl}$$

We then calculate for 500mM EDTA to 1mM EDTA

$$V_1 = \frac{1\text{mM} \times 200\mu\text{l}}{500 \text{ mM}} = 0.4\text{mL (400µl) of 500mM EDTA}$$

Finally we add 2ml of 1M TrisHCl to 0.4mL (400µl) of 500mM EDTA in a measuring cylinder and top up to 200ml.

APPENDIX II

Gene sequences and primer binding sites for *Pfmdr 1* and *Pfcrt*

I. *Pfmdr 1* fragment DNA sequence

FN1/1 → mdr2/1 →

aggttgaaaaagagttgaacaaaaagagtaccgctgaattatttagaaaaataaagaatgagaaaatatcatt

ttttttaccgtttaaatgtttacctgcacaacatagaaaattattatttatatcatttgtatgtgctgtatta

CQ sensitive: N

tcaggaggaacattacctttttttatatctgtgtttggtgtaatattaaagaacatgaatttaggtgatgata

ttaatcctataatattatcattagtatctataggtttagtacaatttatattatcaatgatatcaagttattg

tatggatgtaattacatcaaaaatattaaaaactttaaagcttgaatatttaagaagtgtttttttatcaagat

ggacaatttcatgataataatcctggatctaaattaagatctgatttagatttttatttagaacaagtgagtt

caggaattggtacgaaatttataacaatttttacatatgccagttccttttttaggtttatatatttggtcatt

← newrev1 ← REV/C1

aataaaaaatgcacgtttgactttatgtattacttgcgtttttccgttaatttatgtttgtggtgtcat

CQ resistant: Y

agaacatgtatttaggtgat

II. *Pfcrt* fragment DNA sequence

P1 →

ccgttaataataaatacacgcagtcatattatttattatacattcatttattattttgtttttttaatttct

tacatataacaaaatgaaattcgcaagtaaaaaaaataatcaaaaaaattcaagcaaaaatgacgagcgttat

agagaattagataatttagtacaagaaggaagtaagtatccaaaaatggaaatatggaatgatataaatgaat

agataaatcaacctattggatatatatatatatatatatatatatatatatatatatatatgtatacccatatgt

attaatttttttttttttttttttttttttttttttttttttttcccttgtcgaccttaacagatggctcacgt

D3 →

ttaggtggaggttcttgtcttggtaaatgtgctcatgtgtttaaacttatttttaaagagattaaggataata

CQ sensitive: M N K

ttttttatttatattttaagtattatttatttaagtgtatgtgtaatgaataaaattttttgctaaaagaacttt

← D2 ← P2

aaacaaaattggtaactatagttttgtaacatccg

CQ resistant:

I E T

attgaaacaattt

APPENDIX III

Table showing results for PCR and dot blot compared to parasitaemia data obtained by microscopy for Tiwi 2008 samples *Pfmdr1* gene both mutant (tyrosine) and wild (Asparagine) type probe

Sample code	Microscopy Parasites/fields	Result	Asn (Wild)	Tyr (Mutant)	Mixed
CT01	3200/200	+++	-	++	-
CT02	+	+++	-	++	-
CT03	+	+++	-	++	-
CT04	+	+++	-	++	-
CT05	+	+++	++	++	+
CT06	+	+++	-	++	-
CT07	+	+++	-	++	-
CT08	+	++	+	-	-
CT09	+	+++	-	++	-
CT10	48/200	+++	-	++	-
CT11	+	++	-	+	-
CT12	+	++	-	+	-
CT13	+	++	-	+	+
CT14	+	++	-	+	-
CT15	228/200	+++	-	++	-
CT16	1200/200	+++	-	++	-
CT17	412/200	++	-	+	-
CT18	980/200	+	+	+	+
CT19	1730/200	++	-	+	-
CT20	48/200	++	-	+	-
CT21	312/200	++	-	+	-

CT22	332/200	++	-	+	-
CT23	931/200	++	-	+	-
CT24	1111/200	+++	++	-	-
CT25	312/200	+++	-	++	-
CT28	376/200	+++	++	-	-
CT29	236/200	++	-	+	-
CT30	2135/200	++	-	+	-
CT31	80/200	+++	++	-	-
CT32	690/200	+	+	-	-
CT33	8/200	++	+	-	-
CT34	15/200	+	-	+	-
CT35	5/200	-	-	+	-
CT36	66/200	+++	-	+	-
CT37	28/200	+++	-	+	-
CT38	5/200	+++	+	-	-
CT39	720/200	+++	++	-	-
CT40	1068/200	+++	-	++	-
CT41	94/200	++	-	+	-
CT42	660/200	++	+	-	-
CT43	336/200	++	+	-	-
CT44	46/200	+++	+	-	-
CT45	334/200	++	+	-	-
CT46	3/200	++	+	-	-
CT47	2/200	-	-	+	-
CT48	512/200	-	-	+	-
CT49	460/200	+++	+	-	-
CT50	5/200	+++	+	-	-
CT51	520/200	++	+	+	+

CT52	198/200	+++	-	+	-
CT53	3/200	++	-	+	-
CT54	35/200	-	-	+	-
CT55	194/200	++	-	+	-
CT56	15/200	+++	+	-	-
CT57	6/200	+++	-	+	-
CT58	156/200	+++	-	+	-
CT59	218/200	+++	+	-	-
CT60	6/200	+++	-	+	-
CT61	718/200	+++	+	+	+
CT62	1783/200	++	+	+	+
CT63	370/200	+++	-	+	-
CT64	656/200	+++	+	+	-
CT65	616/200	-	-	+	-
CT66	1560/200	+++	+	-	-
CT67	752/200	+++	-	+	-
CT68	2458/200	+++	+	-	-
CT69	1112/200	+	+	-	-
CT70	873/200	+++	-	+	-
CT71	488/200	+++	-	+	-
CT72	8/200	+++	-	+	-
CT73	+	+++	+	-	-
CT74	+	+++	-	+	-
CT75	832/200	+++	-	+	-
CT76	793/200	+++	-	+	-
CT77	836/200	+++	+	-	-
CT78	456/200	+++	-	+	-
CT79	278/200	+	-	+	-

CT80	-/200	+	-	+	-
Total	77	72	28	49	6

APPENDIX III

Dot blot apparatus showing surface on which the nitrocellulose membrane is layered upon. The membrane takes its exact shape.