MAJOR GENOME REGION UNDERLYING ARTEMISININ RESISTANCE IN MALARIA PARASITE ISOLATES FROM KISUMU COUNTY, WESTERN KENYA.

BIDII STEPHEN NGALAH

MASTER OF SCIENCE

(Molecular Medicine)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

2015

Major genome region underlying artemisinin resistance in malaria parasite isolates from Kisumu County, Western Kenya.

Bidii Stephen Ngalah

A thesis submitted in partial fulfilment for the degree of master of science in molecular medicine in the Jomo Kenyatta University of Agriculture and Technology

2015

DECLARATION

This thesis is my original work and it has not been presented for the award of a degree in any other university.

Signed ….…………………. Date ………………………………

Bidii Stephen Ngalah

This thesis has been submitted for examination with our approval as the supervisors.

Signed ………………………. Date ………………………………

Dr. Major Edwin Kamau

KEMRI-USAMRU-K

Signed ………………………. Date ………………………………

Prof. Zipporah Ng'ang'a

JKUAT, Kenya

Signed ………………………. Date ………………………………

Dr. Ben Andagalu

KEMRI-USAMRU-K

DEDICATION

In memory of my father Eng. Stephen Ngalah Ngenja and Mum Nyamvula Ngome Jefwa. You left fingerprints of grace, wisdom, riches and love in our lives. You shan't be forgotten. To Anne, Sammy, my sisters Santa and Zawadi you are true Joy and love of my life. You always believed that I can do marvellous when I put my mind to.

ACKNOWLEDGEMENTS

The work presented herein has taken the input of many individuals whose precious involvement cannot be fully described. Foremost, I would like to express my sincere gratitude to my Mentor MAJ Dr. Edwin Kamau, for the continuous support through all my study research time, for his leadership, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor.

I would like to express my deepest gratitude to my supervisor, Professor Zipporah Ng'ang'a of JKUAT for her excellent guidance in my write ups, caring, patience, and providing me with an excellent atmosphere to perfect my skills in writing scientific paper. I would also like to thank supervisor Dr. Ben Andagalu, of waltereed Kisumu, for the immense skills on clearance rate determination he imparted on me and for nesting my project under the Invivo clinical trial. To Prof. Mwatelah Kazungu Ziro of Technical university of Mombasa for being my mentor through my career life and for facilitating the financial support that allowed my stay in Kisumu bearable. His wisdom, knowledge and commitment to the highest standards inspired and motivated me.

To Principal investigator Dr.Hoseah Akala, Scientists Charles Okudo, Agnes Cheruiyot and Redemptah Yeda for introducing me in the world of malaria culture techniques. Thanks to laboratory manager Angela Omondi for her good leadership, Dr. John Waitumbi and his team, Irene Otieno, Luiser Ingasia and Dennis Juma for helping me in technical areas. To all colleagues Lorna, Peninah, Jelagat, Ndegwa Mutwiri, Jura, Fred, Wilfred and Mutai who acted as good friends. It would have been a lonely lab without them. To JKUAT library services in particular Mary Wachira, who contributed to the final editing and formatting of the thesis.

Finally to my family members Auntie Clarice Kiti, Umazi Jefwa, Saumu Olotu, Anne Ndunge, Jude, Ferdinard Kiti, Janet, Santa, Hope, Happy, Zawadi, Rita , Stephen Jnr, Kaluwa, Jackson, John, Hassan, Best, Zidini and lucky who have always supported, encouraged and believed in me, in all my endeavors. God bless you all.

TABLE OF CONTENTS

DECLARATION .... **ii**

DEDICATION .... **iii**

ACKNOWLEDGEMENTS ....**iv**

TABLE OF CONTENTS ....**v**

LIST OF TABLES ....**ix**

LIST OF FIGURES ....**x**

ABBREVIATIONS AND ACRONYMS ....**xii**

ABSTRACT ....**xiv**

CHAPTER ONE **1**

INTRODUCTION..**1**

1.1Background information...1

1.2 Problem Statement 2

1.3 Justification...3

1.4 Hypothesis 3

1.4.1 Null Hypothesis...3

1.4.2 Alternative hypothesis 3

1.5 Objectives 4

1.5.1 General objective 4

1.5.2 Specific objectives...4

CHAPTER TWO **5**

LITERATURE REVIEW **5**

2.1 Origin of Artemisinin 5

2.2 Mechanism of Action of Artemisinin and its compounds 5

2.2.1 Artemisinin drug resistance 6

2.3 Life Cycle of Plasmodium falciparum 7
2.3.1 Pathogenesis of Plasmodium falciparum 9
2.4.1 Polymorphism of Plasmodium falciparum genome 10
2.4.2 Mechanisms that generate single nucleotides polymorphism 10
2.4.3 Microsatellite polymorphism of Plasmodia genome 11
2.5 Molecular Markers for Plasmodium falciparum drug resistance 12
2.5.1 Amino-alcohols and sesquiterpene lactones drug resistance markers 12
2.5.2 Plasmodium falciparum malaria drug resistant gene-1 13
2.5.3 Plasmodium falciparum chloroquine resistance transporter gene 13
2.6 Methods used in genotyping Plasmodium falciparum 14
2.6.1 DNA sequencing 14
2.6.2 Restriction fragment length polymorphism (RFLP) 14
2.6.4 Merozoite surface proteins genotyping 15
2.6.5 Real time polymerase chain reaction 16

CHAPTER THREE 17

MATERIALS AND METHODS 17

3.1 Study area 17
3.2 Study Population 17
3.3 Sample size determination 17
3.4 Genotyping 18
3.4.1Genomic DNA Isolation and purification of Plasmodium falciparum 18
3.4.2 Genotyping of Plasmodium falciparum single nucleotide polymorphism 18
3.4.3 PCR-Restriction Length Fragment Polymorphism for genotyping SNPs 20
3.5 Amplification of Plasmodium falciparum MSP-1 and MSP-2. 20
3.5.1 Multiplex primary PCR amplification conditions for MSP1 and MSP2 20
3.5.2 Nested Reaction MSP-1 and nPCR Thermoprofile 21
3.5.3 Nested Reaction MSP-2 and nPCR thermoprofile 21
3.5.4 Gel electrophoresis of PCR products. 22

3.5.5 Genetic Analyzer fragment analysis of MSP-1 and MSP-2 alleles. 22
3.6 Estimation of Pfmdr-1 Copy number 22
3.7 In vivo Clearance of Plasmodium falciparum. 23
3.8 In vitro drug sensitivity tests...23
3.9 Ethical Considerations..24
3.10 Data analysis 24
3.10.1 Sequenome data and clearance rates analysis 24
3.10.2 Restriction fragment length polymorphism analysis....................................25
3.10.4 Pfmdr-1 gene copy number estimation calculations 25
3.10.5 In vitro susceptibilities association with SNPs and copy number 26

CHAPTER FOUR..27

RESULTS...27

4.1 Single nucleotide polymorphism in Pre-ACT and Post ACT samples 27
4.1.1 Restriction Length Polymorphism results for Combined Mal 13-1718319 and Combined Mal 10-688956...29
4.1.2 Population diversity of MSP1 and MSP2 at baseline and day of recurrence ...32
4.2 In vivo Clearance half-life of parasites in response to ACT 34
4.2.1 Association of Single nucleotide polymorphism with In vivo parasite clearance time 36
4.3 Pfmdr-1 Copy number distribution in Pre-ACT period and Post ACT period....40
4.4 Association of multiple copy numbers and In vitro drug susceptibilities 43

CHAPTER FIVE 45

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS 45

5.1 Genetic polymorphism in chromosome 10, 13 and 14 in Plasmodium falciparum45
5.2 In vivo Plasmodium falciparum clearance rate estimation................................46
5.3 Genetic polymorphism and clearance rate association in Plasmodium falciparum47
5.4 Assessment of pfmdr1 copy number before and after ACT introduction 47

5.5 CONCLUSIONS 49
5.6 RECOMMENDATIONS 50
REFERENCES 51
APPENDICES 60

LIST OF TABLES

Table 4.1: Single nucleotide polymorphism profiles and respective minor allele frequencies in different years 28

Table 4.2: Association of P. falciparum SNPs with parasite clearance half-life after Artemisinin ACT treatment. 29

Table 4.3: MSP-1/MSP-2 allele polymorphism of samples at baseline and day of recurrence. 34

Table 4.4: Baseline characteristics, clinical and parasitological outcomes in groups of patients with Plasmodium falciparum malaria followed by genotyping results following day of recurrence 35

Table 4.5: Distribution of Pfmdr1 gene copy number between Pre-ACT and Post-ACT period. 43

Table 4.6: Correlation between IC50 and Pfmdr-1 copy number outcome 44

LIST OF FIGURES

Figure 2.1: Life cycle of Plasmodium species (courtesy of CDC).................. 8

Figure 4.1: The call rate in each SNP in samples assayed..............................27

Figure 4.1.1 A: Digested PCR products (Red) and undigested PCR products (Black) products for MAL13-1718319. 16: 3D7 at 50 pg/µL, 17 (Green) is V1/S at 50 pg/µL 30

Figure 4.1.1 B: Digested PCR products (Red) and undigested PCR products (Black) products for MAL10-688956. 16: 3D7 at 50 pg/µL, 17 (Green) is V1/S at 50 pg/µL 31

Figure 4.2a: Merozoite surface protein 1 allele distribution in recurrence samples 32

Figure 4.2.b: Merozoite surface protein 2 allele distribution in recurrence samples. 33

Figure 4.2.1a: A plot showing, association of Parasite clearance half-life against genotypes 37

Figure 4.2.1 b: A plot showing association of Single clones genotypes against parasite clearance half-life 38

Figure 4.2.1c: A plot showing general parasite Slope half-life against the genotypes. 39

Figure 4.3a: Pfmdr-1 Copy number variations in sample isolates in different years. 41

Figure 4.3b: Pfmdr-1gene copy number distribution between Pre-ACT and Post-ACT periods. 42

APPENDICES

Appendix 1: Pfmdr1 Copy number distribution for isolates from archives (1995-1999) Kisumu. 60
Appendix 2: Pfmdr1 copy number distribution for isolates from archives (2000-2003) 61
Appendix 3: Pfmdr1 copy number distribution in isolates from efficacy clinical trial 2013-2014 64
Appendix 4: Single nucleotide polymorphism results for 1995-1999 isolates from Kisumu. 66
Appendix5: Single nucleotide polymorphism results for 2000-2003 isolates from Kisumu. 70
Appendix 6: Single nucleotide polymorphism results for 2005-2010 isolates from Kisumu 73
Appendix 7: Single nucleotide polymorphism results for 2013-2014 isolates from Kisumu 75
Appendix 8: Ethical clearance letters 82
Appendix 9: List of Publications 88

ABBREVIATIONS AND ACRONYMS

ACTs	Artemisinin based combination therapy
AL	Artemether-Lumefantrine
AS	Artesunate Monotherapy
BLAST	Basic Local Alignment Search Tool
CE	Capillary electrophoresis
CQ	Chloroquine
CRs	Clearance rates
DHFR-TS	Dihydrofolate reductase-thimidylatesynase
DHPS	Dihydropteroate synthetase.
EDTA	Ethyldiaminetetraacetic acid
EHH	Extended Haplotype Homozygosity)
XP-EHH	Cross Population Extended Haplotype Homozygosity
KDH	Kisumu District hospital
MOI	Multiplicity Of Infections
MALDI-TOF	Matrix-Assisted Laser Desorption / Ionization Time-Of-Flight
MSP-1	Merozoite Surface Protein-1
MSP-2	Merozoite Surface Protein-2
MSP-4	Merozoite Surface Protein-4
PCR	Polymerase Chain Reaction
nPCR	nested Polymerase Chain Reaction
POP-7	Performance optimized polymer 7
nRBC	non parasitized Red Blood Cell
pRBC	Parasitized Red Blood cell
RFLP	Restriction Fragment Length Polymorphism
RFU	Relative fluorescent unit
qPCR	Real-time PCR
SD	Standard Deviation
SEA	South East Asia

SP	Sulfadoxine pyrimethamine
SMA	Severe Malaria Anemia
SNPs	Single Nucleotide Polymorphisms
SPSS	Statistical packages for social scientist
SSOP	Sequence Specific Oligonucleotide Probing
TNF	Tumor Necrosis Factor
USA	United States of America
USAMRU	United States Army Medical Research Unit
WHO	World Health Organization
WWARN	WorldWide Antimalarial Resistance Network
ITROMID	Institute of Tropical Medicine and Infectious Diseases
PCR	Polymerase Chain Reaction
Pfcrt	Plasmodium falciparum chloroquine resistance gene
Pfmdr-1	*Plasmodium falciparum* multi-drug resistance gene 1
PfMSP-1	*Plasmodium falciparum* merozoite surface protein-1
PfMSP-2	*Plasmodium falciparum* merozoite surface protein -2
Pgh	P-glycoprotein homologue

ABSTRACT

Clinical resistance to artemisinin and its derivatives is now well established in Southeast Asian (SEA). Studies from SEA have identified single nucleotide polymorphisms (SNPs) which are significantly associated with delayed parasite clearance rates. In this study we screened SNPs and established the SNP profile in Kenyan isolates. DNA was extracted from 129 *Plasmodium falciparum (P. falciparum)* positive patients collected in Kisumu from 2013-2014 and 1995-2010. 25 SNPs were genotyped by Sequenome MassARRAY. *P. falciparum* multi-drug resistance gene 1 (*pfmdr1*) copy number variation was quantified using real time PCR. The in vitro artemether susceptibility of the isolates was analyzed by malaria SYBR Green I assay. Microsatellite analysis of MSP1/MSP2 was used to establish recrudescent verses new infections in 20 follow up samples obtained from the efficacy study. Data showed that pre-ACT samples contained 60% of the SNPs analyzed while the Post-Act samples had 24% of the SNPs. One Pre-ACT Sample (1.4%) contained multiple *pfmdr1* copy number and 7 of post ACT sample (9.9%) contained >1 *pfmdr1* copy number. There was a significant increase in *Pfmdr1* copy number between pre-ACT and post-ACT samples (P<0.001). The average clearance half-life was 2.6 hours. All parasites were sensitive to artemisinin with significance difference in clearance half-life within subject (p= 0.0001). There was no significant association between clearance half-life and genotypes (p> 0.05). In vitro susceptibility to piperaquine reduced for isolates with multiple *pfmdr1* copy number to piperaquine and amodiaquine (P< 0.04). In conclusion there is a possibility of selective sweep of SNP after elimination of previous drug pressure and introduction of ACT. *Pfmdr1* may putatively play a role in ACT resistance.

CHAPTER ONE

INTRODUCTION

1.1Background information

The emergence and spread of resistance to antimalarial drugs threatens the efficacy of existing drug treatments (Phyo *et al.,* 2012). Parasite clearance rate is an important measure of drug efficacy and can be used to assess the *in vivo* responses to treatment with artemisinin derivatives and evaluate new antimalarial drugs (Stepniewska *et al.,* 2010). Slow clearance (CRs) against artemisinin based combination therapies (ACTs) have been shown in South east asia (SEA) , the epicentre for malaria drug resistance. Regions on chromosome 10, 13 and 14 have been identified that are likely to be associated with reduced clearance (Takala *et al.,* 2013; Cheeseman *et al.,* 2012).

The current effort is geared towards assessing the degree of ACT resistance and also determining if the genetic polymorphism of parasites in Kenya with reduced CRs is similar to those in SEA. This was done by determining the genetic polymorphism of the parasite and characterizing region of chromosome 10, 13 and 14 that are associated with resistance to ACTs. Identifying these genetic determinants was crucial for understanding the molecular basis of artemisinin resistance (Cheeseman *et al.,* 2012). Apart from clearance rate; *Plasmodium falciparum* has the ability to generate mutant variants. These mutant variations have led to the emergence of resistance against many of the available antimalarial drugs.

In Kenya, first-line treatment recommended for uncomplicated malaria were changed from chloroquine (CQ) to sulphadoxine pyrimethamine (SP) in 1998, and then from SP to artemether lumefantrine (AL) in (Amin *et al.,* 2007). The emergence and spread of resistance to CQ and SP led to replacement of these drugs with the ACTs in most parts of the malaria endemic regions in SEA and Africa. ACTs have offered much-improved efficacy. However, resistance to ACTs has been confirmed in western Cambodia and

has recently emerged in western Thailand, an epicentre of drug resistance (Takala *et al.,* 2013).

Apart from Cambodia and Thailand-Myanmar border regions where resistance to artemisinin has been confirmed more clinical studies are needed in other areas to map the emergence and spread of artemisinin resistance. The aim of the study was to screen for any emergence of artemisinin resistance and determine genetic polymorphism in chromosome 10, 13 and 14 of *P.falciparum* which were associated with artemisinin slow clearance (Cheeseman *et al.,* 2012) in pre-ACT and post-ACT samples. We also aimed in assessing the *Pfmdr-1* copy number variations before artemisinin was introduced and after artemisinin introduction in western Kenya. This study was part of in the vivo efficacy clinical trial in Kisumu County, western Kenya which assessed the degree of artemisinin resistance in both adult and paediatric subjects presenting with uncomplicated malaria.

1.2 Problem Statement

In vivo studies are the source of crucial data on the efficacy of antimalarial drugs. However, these studies are costly to conduct which makes them not suitable for continuous antimalarial resistance surveillance. Currently clinical resistance to artemisinin and its derivatives have been reported in SEA. SNPs in chromosome 10, 13 and 14 were identified that shows strong association with slow CRs (Cheeseman *et al.,* 2012; Takala *et al.,* 2013). This necessitates a need to confirm and validate these markers in Kenya. To understand the basis of artemisinin resistance, parasite genetics would be useful. Few researches have been done in Kenya to assess genetic factors underlying CRs seen in different subjects. Furthermore data obtained from samples collected before introduction of ACTs in Kenya were used to establish the baseline genetic information of *Plasmodium falciparum* in western Kenya which is currently not sufficient.

1.3 Justification

Development and spread of ACT-resistant parasites would be catastrophic for malaria control. Artemisinin drug resistance has been confirmed in western Cambodia and has recently emerged in western Thailand (Cheeseman *et al.,* 2012; Arjen *et al.,* 2010; Takala *et al.,* 2013). It is critically that the emergence of artemisinin resistance is closely monitored in Kenya. This study was extremely critical and timely for several reasons; it established the genetic baseline for artemisinin resistance by assessing the genetic profile of samples collected in pre-ACTs period, it determined if artemisinin resistance in western Kenya has emerged, and it critically established whether artemisinin resistance genetic markers seen in SEA are similar to those found in Kenyan samples. Molecular marker studies also have several practical advantages over *in vivo* and *in vitro* tests: many isolates can be studied within a short time, and specimens can be collected, stored and transported far more easily than for *in vitro* tests.

1.4 Hypothesis

1.4.1 Null Hypothesis

There is no association between parasite clearance rates and genetic polymorphism in *Plasmodium falciparum* isolates obtained from patients with uncomplicated malaria residing in western Kenya.

1.4.2 Alternative hypothesis

There is an association between parasite clearance rates and genetic polymorphism in *Plasmodium falciparum* isolates obtained from subjects with uncomplicated malaria in western Kenya.

1.5 Objectives

1.5.1 General objective

To determine the genetic polymorphism in *P. falciparum genome* regions of strong selective pressure associated with artemisinin resistance in S.E.A using parasite isolates from western Kenya.

1.5.2 Specific objectives

1. To determine the genetic polymorphism of *Plasmodium falciparum* in chromosome 10, 13 and 14 of sample isolates collected before and after the introduction of ACTs in western-Kenya.
2. To determine and analyze the association of slow CRs and Invitro susceptibilities with polymorphism in *Plasmodium falciparum* in chromosome 10, 13 and 14 of samples collected from an on-going *in vivo* efficacy clinical trial study.
3. To determine the *Pfmdr-1 gene* copy number in sample isolates collected before and after the introduction of ACTS in western- Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin of Artemisinin

In 1970s, Chinese government scientists working on a secret "Project 523" developed a new class of potent antimalarial drugs, the artemisinins or qinghaosu derivatives. In mostly unpublished work that was recognized by a 2011 Lasker Award to Tu Youyou, researchers in China isolated the active compounds from the plant *Artemisia annua*, tested them in mice, analyzed the chemical structure of the artemisinins and demonstrated their high potency and rapid efficacy in human trials (Miller *et al.,* 2011, Arjen *et al.,* 2010). Although they were widely used in China during the 1980s, only in the 1990s did the artemisinins came to wider global attention in the form of artemisinin-based combination therapies. Over the past decade, these highly efficacious treatments, along with other malaria-control measures, have contributed to significant reductions of the malaria burden in many areas of the world, including parts of Africa (Arjen *et al.,* 2010). Some of the ACTs that are currently widely used include AL widely marketed as Coartem by Novartis, piperaquine and pyronandine (Miller *et al.,* 2011).Under guidelines to treat malaria in 2006, WHO recommended artemisinin combination therapies to be the first-line treatment for uncomplicated malaria globally (WHO, 2010).

2.2 Mechanism of Action of Artemisinin and its compounds

Artemisinin is an endoperoxide that are thought to form free radicals that may kill malaria parasites by alkylating essential biomolecules (Cui *et al.,* 2009). Artemisinin may also act by inhibiting the *Plasmodium falciparum* endoplasmic calcium ion ATpase. It is further thought to target the erythrocytic stage of parasites though no consensus on its mode of action has been elucidated (Cui *et al.,* 2009). Studies have shown that artemisinin antimalarial action is by perturbing redox homeostasis in malaria parasites. This ends in reduction of the oxidative stress as a result of haemoglobin consumption by the parasite (Ginsburg *et al.,* 1994). Digestive vacuole

membrane of parasite established at mid-ring stage also suffers damage soon after parasite is exposed to artemisinin (Cui *et al.,* 2009).

2.2.1 Artemisinin drug resistance

World Health Organization defines ‘drug resistance’ as the ability of a parasite strain to survive or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the tolerance of the subject (WHO, 2010) “The form of the drug active against the parasite must be able to gain access to the parasite or the infected erythrocyte for the duration of the time necessary for its normal action”. Artemisinin resistance is characterized by slow parasite CRs in efficacy clinical trials. Clearance rates are assessed by microscopy. Artemisinin-resistant infections remain slide-positive for 2 or more days. In Thailand, where treatments with artesunate and mefloquine are the first-line regimen, concerns over potential artemisinin resistance were voiced when efficacy against the two day standard regimen were reported in the early 2000s and subsequently published in 2008 (Stepniewska *et al.,* 2010). To investigate the possibility that failure of the artesunate-mefloquine combination regimen might be attributable to artesunate, a clinical trial using artesunate alone was conducted in 2006 attempting to identify clinical artemisinin resistance using prospectively defined criteria. Subjects with uncomplicated *Plasmodium falciparum* were enrolled and randomized in 2:1 allocation to 7 days of AS at 4 mg/kg or 7 days of quinine and tetracycline with 28 day follow-up in a non-transmission area (Noedl *et al.,* 2010). Two subjects receiving AS had recrudescence despite what were considered to be adequate plasma drug levels with DHA [IC50] approximately four times higher than those in volunteers who were cured. These two patients had parasite clearance times of 133 and 95 hours, markedly longer than the median PCT of 52.2 hours. These two cases have been acknowledged by World Health Organization as the first documented cases of artemisinin resistance (WHO, 2010). Although slower parasitological clearance does not constitute direct evidence that ACT regimens are currently failing, these altered parasitological responses are regarded as an

early warning that this may occur in the future. Genetic studies have shown that the prolonged parasite phenotype appears to be a heritable trait in Cambodia, further emphasizing the need to systematically target geographical areas and populations for aggressive malaria control and elimination operations to prevent the spread of resistant strains (Anderson *et al.,* 2010).

2.3 Life Cycle of *Plasmodium falciparum*

There are five species of *Plasmodium* that infect humans namely *Plasmodium falciparum, P. vivax*, *P. malariae*, *P. ovale* and more recently *P. knowlesi*. They share a common life cycle with only minor differences. *Plasmodium falciparum* causes the most fatal form of the disease, which is due to the ability of the parasite to sequester in brain causing cerebral malaria. *P. vivax* has the most widespread distribution and is responsible for majority of malaria cases outside of Africa and about half of all clinical malaria (Rosenberg *et al.,* 1990) in the world.

The life cycle of the malaria parasite consists of two phases: asexual phase in the vertebrate host and a sexual phase that occurs in the mosquito mid gut. Sexual stage in mosquito midgut includes a zygote, the only diploid stage in the life cycle of the parasite. After a bite by an infected mosquito, sporozoites enter the blood stream of the vertebrate host and within 30 to 45 minutes, they migrate to the liver parenchyma cells and develop into exoerythrocytic schizonts. Thereafter, they divide by multiple fission to form invasive merozoites which after the host cell ruptures; they attach to and enter circulating erythrocytes. The merozoite stage is short-lived and must invade host red blood cells in a rapid manner .Within the erythrocyte; the merozoite undergoes development and multiple divisions to become a mature schizont that contains 8-32 merozoites. The erythrocytic schizont ruptures releasing infective merozoite that can invade new erythrocytes. The release of merozoite from ruptured schizont is a synchronized event and is responsible for the cyclic chills and fever classically associated with clinical malaria. Merozoite develops into male or female gametocytes, which are taken up in subsequent Anopheline mosquito blood meals. Within the

mosquito midgut the gametocytes quickly differentiate into male microgametes or a female macrogamete. Upon fertilization, a motile zygote or oökinete is produced, which is the only diploid stage in the life cycle of the parasite. The oökinete then penetrates the peritrophic membrane and moves to the outer wall of the midgut epithelium where it develops into an oöcyst. After meiosis within the oöcyst, several thousand sporozoites are formed that differentiate and migrate through the hemocoel to the salivary glands of the mosquito where they become infective for their vertebrate host. Less than 25% of oöcyst-produce sporozoites successfully and invade the salivary glands (Rosenberg *et al.,* 1990).The parasite's ability to complete its life cycle in two different hosts is dependent upon its active invasion of host cells as illustrated in Figure 2.1 below. The infection of host erythrocytes by the parasite is responsible for the mortality and morbidity caused by this disease.

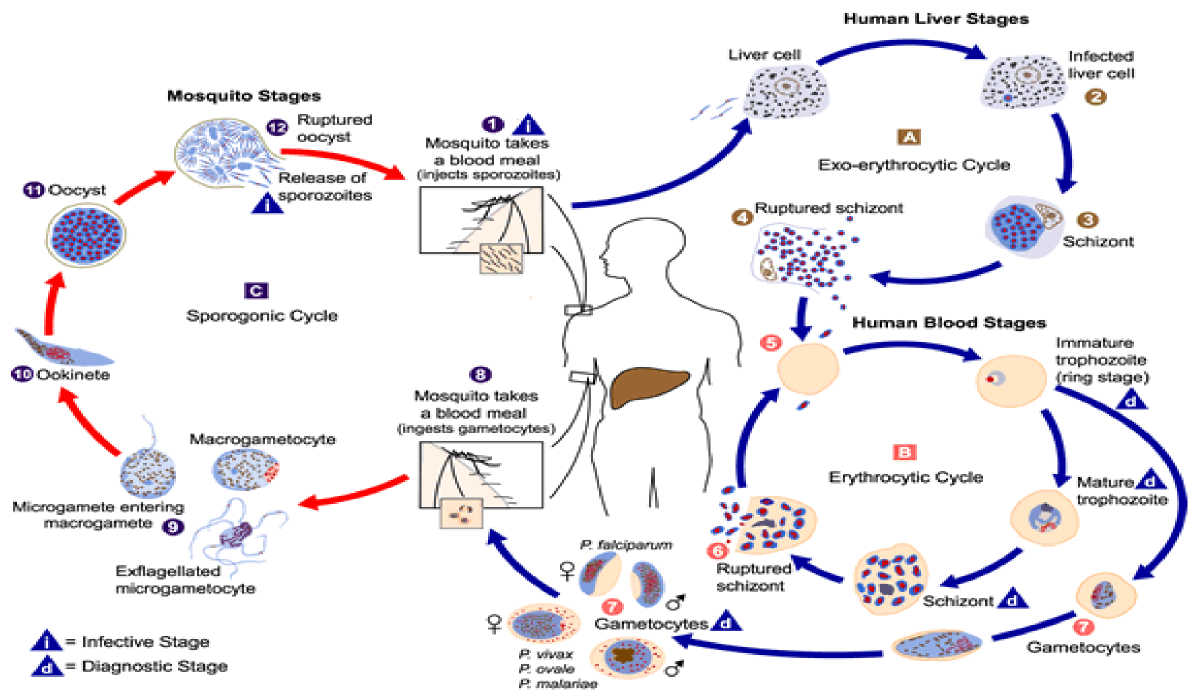

Figure 2.1: Life cycle of *Plasmodium* species (courtesy of CDC)

2.3.1 Pathogenesis of *Plasmodium falciparum*

Pathogenesis of *Plasmodium falciparum* infection is associated with erythrocytes rupture and the release of parasite hemozoin. These metabolite and cellular debris induce an increased activity of the reticulo-endothelial system, particularly in the liver and spleen leading to hepatomegaly and spleenomegaly. Severe complications of malaria are associated with cerebral malaria characterized by an impaired consciousness (Coma), followed by severe headache. These manifestations due to the sequestration of the infected erythrocytes in the cerebral microvasculature (Rosenberg *et al.,* 1990).

2.4 Genome of the *Plasmodium* genus

The complete *Plasmodium falciparum* genome is a 23 Mb, 80% A-T rich genome consisting of around 5000 genes on 14 chromosomes, a 6 Kb mitochondrial repeat element and a circular 35 Kb apicoplast segment. The putative function assignments of the bulk of the genes (60%) have not yet been accomplished (Gardner *et al.,* 2002). However, comparative genomics studies with other genomes has predicted the possible roles of a large number of genes in cell-to-cell adhesion and host cell invasion (1.3%), evasion of host immunity (3.9%), cellular metabolism (8%), apicoplast-related genes (10%) and sub-telomeric gene families (5 – 10%) (Koiij *et al.,* 2006). The *Plasmodium falciparum* genome has an abundant nucleotide resource which allows mutations and rearrangements of DNA segments that together drive an ever expanding interspecies diversity. DNA sequence alterations may be present as tandem repeats differing in the number and length or as point mutations. Certain multiple copy genes further add to the genetic complexity of parasite genotypes by regulated expression of a single member gene at any given time (Gardner *et al.,* 2002).

2.4.1 Polymorphism of *Plasmodium falciparum* genome

Polymorphism of the *Plasmodium falciparum* genome is thought to have evolved through DNA rearrangements such as gene duplication events, gene conversions, translocations, deletions and insertions (Kemp, 1992). Genes encoding proteins that are expressed on the surface of sporozoites or merozoite are in general more variable than housekeeping genes or genes expressed during the sexual stages. This constitutes a major obstacle in targeting them in the design of vaccines. Comparison of the rather selectively neutral synonymous and the more frequent non-synonymous substitutions indicate that natural selection may account for most polymorphisms observed at functional gene loci (Escalante *et al.,* 1998). Non-synonymous SNPs are found at high frequencies in genes that are under strong selective pressure, in particular those genes encoding proteins linked to immune evasion or drug resistance. Point mutations affecting, for example, drug resistance have been found in the DHFR and DHPS. These genes are associated with resistance to pyrimethamine and Sulfadoxine, respectively (Cowman *et al.,* 1988; Wang *et al.,* 1997). SNPs may also be responsible for artemisinin resistance. Genes where polymorphisms have arisen through intragenic recombination in repetitive segments are characterized by repeat motifs with length variability differing between strains. Among these genes are those encoding the *Plasmodium falciparum* circuMSPorozoite protein(Arnot *et al.,* 1993), GLURP (Borre *et al.,* 1991), two merozoite surface proteins *MSP-1* and *MSP-2* (Kimura *et al.,* 1990) and the apical membrane antigen (Marshall *et al.,* 1996).

2.4.2 Mechanisms that generate single nucleotides polymorphism

Nucleotide recombination which occurs during DNA replication may result in single nucleotide polymorphisms (SNPs). Even though they are relatively rare (10^{-9} replacements/nucleotide/replication) (Ferreira *et al*., 2004) they can have a profound phenotypic effect. For example, mutation seen in parasites resistant to the antimalarial drug, pyrimethamine is conferred by a single nucleotide change at position 108 of the *Plasmodium falciparum pfdhfr* gene resulting in a shift from serine (S) to asparagine (N)

(Jelinek *et al.,* 1999). SNPs are differentiated on the basis of frequency of occurrence in the population. Random nucleotide replacements give rise to the four categories of SNPs which are defined on the basis of where they occur and their impact. Foremost we have Coding and non-coding SNPs which distinguish the location of the polymorphism. Moreover Nucleotide alterations that result in the translation of a different amino acid are referred to as non-synonymous SNPs while those that retain the original amino acid are synonymous SNPs. Many non-synonymous SNPs are found clustered in genes encoding B and T cell epitopes of malaria antigens probably due to positive selection for evasion of host immune responses (Ferreira *et al.,* 1999).

2.4.3 Microsatellite polymorphism of Plasmodia genome

RFLPs and microsatellite polymorphisms have been used for the determination of intergenic diversity in Plasmodia (Su & Wellems 1996). Microsatellites evolved through sequential mutational processes. Sequence repeats of microsatellites are found frequently in the *Plasmodium falciparum* genome and occur every 2–3 kb in coding and non-coding regions (Su and Wellems, 1996). Advantages of microsatellite markers over RFLP determination are rapidity and automatization of analysis, but also smaller amounts of DNA required for typing. Typing of microsatellite markers allow for screening for loci which are under selective pressure and to characterize population structures and divergent evolution of the parasites. Microsatellites have been, for example, applied in describing inter-population diversity in allele and haplotype frequencies of the Pfs48/45 gene on chromosome 13 (encoding a candidate for a transmission blocking vaccine) (Conway *et al.,* 2001), in the determination of the recent origin of *Plasmodium falciparum* from a single ancestor (Volkman *et al.,* 2001), in the analysis of mutation patterns of the *Plasmodium falciparum* genome (Anderson *et al.,* 2000a), and in the detection of drug-resistance promoting loci.

2.5 Molecular Markers for *Plasmodium falciparum* drug resistance

Molecular markers of drug resistance characterization are an important aspect of understanding resistance to antimalarial treatment (Ariey *et al.,* 2014). After the genetic changes associated with resistance are identified, drug resistance can be confirmed with molecular techniques.

A limited number of genes involved or potentially involved in *Plasmodium falciparum* antimalarial drug resistance have been identified: the genes encoding dihydrofolate reductase (*Pfdhfr*), dihydropteroate synthase (*Pfdhps*), the chloroquine resistance transporter (*Pfcrt*), the multidrug resistance 1 protein (*Pfmdr-1*),Na+/H+ exchanger (*Pfnhe-1*) and cytochrome *b*. While there has been some success in identifying the molecular markers of resistance for many antimalarial medicines, more research is needed to identify those for other antimalarial drugs, in particular artemisinins (WHO, 2010). Recently a molecular marker for artemisinin resistance was discovered following whole-genome sequencing of an artemisinin-resistant parasite line from Africa and clinical parasite isolates from Cambodia. Mutations in the PF3D7_1343700 kelch propeller domain ('K13-propeller') were associated with artemisinin resistance *in vitro* and *in vivo* (Ariey *et al.,* 2014)

2.5.1 Amino-alcohols and sesquiterpene lactones drug resistance markers

Amino- alcohol is one of the chemical families of antimalarials which include drugs like; Mefloquine, lumefantrine, quinine and halofantrine (Price *et al.,* 2004; 2006). In this family *Pfmdr1* gene has been implicated to cause resistance to different antimalarial drugs. Just like artemisinins which belong to sesquiterpene lactones family. Studies conducted in the Greater Mekong sub region (Cambodia and Thailand) showed that increase in *Pfmdr1* copy numbers are responsible for resistance to mefloquine and the increased risks for treatment failure with artesunate–mefloquine and artemether–lumefantrine (Price *et al.,* 2004; 2006). *In vitro* susceptibility to mefloquine, quinine, halofantrine and artemisinin increased when the *Pfmdr1* copy

numbers were reduced or when the parasites carried *Pfmdr1* mutations (Sidhu *et al.,* 2006; Nkhoma *et al.,* 2009). In South-East Asia, the presence of the Asn86Tyr mutation is a negative marker for gene amplification. *Pfmdr1* amplification and de-amplification are relatively frequent events related to the rapid evolution of mefloquine resistance when the drug is used as monotherapy (WHO, 2010)

2.5.2 *Plasmodium falciparum malaria drug resistant gene-1*

Pfmdr-1 gene codes for a parasite homologue Pgh of the human Pgp (P-glycoprotein), an antigen binding cassette transporter (Foote *et al.,* 1989; Wilson *et al.,* 1989). The protein is mainly localized in the membrane of the digestive vacuole. Furthermore, the *Pfmdr-1* gene is polymorphic with SNPs at positions N86Y, Y184F and S1034C. (Foote *et al.,* 1990).These polymorphisms and over expression of *Pfmdr-1* have been postulated to affect transport of solutes and promote the export of antimalarials (especially quinoline based) from the food vacuole hence reducing their intracellular concentration at their site of action. Current models, supported by results of parasite transfection studies, suggest that *Pfmdr-1* might be a secondary factor in chloroquine resistance (Fidock *et al.,* 2000), but possibly a more important one in quinine, mefloquine and halofantrine sensitivity (Reed *et al.,* 2000).The role of this gene in the modulation of a potential artemisinin derivative resistance are of interest (Duraisingh *et al.,* 2000a, b; Reed *et al.,* 2000). Other types of polymorphisms have been identified in this locus, in particular a repetitive motif at the untranslated region of the gene (Foote *et al.,*1990) and a full tandem amplification of the gene (Foote *et al.,*1989).The tandem sequence appears to correlate with quinine, mefloquine and halofantrine resistance (Barnes *et al.,* 1992; Cowman *et al.,* 1994), leading to the view that this might be a contributing, but not exclusive event for the development of these phenotypes (Lim *et al.,* 1996).

2.5.3 *Plasmodium falciparum* chloroquine resistance transporter gene

Plasmodium falciparum chloroquine resistance transporter gene is located on chromosome 7 with a locus tag of MAL7P1.27 (Fidock *et al.,* 2000). This gene have

424 amino acid protein located in the digestive vacuole membrane of the *Plasmodium* where chloroquine has been suggested to act by binding to haematin, a toxic by product from the digestion of haemoglobin. This binding prevents synthesis of non toxic hemozoin. The gene has 13 exons which are unusually complex for *P.falciparum.* Twelve SNPs in the coding region of the *Pfcrt* have been recognized with mutation Lys76Thr linked to chloroquine resistance with 100% concordance in *P.falciparum* lines from diverse geographical locations (Fidock *et al.* 2000). Polymorphisms of the Pfcrt and Pfmdr-1 gene have been linked to chloroquine resistance. Although the K76T mutation in the pfcrt gene has been shown to be a key determinant in chloroquine resistance, evidence suggests that the Pfmdr-1 gene could modulate the level of chloroquine resistance.

2.6 Methods used in genotyping *Plasmodium falciparum*

2.6.1 DNA sequencing

DNA sequencing has been the 'gold standard'. This method enables the accurate determination of the presence of alleles and their exact position within a particular locus. Recently it was used to locate SNPs in chromosome 10, 13 and 14 of malaria parasite which are associated with slow clearance (Cheeseman *et al.,* 2013). This makes direct sequencing dependable for the identification of novel alleles and haplotypes which cannot be accomplished by other methods many of which require prior knowledge of the polymorphism. However existence of multiple infections necessitates PCR cloning prior to sequencing and primer design for A-T rich segments. This makes this technique extremely laborious.

2.6.2 Restriction fragment length polymorphism (RFLP)

This is a DNA fingerprinting technique that discriminates DNA species on the basis of polymorphisms that add or remove a restriction site. First an amplicon of the locus under investigation is generated by Primary PCR reaction. Specific allele is then amplified in the secondary PCR. Final the amplification product is digested with

appropriate enzymes and the fragments separated by agarose gel electrophoresis. Digestion is usually based on presence or absence of the restriction site. Digested product is usually small and moves longer distance on the gel. This method was applied effectively to genotype chromosome 10 and 13 SNPs of malaria parasite (Takala *et al.,* 2013).

2.6.3 Microsatellite marker analysis

Microsatellites are regions comprising eight or more consecutive repeats of any sequence of 1–8 bp. Over 50% of the *Plasmodium falciparum* microsatellites display length polymorphisms which enables the distinguishing of different isolates on the basis of alleles consisting of different numbers of repeat units. Replication slippage (also known as slip-strand mispairing) occurs during DNA replication and generates these repeat units. Detection of microsatellites typically involves the use of primers flanking to amplify the repeat locus by PCR followed by analysis by electrophoresis on sequencing grade polyacrylamide gels (Monis *et al.,* 2002).

2.6.4 Merozoite surface proteins genotyping

This technique involves a nested PCR amplification of the polymorphic loci. It begins with primary PCR which uses primer pairs that target conserved sequences, spanning the polymorphic regions of block 2 of *MSP-1* gene and block 3 of *MSP-2* gene. PCR products from the primary PCR are used as templates for the secondary PCR reaction. In the secondary reaction allele specific primer sets are used to amplify alleles, K1, MAD20, and RO33 alleles of the *MSP-1* gene and the FC27 and IC3D7 alleles of *MSP-2* alleles. Allelic discrimination is usually achieved by running the PCR amplicon in agarose gel electrophoresis after staining gel with Ethidium bromide whereby amplicon are distinguished from each other based on fragment size. Migration along the gel is dependent on fragment size; with smaller fragments moving faster compared to larger molecular weight fragments. By using molecular ladders of known size, the size of the PCR amplicon can be determined. However, due to limited resolving power of gel

electrophoresis, adequate discrimination of alleles is achieved by Capillary electrophoresis. (Liljander *et al.,* 2009)

2.6.5 Real time polymerase chain reaction

Real Time PCR works like conventional PCR but it uses fluorogenic probes to detect allele polymorphisms. During the course of PCR amplification the probe binds to the amplicon and emits fluorescence proportional to the product. This allows the "real time" monitoring of the amplification reaction. This method has rapid cycling reaction which provides results within short period of time. It is also sensitive, specific and highly reproducible. However substantial optimization is required prior to sample analysis and this type of PCR amplification is not suited for multiplexing. Quantitative Real Time PCR has found many applications including measuring mRNA expression levels, levels, DNA copy number, transgenic copy number and expression analysis, allelic discrimination, and measuring viral titters (Bell *et al.,* 2002). Calculation of the initial amount of the nucleotide template is achieved either by absolute quantitation (involving comparison with a standard curve with serial dilutions of standards of known concentration) or more often by relative quantitation using an internal control (for normalization) and a reference calibrator that yields fold differences between the target and the reference (Wong *et al.,* 2005). Absolute quantitation is preferred for viral load determination while relative quantitation using a calibrator is routinely applied in gene expression studies (Gabriel *et al.,*2004). Another approach that is also used for mRNA quantitation is the comparative Ct ($2^{-\Delta\Delta C_T}$) method.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study area

This study was carried at Kombewa, in Kisumu County, a malaria endemic region in Kenya.

3.2 Study Population

The study population comprised of patients residing within the study area, presenting with uncomplicated malaria. Historical samples obtained through malaria surveillance activities in western Kenya from the period before the introduction of ACT were retrieved and analyzed.

Inclusion criteria

Adult/child aged between 3 years and 65 years inclusive (minimum weight 11kg), presenting with a measured temperature of $\geq 37.5^{0}$C, or history of fever within 24 hours prior to presentation were consented and included in the study. Archived *P.falciparum* blood collected in western Kenya between 1995 to 2003, Kisumu county.

Exclusion criteria

Patients presenting with signs of severe malaria or with severe anemia defined as hemoglobin below 6 g/dl. *Plasmodium falciparum* archived blood collected from other county rather than western Kenya.

3.3 Sample size determination

One hundred and forty samples were used in this study. The minimum number of samples size was 70 as determined using Fisher's exact formula (2000).

Sample size; $n \geq Z^2_{\alpha/2}\ (p\ (1-p))/d^2$

$$n \geq 1.96^2\ (0.1(1-0.1)/0.05^2 = 70$$

Where $Z_{\alpha/2}$ is the corresponding value to the 95% confidence interval (1.96)

p=10% estimated malaria prevalence among highly risk group Kenya (Malaria survey indicator 2010). Confidence interval= 95% and absolute precision d=0.05

3.4 Genotyping

3.4.1Genomic DNA Isolation and purification of *Plasmodium falciparum*

Archived whole blood samples containing parasites were identified from the sample repository and thawed according to standard procedures. A total of 200ul of sample isolate from each subject was aliquoted for the assays. Blood was used in DNA extraction according to QIAamp Blood mini Kits spin protocol (Qiagen, CA). Briefly, 200ul blood sample was added into the microcentrifuge tube followed by addition of 200µL lysis buffer and 20ul protease enzyme that digest proteins. The mixture was mixed by pulse vortexing for 15 second followed by incubation at 50 to 57°C for 10 minutes to ensure complete lysis of the plasmodium particle and inactivate protein. This was followed by centrifugation processes. The mixture above was placed in a sterile 1.5ml. The mixture was transferred to a spin column and equal volume of ethanol was added to purify it. Purification was achieved by micro-centrifugation of the column at 8000 rpm for 1 minute. Filtrate was discarded and spin column was then transferred into another sterile 1.5 mL collection tube and 500µl of buffer AW1 was added, centrifuged at 8000 rpm and filtrate discarded. This was followed by addition of 500µl of buffer AW2 into the spin column and the preparation was centrifuged at 14,000 rpm for 3 minutes. Elution of the DNA from the spin column was achieved by addition of 200µl elution buffer (AE) followed by centrifugation at 8000rpm for 1 minute. The filtrate contained the purified DNA which was stored at –20 to -80°C until time of amplification.

3.4.2 Genotyping of *Plasmodium falciparum* single nucleotide polymorphism

Single nucleotide polymorphism in chromosome 10, 13 and 14 were analyzed by Sequenom Mass Array SNP analysis as described in iPLEX® Gold Application Guide and elsewhere (Gabriel *et al.,* 2006). In brief genomic DNA concentration was determined using nanodrop. DNA concentration was diluted into 5 to 10 ng/ µL using Elution buffer .The DNA was divided into aliquots in a 384-well PCR reaction plate

from a deep-well PCR plate. DNA was then amplified by PCR prior to performing an extension reaction of the SNP of interest. The amplification was done as described in the iPLEX® Gold Application Guide.

In post PCR Shrimp alkaline phosphatase (SAP) was used to dephosphorylates unincorporated dNTPs by cleaving the phosphate groups from the 5' end. This removes all non-incorporated dNTPs from the amplified product. To do this we added 2 µL of a SAP cocktail into each individual well of the 384-well post-PCR reaction plate. We further placed them in a 384- well block thermo cycler at 37◦C for around 15 minutes for incubation followed by 85 degrees celcius for inactivation of SAP as shown in iPLEX® Gold Application Guide.

In extension step, the PCR assay pool plexes consisted of the multiplexed forward and reverse PCR Oligonucleotide primers which we prepared using the three-Tier method. In this method we divided the plexes into low, medium and high mass. We pooled the plexes at 5 µM, 10 µM and 15 µM based on the masses respectively. We did this because of the inverse relationship between peak intensity and analyte mass and to ensure that each extension primer is equally in intensity as possible. The iPLEX extension mix contained 0.940 µL of the adjusted oligos pool mix, which was added to 0.619 µL water, 0.2 µL iPLEX termination mix, 0.20 µL 10 x iPLEX buffers and 0.041 µL iPLEX enzyme. This made up mix of 2 µL per each reaction. We added overhang of 38%, mixed well and made Aliquot of 2 µL to several wells of a 384-well PCR plate with amplified DNA (one pool per row). Thermo cycling conditions used are as described in iPLEX® Gold Application Guide.

The Single Base Extension pool plexes consisted of the multiplexed Oligonucleotide primers, which annealed adjacent to the polymorphic site for each reaction present together in the multiplexed assay pool. By pooling locus-specific primers, many individual loci of DNA corresponding to the SNP sites were analyzed in a one-well reaction. Extension oligos pools were spotted on SpectroCHIP and run on detectors to

verify uniformity of intensities of low/medium/high mass and accuracy of the manual pooling procedure. All masses matched the expected spectra. Any oligos of low-peak intensity were spiked in by adding volume to equalize the peak intensities. All diluted working oligos were stored at 4◦C and concentrated stocks at −20◦C until time of resin addition.

We then defined the plate in the Sequenom Typer Plate Editor Database as to which pools are in which wells. Finally we added resin, centrifuged and run the plates on the Mass Spectrometer as described in iPLEX® Gold Application Guide.

3.4.3 PCR-Restriction Length Fragment Polymorphism for genotyping SNPs

SNPs in chromosome 10 (MAL10-688956) and 13 (MAL13-1718319) have been identified that are associated with artemisinin resistance (Takala-Harrison *et al.,* 2013). These SNPs were genotyped as described in WWARN PCR_RFLP for genotyping candidate *Plasmodium falciparum* artemisinin resistance SNPs MAL10-688956 and MAL13-1718319 v1.0 protocol available online.

3.5 Amplification of *Plasmodium falciparum MSP-1* and *MSP-2.*

Genomic variation between parasites strains was determined by use of two step nested PCR. Multiplex primary PCR method was done using primer pairs specific for two independent genes *MSP-1* and *MSP-2* as described in (Liljander *et al.,* 2009).

3.5.1 Multiplex primary PCR amplification conditions for *MSP1* and *MSP2*

The master mix concentration consisted of 1X PCR buffer, 2mM M_gCl_2, 1× PCR buffer, 1.0mM M_gCl_2, 125µM dNTP and 0.05units/µl of AmpliTaq® DNA polymerase (Applied Biosystems), and 250nM each of the forward and Reverse primer pairs. Extracted DNA (2uL) was used as the template and a total of 25ul master mix was used per reaction. Thermocycle conditions included initial denaturation at 95 °C for 5 min followed by 30 cycles of annealing at 58 °C for 1min, extension at 72 °C for 2 min,

denaturation at 94 °C for 1 min with a final round of amplification at 58 °C for 2 min and 72 °C for 5 min (Viriyakosol *et al.,* 1995].

3.5.2 Nested Reaction *MSP-1* and nPCR Thermoprofile

The final concentration of the master mix consisted of 1× PCR buffer, 1 mM MgCl2, 125 µM dNTP and 0.02 units/ µl of AmpliTaq® DNA polymerase, and 125 nM of the respective *MSP-1* allelic type-specific primers (K1, MAD20, and RO33 types) . From primary amplification, 1µl product from each reaction was used as a template for amplification in the nested PCR and final reaction volume was 25ul. Thermoprofile conditions for the amplification of the *MSP-1* KI, MD20 and R033 allelic types were as follows; denaturation at 95 °C for 5 min then 30 cycles of annealing at 61°C for 2 min. This were followed by extension at 72 °C for 2 min, denaturation at 94 °C for 1 min, and a final step at 61 °C for 2 min and 72 °C for 5 min (Liljander *et al.,* 2009).

3.5.3 Nested Reaction MSP-2 and nPCR thermoprofile

We targeted block 3 *MSP-2* in the allelic amplification. The final concentration of the master mix consisted of 1× PCR buffer, 1mM MgCl2, 125µM dNTP and 0.02 units/µL of AmpliTaq® DNA polymerase, and 125nM of the FC27 allelic primers. For IC3D7 allele amplification was used 0.05 units/ µL of AmpliTaq® DNA polymerase and raised the concentration of the primer pair to 300 nM. From primary amplification 1µl product from each reaction was used as a template for amplification in the nested PCR. For the IC3D7 nested reaction we used 2µl product from each primary reaction. Total reaction volume was raised to 25uL.Thermoprofile conditions for MSP-2 amplification and cycle conditions was as follows: Denaturation at 95 °C for 5 min, then 30 cycles of annealing at 58 °C for 1 min, followed by extension at 72 °C for 2 min. Final denaturation at 94 °C for 1 min, and a final round at 58 °C for 1 min and 5 min at 72 °C .The PCR products was stored away from sunlight exposure till time of electrophoresis (Liljander *et al.,* 2009).

3.5.4 Gel electrophoresis of PCR products.

Nested PCR amplified product was checked on a 2% agarose gel. Gels were stained using ethidium bromide staining before solidification. I loaded 5µL of nested PCR product and mix with 5µL loading dye. Then we visualized in a 312nm ultraviolet transilluminator.

3.5.5 Genetic Analyzer fragment analysis of *MSP-1* and *MSP-2* alleles.

Fragment analysis was done using capillary electrophoresis in a Genetic Analyzer (Applied Biosystem) as described in the manufacturer user guide. The Principle behind this analyzer is that, both the sample and the size standard DNA are co-loaded into the capillary electrophoresis system. Upon analysis, DNA fragments were displayed as peaks, with fragment size calculated in base pairs, a height is calculated in rfu and area is calculated underneath each associated peak. Stutter peaks were eliminated by setting a background cut-off 300 rfu (Liljander *et al.,* 2009).

3.6 Estimation of *Pfmdr-1* Copy number

Assessment of *Pfmdr-1* copy number analysis was performed with TaqMan probes as previously described (Price *et al.,* 2004). The copy number of *Plasmodium falciparum Pfmdr-1* was determined by quantitative PCR. Primers MDR1 (5_-GCATTT-GTG-GGA-GAA-TCA-GG) and MDR2 (5_-CAA-CTC-CAA-TTT-TTGATC-TCC-A) and primers Tubulin forward primer (5_-ACA-ACG-AAG-CAA-CAG-GAG-GT) and Tubulin reverse primer (5_-AGC-CCA-ATT-ATT-TCC-TGC-AC) were designed to amplify *Pfmdr-1* and the gene for _-tubulin (a single-copy housekeeping gene), respectively.The real-time quantitative PCR (qRT-PCR) method was used on a 7500 fast qPCR system .Reference strains Dd2 (which carries two to three *Pfmdr-1* copies) and 3D7 (which carries one *Pfmdr-1* copy) were used as controls. Each reaction was performed in triplicate, and the experiment was repeated three times (Benoit *et al.,* 2010).

3.7 *In vivo* Clearance of *Plasmodium falciparum.*

Parasitemia was measured from Giemsa-stained thin and then thick blood films every 6 hours until parasite negativity (two consecutive negative blood smears) by counting the proportion of parasite infected RBCs .Clearance rates (CR) were estimated by Tobit regression of log (parasite density) over time after correcting for an initial lag phase and accounting for uncertainty in low Parasitemia estimates *(*Flegg *et al., 2011).* Parasite half-lives were calculated as log (2)/CR. To account for non-normality in the distribution of CRs these were log-transformed for association analysis. Association between CRs and genetic polymorphisms in chromosomes 10, 13 and 14 was investigated using genotyping results from ACT efficacy trials, while the historical samples provided background information that enabled the interpretation of any genetic changes observed.

3.8 *In vitro* drug sensitivity tests

Dihydroartemisinin (DHA), Piperaquine (PPQ), Amodiaquine, lumefantrine, artemether, artesunate and mefloquine were obtained from Walter Reed Army Institute of Research (WRAIR), Silver spring, MD. Parasites were maintained in continuous culture as described in (Trager *et al.,* 1976). SYBR Green I- based drug sensitivity assay was used for *in vitro* drug sensitivity testing (Rason *et al.,* 2008). *P. falciparum* parasites in continuous culture attaining 3 to 8% parasitemia (field isolates or reference strains) were adjusted to 2% hematocrit and 1% parasitemia. Drug panel mentioned above were prepared in 5 mL of 70% ethanol to attain 5 mg/mL which were lowered to 2554 nM in tissue culture medium. This was followed by serial dilution across 10-concentrations ranging from 2554 nM to 5 nM and pre-dosed on 96-well microliter plates as previously described in (Tyner *et al.,* 2012). The assay was initiated by addition of 100 μL reconstituted parasite components to each well of the drug plates and incubation at 37 ºC as previously described (Johnson *et al.,* 2007). The assay was terminated after 72 hours and 100 μL of lysis buffer containing SYBR green I (1x final

concentration) was added directly to the plates and kept at room temperature in the dark, for 24 hours. Parasite replication inhibition was quantified by measuring the per well relative fluorescence units (RFU) of SYBR green 1 dye using the Tecan Genios Plus® with excitation and emission wavelengths of 485 nm and 535 nm. The 50% inhibition concentration (IC_{50}) values for each drug was calculated as described previously (Eyase *et al.,* 2013)

3.9 Ethical Considerations

Consenting procedures, study protocols, and collection of malaria infected blood samples in Kisumu , western County was obtained from the Ethical Review Committee of the Kenya Medical Research Institute, Nairobi (SSC protocol No 2518) and the Walter Reed Army Institute of Research, Silver Spring, Maryland, USA (WRAIR # 1935,HRPO Log # A-17502,SSC # 2518). Refer appendix 8.

3.10 Data analysis

3.10.1 Sequenome data and clearance rates analysis

Mass ARRAY Workstation software was used to process and analyze iPLEX Spectro CHIP bioarrays. Genotype calls was made in real time during chip detection. Spectro TYPER-RT (Sequenom) software was used to calculate the expected position of the correct analyte peaks in the spectra Call. The frequency of the molecular markers of interest in the SNPs with MAF of <1% were removed. SNPs with MAF >5% and <10% missing data were used for analysis (Cheeseman *et al.,* 2013). Differences in clearance based on suspected genotype profiles were compared using the non-parametric (1- way ANOVA, Kruskal-Wallis with Dunn's multiple comparison post test, t-test and Mann-Whitney test as appropriate. To test the utility of the SNPs profile shown in Table 4.1 as surveillance tools for tracking parasites with reduced parasite clearance, odds ratios (ORs) were estimated for 2013-2014 SNPs by comparing the log odds of an infection having clearance half-life >2.55 h (the median clearance half-life in our dataset) in parasites with a given allele to those with the alternative allele (Table

4.2).Parasites were partitioned into Faster and fastest using our lower 25th percentile for the purpose of this analysis. (Fastest group<3.0 hrs>= Faster clearance) of the Kombewa In vivo clinical trial *Plasmodium falciparum* distribution of clearance half-life values. A difference in these two populations was carried out using Kolmogorov-Smirnov test. Similar approach was used by Takala *et al.,* 2013; Cheeseman *et al.* 2012. There was a small population of genetically unique single clone infections (n=24). A SNP profile for *Plasmodium falciparum* isolates from western Kenya was established (Table 2.)

3.10.2 Restriction fragment length polymorphism analysis

All the samples from pre-historic and current samples were genotyped and classified as either mutants or wild type based on ability of the restriction enzyme to digest the DNA. The rationale was, if SNP was present then the restriction enzyme won't be able to digest the strand. This is detected on gel electrophoresis as digested fragments are small (figure 4.1.1a and b).

3.10.3 Recrudescent and re-infection

Polymorphisms in *MSP-1* and MSP-2 were analyzed using the genetic analyzer. Parasite isolates were classified as recrudescent or re-infection based on the genetic polymorphism in *MSP-1* and MSP-2. In brief those with same allele in MSP1-1 and MSP-2 both in day 0 and during follow up visit were considered recrudescence parasites while those with different allele sizes in MSP1 and *MSP-2* during follow up were considered as due to re-infection

3.10.4 *Pfmdr-1* gene copy number estimation calculations

Comparative cycle threshold method ($2^{-\Delta\Delta CT}$ relative quantification technique) was used to analyze the copy number variation. In brief the gene amplification copy number was estimated by comparison of the threshold cycle (*CT*) value of _tubulin with the *CT* value of *Pfmdr-1* by the $2^{-\Delta\Delta CT}$ method. The difference in copy number variation

between current and pre-historic samples was tested using t-test. Analyses were conducted using Graph pad Prism, version 5.0.2; (Graph pad Software, Inc., San Diego, CA). Isolates with copy number >= 1.75 were considered to have true *Pfmdr-1* gene duplicate copy number as previously described (Nahla *et al.,* 2011). We grouped the three periods into Pre-ACT samples (1995-2003) and Post-ACT samples (2013-2014) for purposes of this analysis. *Pfmdr-1* copy number variations were compared using the non-parametric tests (1- way ANOVA Kruskal-Wallis with Dunn's multiple comparison post test and Mann-Whitney test).

3.10.5 *In vitro* susceptibilities association with SNPs and copy number

The 50% inhibitory concentrations (IC50s) were successfully determined for Dihydroartemisinin (DHA), Piperaquine (PPQ), Amodiaquine, lumefantrine, artemether, artesunate and mefloquine. The 7 *Plasmodium falciparum* strains with true duplicate copy number and 20 randomly selected *Plasmodium falciparum* with single *Pfmdr-1* gene copy number from the *in vivo* efficacy study were cultured. Association of IC50s between isolates with multiple copy number and single copy number was done using Kruskal-wallis Mann-Whitney test while Association of SNP patterns and IC50s was done using 1-way ANOVA.

CHAPTER FOUR

RESULTS

4.1 Single nucleotide polymorphism in Pre-ACT and Post ACT samples

Genetically-unique parasites were genotyped by sequenome MassARRAY that scores SNPs based on mass. The call results of each SNP are shown in figure 4.1. 129 Samples with minor allele frequency (MAF) > 1% were used in the analysis. For Post ACT samples, 71 were collected between 2013 and 2014. 10 were collected between 2005 and 2010. For Pre-ACT samples, 30 were collected between 1995 and 1999 while 18 were collected between 2000 and 2003. 25 SNPs were screened in each sample. 60% of the 25 SNPs (15 SNPs) were found in pre-ACT samples (1995-2003) while in Post ACT samples (2013-2014) were found 23% of the 25 SNPs (6 SNPs) as shown in Table 4.1

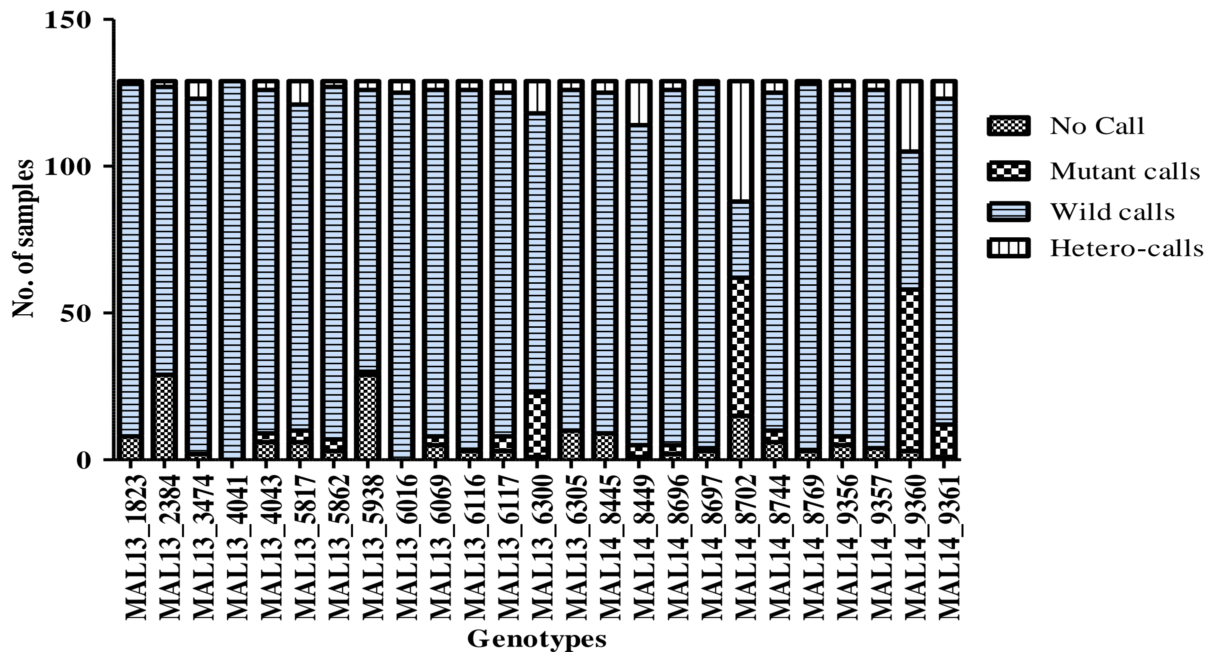

Figure 4.1: The call rate in each SNP in samples assayed.

Table 4.1: Single nucleotide polymorphism profiles and respective minor allele frequencies in different years

Combined SNP-ID	Kisumu samples 1995-1999 n= 30 Allele (MAF)	Kisumu samples 2000-2003 n=18 Allele (MAF)	Kisumu samples 2005-2010 n=10 Allele (MAF)	Kisumu clinical trial samples 2013-2014 n=71 Allele (MAF)
SNP_MAL13_6300	C(0.3)	C(0.17)	C(0.1)	C(0.13)
SNP_MAL14_8697		C(0.06)		
SNP_MAL14_9360	T(0.57)	T(0.5)	T(0.3)	T(0.38)
SNP_MAL13_5862	G(0.07)	G(0.12)		
SNP_MAL13_5938	G(0.03)			
SNP_MAL14_8449		A(0.11)		A(0.029)
SNP_MAL13_6117	G(0.07)	G(0.17)		
SNP_MAL13_5817	T(0.1)			T(0.014)
SNP_MAL13_4041				
SNP_MAL14_9361	T(0.014)	T(0.014)	T(0.4)	T(0.014)
SNP_MAL14_8702	C(0.4)	C(0.5)	C(0.4)	C(0.31)
SNP_MAL13_6069	C(0.07)	C(0.07)		
SNP_MAL14_8696	C(0.07)	C(0.06)		
SNP_MAL13_4043	C(0.07)	C(0.07)		
SNP_MAL14_9356	G(0.07)	G(0.07)		
SNP_MAL14_8744	G(0.07)	G(0.13)		

Table 4.2: Association of P. falciparum SNPs with parasite clearance half-life after Artemisinin ACT treatment.

Chromosome	SNP (Allele) Position	OR(95% confidence interval)
14	Combined SNP mal14.8702	0.80(0.25-2.52)
14	Combined SNP mal14.9360	0.98(0.33-2.88)
13	Combined SNP mal13.6300	0.91(0.22-3.86)
14	Combined SNPmal14.8702/mal14.9360	1.25 (0.35-4.49)
14 and 13	Combined SNmal14.8702/mal13.6300	7.67 (0.39-150.5)

4.1.1 Restriction Length Polymorphism results for Combined Mal 13-1718319 and Combined Mal 10-688956.

The pre-ACT and Post ACT samples were screened for single nucleotide polymorphism at mal13-1718319 and mal10-688956. All 104 samples were digested by the enzyme indicating they were wild type. Figure 4.1.1 A, B

A

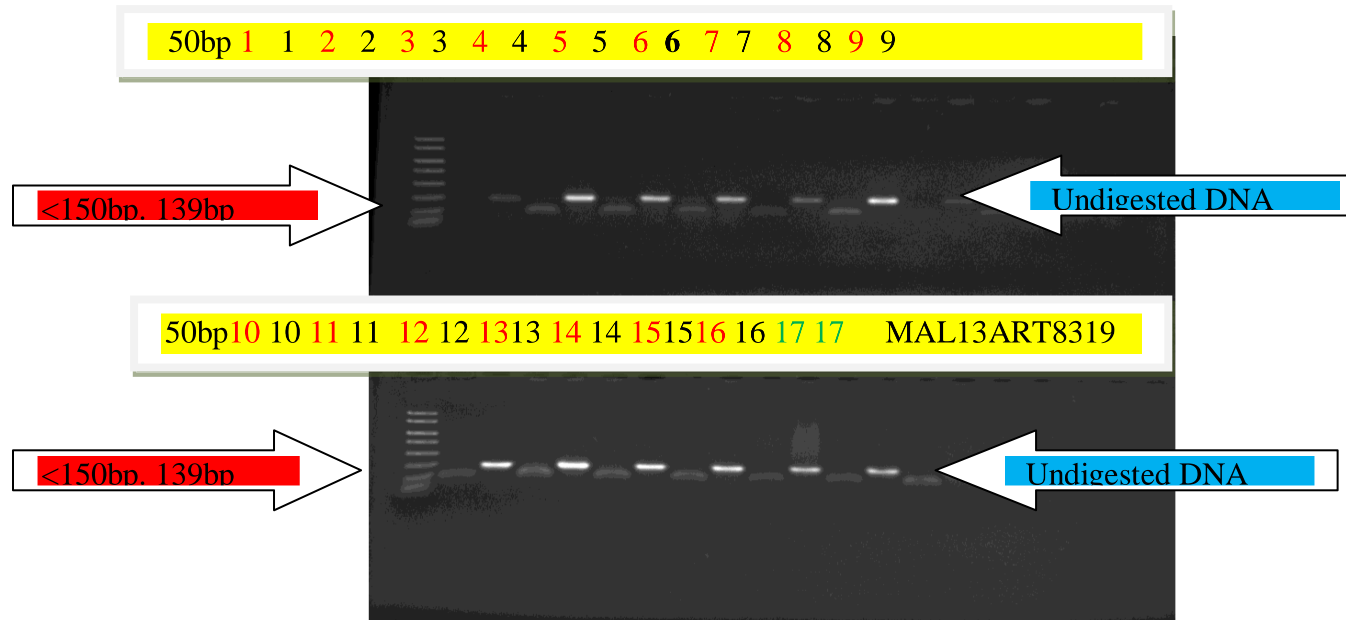

Figure 4.1.1 A: Digested PCR products (Red) and undigested PCR products (Black) products for MAL13-1718319. 16: 3D7 at 50 pg/µL, 17 (Green) is V1/S at 50 pg/µL

Figure 4.1.1 B: Digested PCR products (Red) and undigested PCR products (Black) products for MAL10-688956. 16: 3D7 at 50 pg/µL, 17 (Green) is V1/S at 50 pg/µL

4.1.2 Population diversity of *MSP1* and *MSP2* at baseline and day of recurrence

10 subjects with genotyped data presented with recurrence parasite. Genotyping results of MSP1 and MSP2 block proteins showed that K1 allele had high prevalence in *MSP1* (22 alleles) followed by RO33 (10 alleles) and MAD20 (6 alleles). (Figure4.1.2 a). In *MSP2,* IC3D7 allele was more prevalent (13 alleles) and FC27 allele had (12 alleles). (Figure4.2b). The length variations of the *MSP1*-amplified product were approximately 184-246 bp for K1 (22 alleles), 170-227 bp for MAD20 (6 Alleles) and 158-160 bp for RO33 (10 Alleles). For *MSP2*, the length variations of the amplified product were approximately 295-415 bp for FC27 (12 Alleles) and 474-621 for IC3D7 (13 Alleles). All 20 follow up samples were associated with re-infection based on the merozoite surface protein polymorphisms. Multiplicity of infection was 2 for most samples (Table4.3)

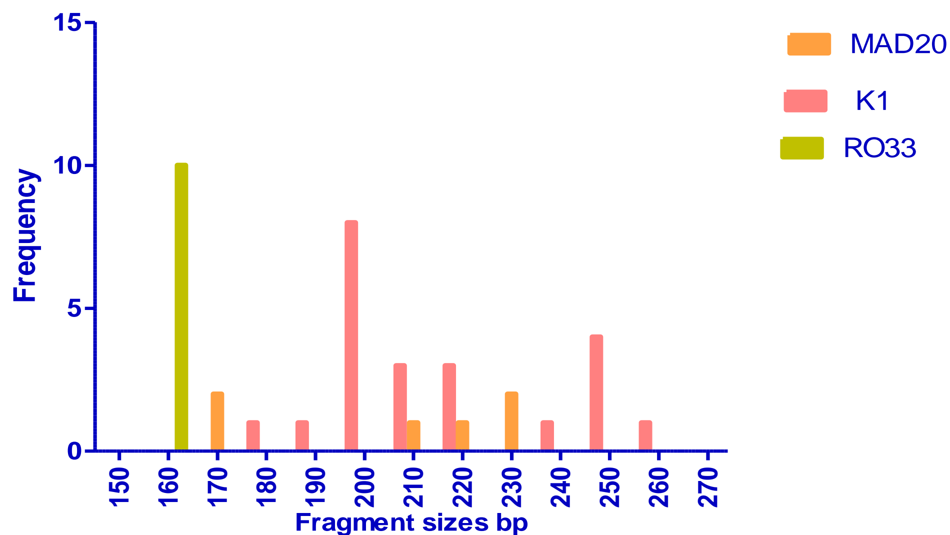

Figure 4.2a: Merozoite surface protein 1 allele distribution in recurrence samples

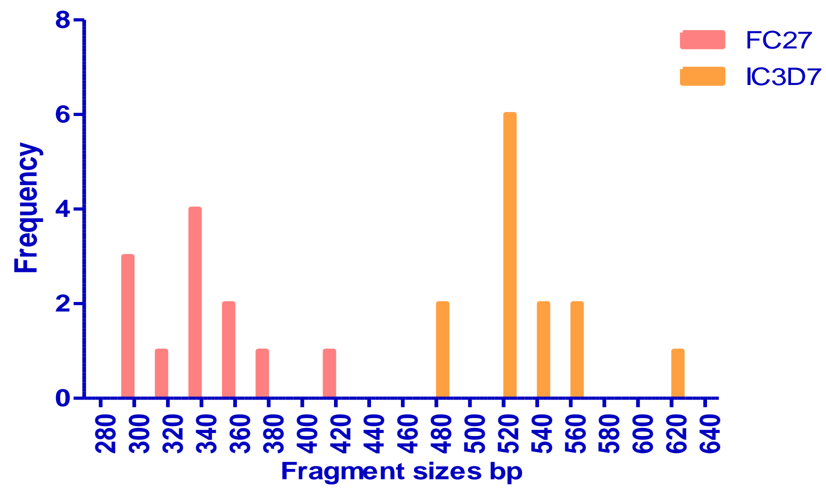

Figure 4.2.b: Merozoite surface protein 2 allele distribution in recurrence samples.

Table 4.3: MSP-1/MSP-2 allele polymorphism of samples at baseline and day of recurrence.

Sample ID	K1 (bp)	MAD20 (bp)	R033(bp)	FC27(bp)	IC3D7(bp)	MOI
M002 D0	201			296	518	1
002 D41	184, 185	225		380	553	2
007 D0	219				515	1
007 D28	201		158,159		518, 535	2
018D0			158, 159		516	
018D21	245, 246			342	565	2
045D0	219, 220		159		520	2
045D35			159	334		1
046D0	201		159		621	1
046D21	197		158		530	1
076D0	262			415		1
076D45			158,159	367		2
088D0	201, 236	224			474	2
088D42	198	170, 227			479	2
094D0	210,208	206		350		2
094D35	202,201				510	2
020D0	245			329		1
020D21	245			342		1
077D0	210			295,334		2
077D42		170		306		1

4.2 *In vivo* Clearance half-life of parasites in response to ACT

Subjects from *in vivo* efficacy study with uncomplicated malaria provided samples to assess clearance half-life. All *P. falciparum* strains were sensitive to artemisinin with clearance mean half-life of 2.63 95% CI (2.44-2.81) median clearance half life was 2.61 IQR (1.99, 3.21 as shown in table 4.4 below.

Table 4.4: Baseline characteristics, clinical and parasitological outcomes in groups of patients with *Plasmodium falciparum* malaria followed by genotyping results following day of recurrence

	Fastest parasite clearance Artemisinin sensitive	**Faster parasite clearance Artemisinin sensitive**	**Recurrence parasites**	**Overall Study population**	**P. value**
	n=46	n=17	n=10	n=73	
Age (y) Median(IQR)	8 (5-10)	3 (2-5)	3 (2-6)	6.0 (3-9)	0.0001
Parasitemia(ul) geometric mean (95%CI)	30,680 (24,209-37,151)	10,154 (2,569-17,739)	33,317 (14,071-52,564)	26,261 (20,960-31,563)	0.0002
Parasite clearance (hrs) geometric Mean (95%CI)	2.24 (2.09-3.44)	3.52 (3.21-3.83)	2.82 (2.20-2.81)	2.63 (2.44-2.81)	0.0001
Parasitological outcome (%)					
Cured	46 (100)	17 (100)	10 (100)	63(86)	
Re-infection	0	0	10 (100)	10(14)	
Recrudescence	0	0	0	0	
No outcome	0	0	0	0	

4.2.1 Association of Single nucleotide polymorphism with *In vivo* parasite clearance time

There was no significant association between parasites with SNPs and the *In vivo* clearance slope half of parasite (P= 0.172 Wilcoxon rank test (Figure 4.2.1a). There was a significant difference in age between those subjects who cleared parasites fastest and those who cleared parasite faster.(P=0.0001). 24 single clone parasites were isolated. The association of SNPs

from the single clones and clearance half-life was not significant (P=0.315) Wilcoxon rank sum (Mann Whitney) test (Figure4.2.1b). There were no significant differences between genotypes with clearance half-life. (Figure 4.2.1c) .Using Cox regression analysis there was association of prolonged clearance slope half life with having minor allele A at Combined SNP Mal14.9360 and having a SNP at Combined SNP Mal 13.6300 (P=0.007 and P=0.016)

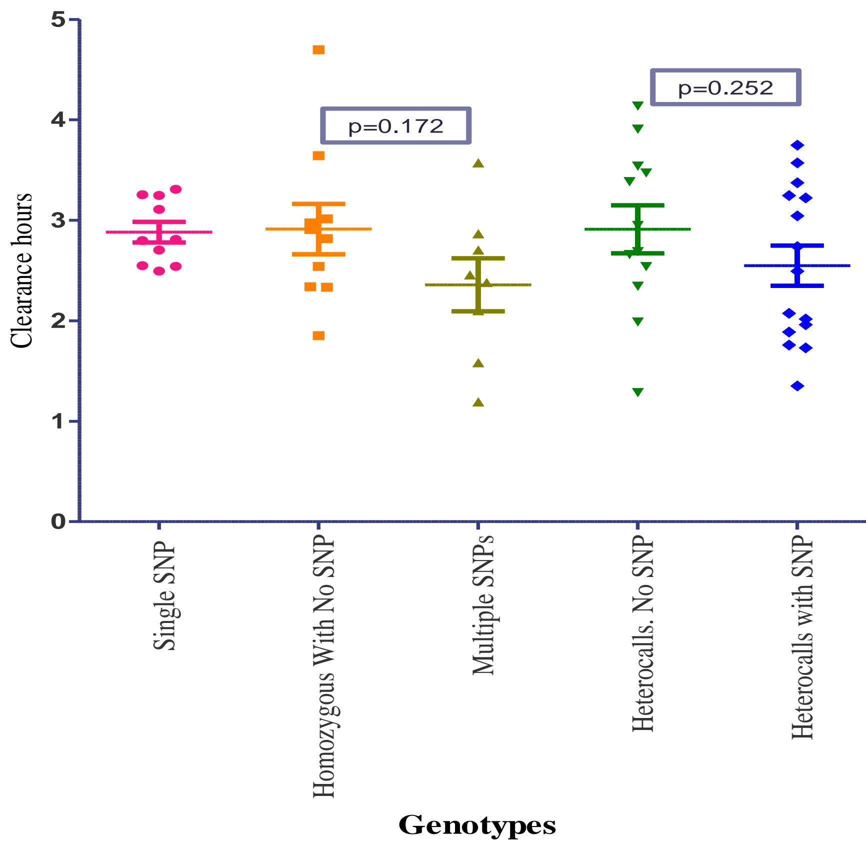

Figure 4.2.1a: A plot showing, association of Parasite clearance half-life against genotypes

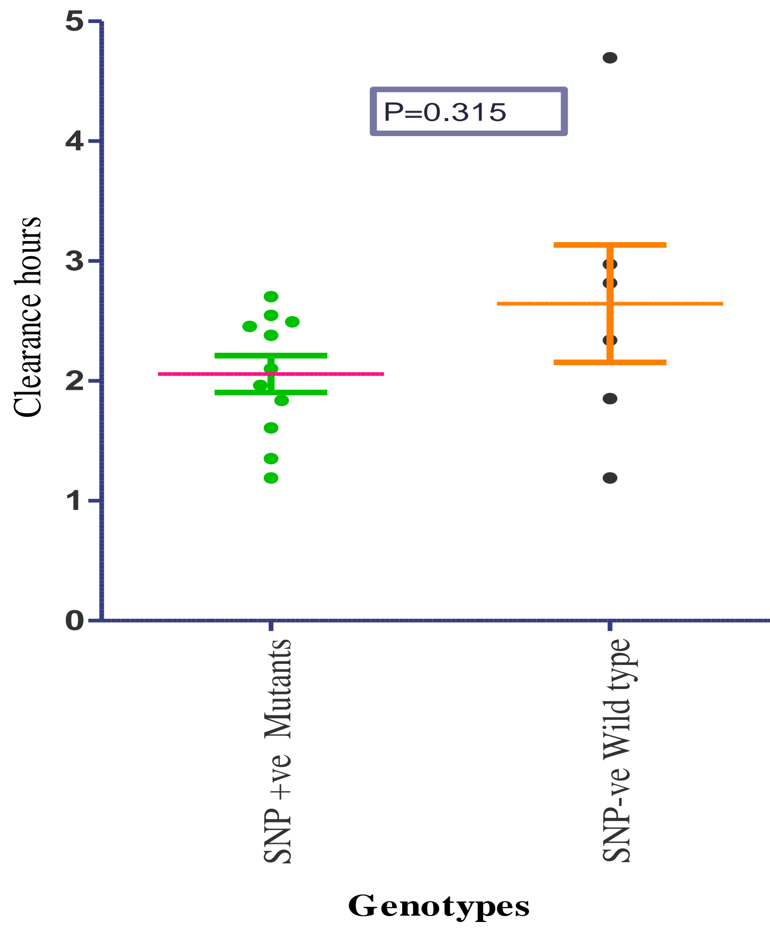

Figure 4.2.1 b: A plot showing association of Single clones genotypes against parasite clearance half-life

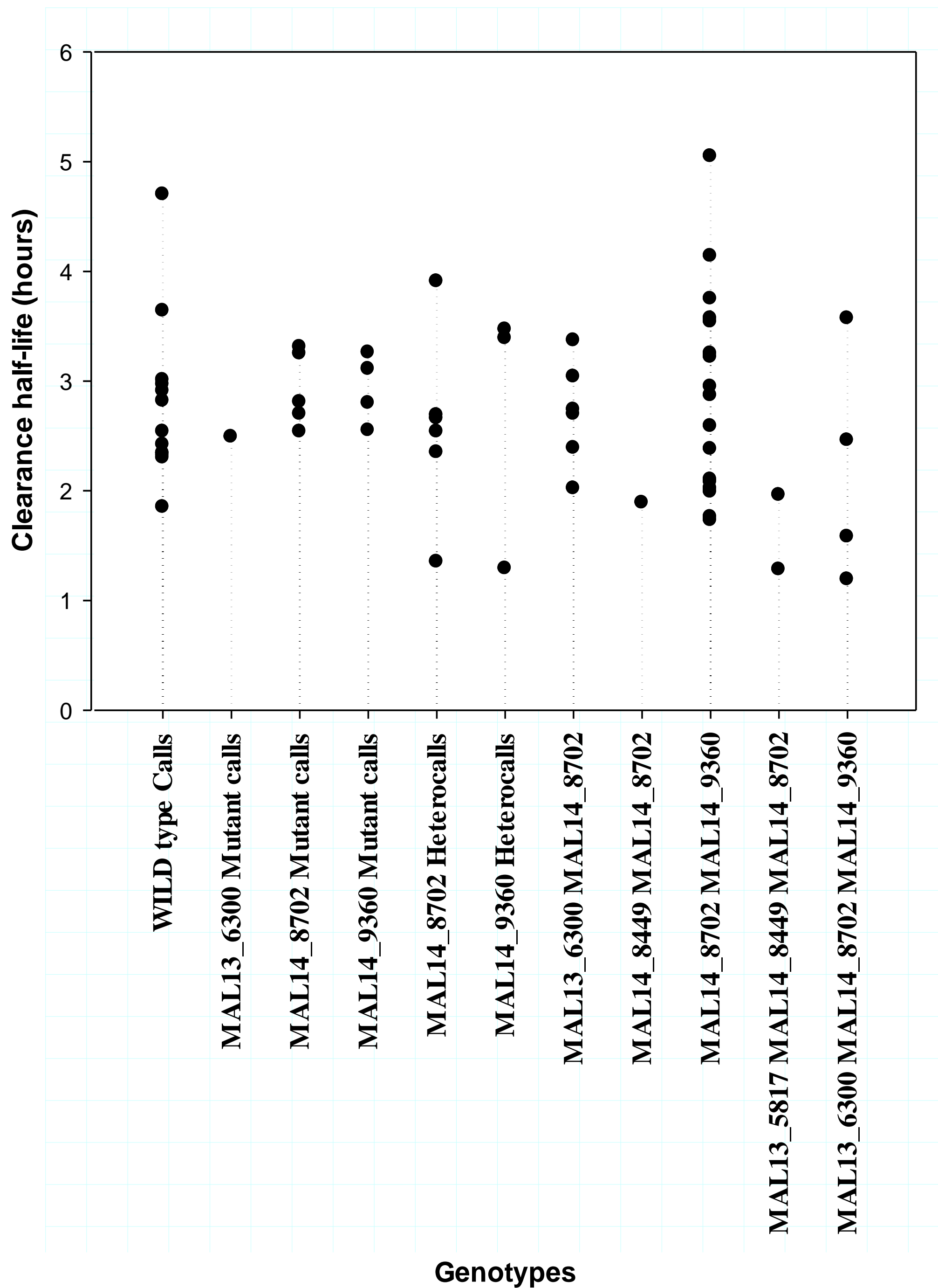

Figure 4.2.1c: A plot showing general parasite Slope half-life against the genotypes.

4.3 *Pfmdr-1* Copy number distribution in Pre-ACT period and Post ACT period

One-hundred and forty-two (142) samples were assayed for *pfmdr-1* copy number variation. 30 samples (21.1%) were collected in 1995–1999, 41 samples (28.9%) n 2000–2003 and 71 samples (50%) in 2013–2014. Out of the 142 strains analyzed, 8 carried increased *Pfmdr-1* copy number. Specifically, 7 strains from the 2013-2014 samples carried two copies of *Pfmdr-1* gene and 1 strain harbored two copies of *Pfmdr-1* gene from the 1995-1999 samples. The mean distribution of the *Pfmdr-1* gene were 1.05 95%CI(0.97-1.14), 1.29 95%CI (1.2-1.35) and 1.31 95% CI(1.24-1.37) for the periods 1995-1999, 2000-2003 and 2013-2014 respectively.(Table 4.3) A significant difference in *Pfmdr-1* mean distribution between the three periods was found.(P<0.0001 One way Anova.). (Figure 4.3a). There was significant increase in *Pfmdr-1* gene amplification between the two periods (P=0.0091) (Figure4.3b)

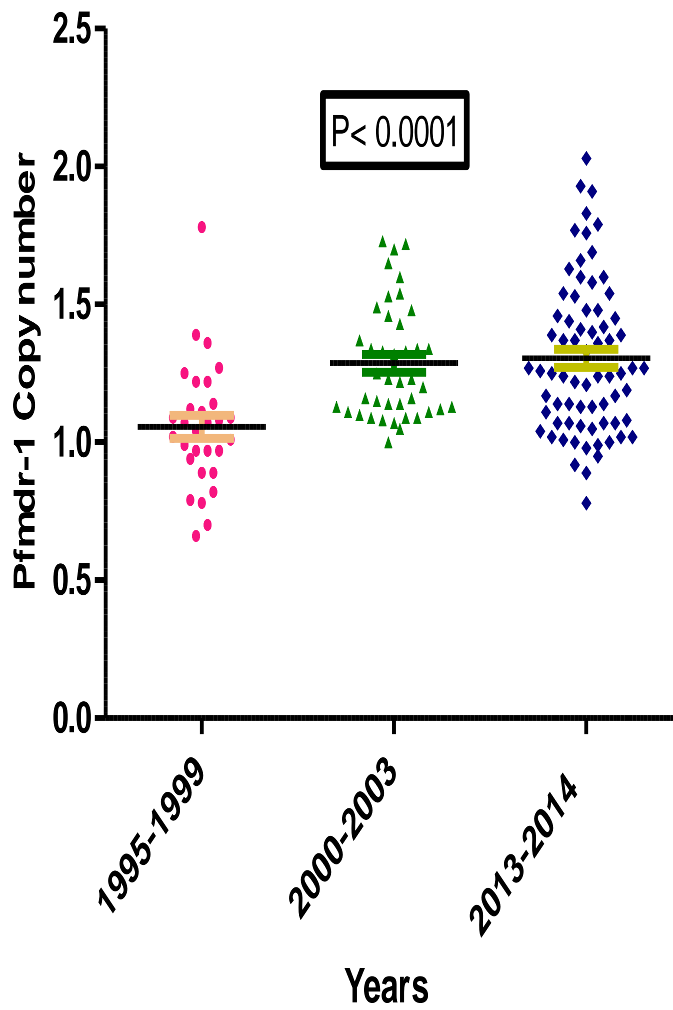

Figure 4.3a: ***Pfmdr-1*** **Copy number variations in sample isolates in different years.**

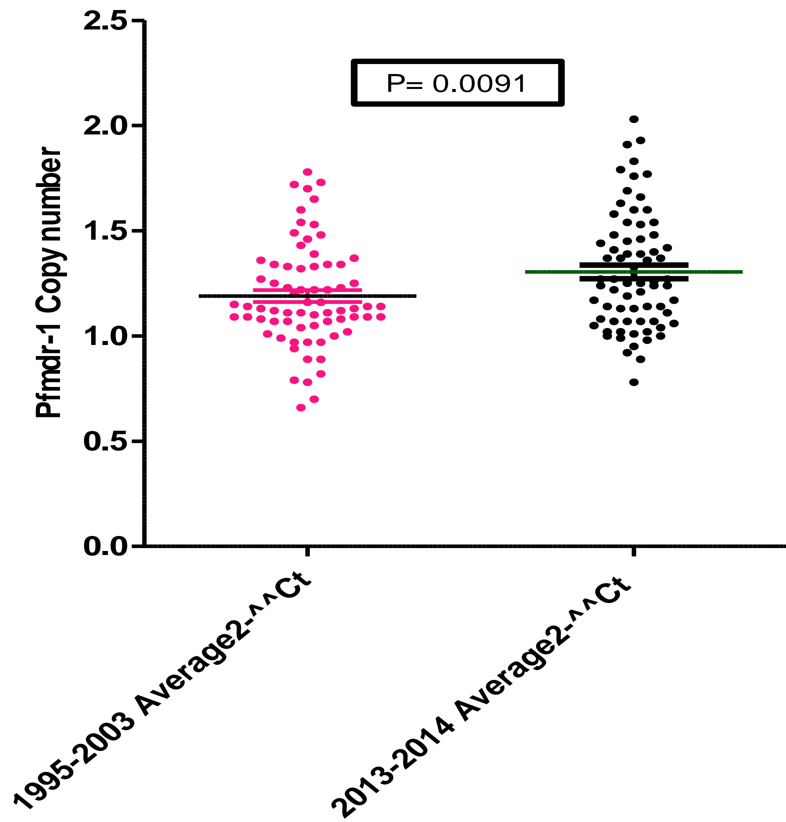

Figure 4.3b: ***Pfmdr-1gene*** **copy number distribution between Pre-ACT and Post-ACT periods.**

Table 4.5: Distribution of *Pfmdr1* gene copy number between Pre-ACT and Post-ACT period.

Years	1995-1999	2000-2003	2013-2014
	n=30	n=41	n=71
Minimum	0.6600	1.000	0.7800
25% Percentile	0.9275	1.115	1.070
Median	1.055	Z	1.250
75% Percentile	1.160	1.445	1.480
Maximum	1.780	1.730	2.030
Mean	1.057	1.287	1.305
Std. Deviation	0.2250	0.2055	0.2772
Std. Error	0.04107	0.03210	0.03289
Lower 95% CI	0.9727	1.222	1.239
Upper 95% CI	1.141	1.351	1.370

4.4 Association of multiple copy numbers and *In vitro* drug susceptibilities

A large range of sensitivities was recorded for PPQ, with a median IC50 of 26.37nM ranging from 0.76 to 35.46nM for *Pfmdr-1* single copy number. In strains with multiple copy number the median was 37.29 ranging from 27.97 to 177.8 nM (Table 4.4). There was significant increase in IC50s in PPQ and Amodiaquine between strains with single *Pfmdr-1* copy number and those with multiple copy numbers (P= 0.038) and (P=0.009) respectively. Wilcoxon rank sum (Mann-Whitney) test.

Table 4.6: Correlation between IC50 and *Pfmdr-1* copy number outcome

	Single *Pfmdr-1* copy number		Increased *Pfmdr-1* copy number		
Drug Name	**Number**	**Median IC50(IQR)nM/L**	**Number**	**Median IC50(IQR)nM/L**	**P value**
Amodiaquine	20	3.93 (0.33-5.67)	7	8.62(6.76-18.17)	0.009
Artemether	20	3.77 (2.7-5.49)	7	4.21 (3.21-5.8)	0.361
Lumefantrine	20	29.9(0.74-43.12)	7	56.12 (28.11-48.35)	0.26
Artesunate	20	15.01 (4.39-22.15)	7	11.51 (9.93-16.53)	0.80
Mefloquine	20	2.14 (0.98-3.14)	7	4.02 (1.79 -8.22)	0.21
Dihydroartemisinin	20	5.83(0.0-12.09)	7	7.071(5.62-11.35)	0.59
Piperaquine	20	26.37 (0.72-35.46)	7	37.29 (24.97-177.8)	0.038

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Genetic polymorphism in chromosome 10, 13 and 14 in *Plasmodium falciparum*

Here we report a different profile in *Plasmodium falciparum* SNP associated with artemisinin resistance after introduction of ACT in Kenya compared to SNPs associated with delayed clearance rate in South East Asia (Cheeseman *et al.,* 2012). This suggests the existence of different population of parasites from those of south East Asia. It further shows response rates of *Plasmodium falciparum* infections to treatment with ACTs after 9 years of their use in western Kenya. Studies have shown that genetic variations may be brought about by geographical bias which is partially understood (Eastman *et al.,* 2009). Ecological, behavioral and biological factors may also play a role including high rates of inbreeding in the mosquito vector, which reduce competition and favor clonal expansion of emerging genetic variants under drug pressure (Eastman *et al.,* 2009) , indiscriminate use of poor-quality drugs (Nayyar *et al.,* 2012) and possibly, ''hyper-mutant'' parasite strains (Rathod *et al.,*1997) contribute to the variations.

The parasite SNP prevalence rate before introduction of ACT was 60% (15/25) and reduced to 23% (6/25) after introduction of ACT. There was absence of delayed *Plasmodium falciparum* infections after treatment despite the presence of 6 SNP in moderate frequency in the 2013-2014 western Kenya *Plasmodium falciparum* isolates. This suggest that there is yet unknown causal genetic variants in western Kenya. This 6 SNP (Combined SNP mal13.6300, Combined SNP mal14.9360, Combined SNP mal14.8449, Combined SNP mal13.5817, Combined SNP mal14.9361 and Combined SNP mal14.8702) on chromosome 13 and 14 are required but may not be sufficient to suggest *In vivo* artemisinin tolerance. The high prevalence of SNPs in pre- ACT period and low prevalence in Post ACT period may be due to selective sweep and change in

the drug pressure. Drug resistance in *Plasmodium falciparum* has been reported as single locus effect, with which beneficial alleles rapidly going to fixation by selective sweeps living a characteristic low diversity scars in genome of parasite (Anderson *et al.,* 2010). Nevertheless in another study a SNP mal 13_1644675 was found in coast region of Kenya, which was associated with delayed clearance in south East Asia (Borman *et al.,* 2011). The absence of this SNP in this study could be because of the study set-up which was restricted to SNPs reported in Cheeseman *et al.,* 2012 and Takala *et al.,* 2013.

5.2 *In vivo Plasmodium falciparum* clearance rate estimation

Genomic comparisons of slow and first clearance parasite from *In vivo* studies offer a means to identify genetic basis of drug resistant maker and genes undergoing natural selection (Takala *et al.,* 2013). In this study we determined clearance rate of *Plasmodium falciparum* populations in Kombewa western Kenya. Differences in clearance half-life within subjects following ART-treatment was observed though all parasites were sensitive since they cleared within the expected 48hours (Table 4.1). The mean clearance half-life was 2.6 hours which is still low compared to 3.6 hours in Myanmar border 2010 and 6.2 in western Cambodia 2010 (Phyo *et al.,* 2012). This difference in clearance half life in western Kenya was probable due to age differences since we found out that there was significant differences in age groups between those subjects who cleared faster as compared to those who cleared fastest (P=0.0001). Similar studies have shown that parasite clearance half-life is not only determined by the intrinsic susceptibility of a parasite isolate to ART, but also by its developmental stage at the time of ART treatment and host-related parameters such as pharmacokinetics, age and immunity (Lopera-Mesa *et al.*.2013).

To distinguish recrudescent (true failures) from new infections and to determine the multiplicity of infection (MOI) index, matched pairs of 20 parasite isolates obtained at baseline and recurrence were compared by genotyping MSP2 and MSP1 gene as

described in (Lijandelar *et al.,* 2009). Results were verified using genetic analyzer, a semi-automated capillary electrophoresis. All follow up samples were associated with re-infection and not recrudescence. This was expected since there has been no reported case of *Plasmodium falciparum* resistance to ACT in Kenya. We had multiplicity of infection of 2. This indicates there is high transmission intensity due to successfully bites of mosquitoes but the malaria parasites are still sensitive to ACT.

5.3 Genetic polymorphism and clearance rate association in *Plasmodium falciparum*

We determined the differences in the phenotypic characteristics with molecular makers in chromosome 10, 13 and 14 that were associated with slow clearance in South East Asia (Cheeseman *et al.,* 2012). The hypothesis was that that if the six screened SNPs are associated with clearance rate then there would be differences in clearance half- life between populations of parasites with the SNPs (mutant) against those without the SNPs (wild type.).Using logic regression test we got odds ratio of 1 to 7.6 (Table 4.2). The differences between each genotype were tested using appropriate non-parametric independent t-test and ANOVA appropriately. SNPs genotypes were not significantly associated with the differences in Invivo parasite clearance half life. Genome wide study at the Kenyan coast reported small, decline through time of parasitological response rates to treatment with ACTs. This was attributed to the possibility of emergence of parasites with reduced drug sensitivity (Bormann *et al.,* 2011). We could not rule out this possibility in this study.

5.4 Assessment of *pfmdr1* copy number before and after ACT introduction

Apart from single nucleotide polymorphisms, *Pfmdr-1* amplifications affect susceptibility to structurally unrelated antimalarial drugs, including mefloquine, artesunate, lumefantrine and quinine [Price *et al.,* 1999; 2004; Duraisingh *et al.,* 2000]. In this study there was an increase in *pfmdr1* gene amplification after the introduction of ACT in Kenya. Currently there is no reported case of parasite resistance to ACT in

Kenya. Nevertheless all follow up samples were associated with re-infection based on MSP-1/ MSP-2 genotyping. The data showed that before introduction of ACT in Kenya (1995-2003) only 1.4% parasite isolate (1 sample out of 71) had *Pfmdr1* copy number of 2. In the period 2013-2014, we found out 10% of the parasite isolates (7 samples out of the 71) had increased *pfmdr1* gene copy number . *Pfmdr1*copy number distribution was statistically different ($P< 0.001$) between Pre-ACT period and post ACT period. This suggests a possibility of existence of *Plasmodium falciparum* clones with multiple copy number in circulation which may putatively contribute to reduced clearance after ACT dosage in the country. This outcome shows parasite response due to introduction of artemisinin drug pressure and elimination of the previous drug pressure.

A previous study in Ghana reported increase in *pfmdr1* amplification as drug regimen changes (Duah *et al.,* 2013). In that study they attributed the increased copy number to artesunate drug pressure, a component in the ACT (Duah *et al.,* 2013). Other studies have assertion that *pfmdr1* gene copy number rather than the SNPs exercises greater influence in mediating anti-malarial drug resistance to some compounds (Anderson *et al.,* 2013). This was reported due to the fact that many transporter proteins mandate concerted complementary attention to copy number variations (CNV) in mediating anti-malarial activity (Anderson *et al.,* 2013).

Studies in Africa have shown probability of parasites developing resistance to Lumefantrine in Africa due to high transmission rates in Africa and subsequent exposure of many parasites to sub-therapeutic concentrations of the drug (Bloland *et al.,* 2000). Association of *in vitro* test and *Pfmdr1* copy number variations have shown in increase in IC_{50} susceptibility in parasite with multiple *pfmdr1* copy number (Table 4.4). However the differences were not significant apart from Amodiaquine and piperaquine. The median IC50 (Interquartile range) for isolates with single and multiple copy number from our study was 3.93 nM (0.33–5.67); 8.62nM (6.76-18.17) for amodiaquine ($p=0.009$) and 26.37nM (0.72-35.46); 37.29nM (24.97-177.8) for

piperaquine (p=0.038). Other *In vitro* investigations have also reported some cross-resistance between Lumefantrine and Mefloquine and have identified copy number changes and SNPs in *pfmdr1* that can decrease parasite susceptibility to both agents (Sidhu *et al.,* 2006; Price *et al.,* 2006). Artemether lumefantrine treatment failure has been associated in Asia with *pfmdr1* amplification and in Africa with selection for certain *pfmdr1* polymorphic residues (Price *et al.,* 2006; Dokomajilar *et al.,* 2006). In a study from Zanzibar, selection for *pfmdr1* variants seemed to be operating mainly in re-infections that occurred during the elimination phase of Lumefantrine (Sisowath *et al.,* 2007).

5.5 CONCLUSIONS

1. There is a different malaria parasite single nucleotide polymorphism profile in western Kenya compared to the recently published 30 SNPs found in SEA. Kenya has a different geographical landscape, different malaria ecologies, varying malaria endemicity and different parasite population structures as compared to SEA. This may explain the difference in SNPs detected and possible different makers may be required in future for resistance surveillance.
2. We have also shown low prevalence of the SNP after artemisinin introduction as compared to periods before artemisinin introduction indicating a possible selective sweep after artemisinin introduction.
3. There is a significant increase in *Pfmdr1* gene copy number after ACT introduction which gives an alarm for continued monitoring of artemisinin resistance and calls for more studies to be carried to understand the role played by this gene in determining ACT resistance.
4. Subjects had different clearance slope- half life after artemisinin treatment but none presented with recurrence. This indicates that the western Kenya P. falciparum isolates are still sensitive to artemether lumefantrine.

5.6 RECOMMENDATIONS

1. There is need for more clinical trials to validate molecular makers associated with delayed clearance rates in SEA.
2. Continued close monitoring of ACT efficacy is recommended due to its direct application in drug policy implementation.
3. There is need for continued surveillance of the molecular makers underlying resistant so that we come up with cost effective tools to screen resistance in Africa.
4. The role played by increased copy number of *Pfmdr1* gene in moderating ACT resistance need to be further studied.

REFERENCES

Amin AA., Zurovac D., Kangwana BB., Greenfield J., Otieno DN., Akhwale WS & Snow RW. (2007) The challenges of changing national malaria drug policy to artemisinin-based combinations in Kenya. *Malar J **6,**72.*

Anderson TJ., Haubold B., Williams JT., Estrada-Franco JG., Richardson L., Mollinedo R., Bockarie M., Mokili J., Mharakurwa S., French N., Whitworth J., Velez ID., Brockman AH., Nosten F., Ferreira MU & Day KP. (2000) Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol Biol Evol **17,**1467-1482*.

Anderson TJ., Nair S., Nkhoma S., Williams JT., Imwong M., Yi P., Socheat D., Das D., Chotivanich K., Day NP., White NJ & Dondorp AM. (2010) High heritability of malaria parasite clearance rate indicates a genetic basis for artemisinin resistance in western Cambodia. *J Infect Dis **201,**1326-1330.*

Ariey F., Witkowski B., Amaratunga C., Beghain J., Langlois AC., Khim N., Kim S., Duru V., Bouchier C., Ma L., Lim P., Leang R., Duong S., Sreng S., Suon S., Chuor CM., Bout DM., Menard S., Rogers WO., Genton B., Fandeur T., Miotto O., Ringwald P., Le Bras J., Berry A., Barale JC., Fairhurst RM., Benoit-Vical F., Mercereau-Puijalon O & Menard D. (2014) A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature **505,**50-55.*

Arnot DE., Roper C & Bayoumi RA. (1993) Digital codes from hypervariable tandemly repeated DNA sequences in the *Plasmodium falciparum* circuMSPorozoite gene can genetically barcode isolates. *Mol Biochem Parasitol **61,**15-24*.

Bacon DJ., Latour C., Lucas C., Colina O., Ringwald P & Picot S. (2007) Comparison of a SYBR green I-based assay with a histidine-rich protein II enzyme-

linked immunosorbent assay for in vitro antimalarial drug efficacy testing and application to clinical isolates. Antimicrob Agents Chemother **51,**1172-1178.

Barnes DA., Foote SJ., Galatis D., Kemp DJ & Cowman AF. (1992) Selection for high-level chloroquine resistance results in deamplification of the *pfmdr1* gene and increased sensitivity to mefloquine in *Plasmodium falciparum*. *EMBO J* ***11,****3067-3075.*

Bell AS & Ranford-Cartwright LC. (2002) Real-time quantitative PCR in parasitology. Trends Parasitol **18,** 337-342.

Borre MB., Dziegiel M., Hogh B., Petersen E., Rieneck K., Riley E., Meis JF., Aikawa M., Nakamura K & Harada M. (1991) Primary structure and localization of a conserved immunogenic *Plasmodium falciparum* glutamate rich protein (GLURP) expressed in both the preerythrocytic and erythrocytic stages of the vertebrate life cycle. Mol Biochem Parasitol **49,**119-131.

Cheeseman IH., Miller BA., Nair S., Nkhoma S., Tan A., Tan JC., Al Saai S., Phyo AP., Moo CL., Lwin KM., McGready R., Ashley E., Imwong M., Stepniewska K., Yi P., Dondorp AM., Mayxay M., Newton PN., White NJ., Nosten F.., Ferdig MT & Anderson TJ. (2012) A major genome region underlying artemisinin resistance in malaria *Science* ***336,*** *79-82.*

Conway DJ., Machado RL., Singh B., Dessert P., Mikes ZS., Povoa MM., Oduola AM & Roper C. (2001) Extreme geographical fixation of variation in the *Plasmodium falciparum* gamete surface protein gene Pfs48/45 compared with microsatellite loci. *Mol Biochem Parasitol* ***115,****145-156.*

Cowman AF., Morry MJ., Biggs BA., Cross GA & Foote SJ. (1988) Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* ***85,****9109-9113.*

Cui L & Su XZ. (2009) Discovery, mechanisms of action and combination therapy of artemisinin. *Expert Rev Anti Infect Ther* ***7,****999-1013.*

Duah, N. O. (2013). Increased pfmdr1 gene copy number and the decline in pfcrt and pfmdr1 resistance alleles in Ghanaian Plasmodium falciparum isolates after the change of anti-malarial drug treatment policy. Malar J 12, 377

Duraisingh MT., Roper C., Walliker D & Warhurst DC. (2000) Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the *pfmdr1* gene of *Plasmodium falciparum*. *Mol Microbiol* ***36,****955-961.*

Eastman RT & Fidock DA. (2009) Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. *Nat Rev Microbiol* ***7,****864-874.*

Escalante AA., Lal AA & Ayala FJ. (1998) Genetic polymorphism and natural selection in the malaria parasite *Plasmodium falciparum*. Genetics **149,**189-202.

Eyase FL., Akala HM., Ingasia L., Cheruiyot A., Omondi A., Okudo C., Juma D., Yeda R., Andagalu B., Wanja E., Kamau E., Schnabel D., Bulimo W., Waters NC., Walsh DS & Johnson JD. (2013) The role of *Pfmdr1* and Pfcrt in changing chloroquine, amodiaquine, mefloquine and lumefantrine susceptibility in western-Kenya P. falciparum samples during 2008-2011. *PLoS One* ***8:****e64299.*

Ferreira MU., da Silva Nunes M & Wunderlich G. (2004) Antigenic diversity and immune evasion by malaria parasites. *Clinical and diagnostic laboratory immunology* ***11,****987-995.*

Fidock DA., Nguyen TV., Ribeiro JM., Valenzuela JG & James AA. (2000) *Plasmodium falciparum*, generation of a cDNA library enriched in sporozoite-specific transcripts by directional tag subtractive hybridization. *Exp Parasitol* ***95,****220-225.*

Fidock DA., Nomura T., Talley AK., Cooper RA., Dzekunov SM., Ferdig MT., Ursos LM., Sidhu AB., Naude B., Deitsch KW., Su XZ., Wootton JC., Roepe PD &

Wellems TE. (2000) Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell **6,**861-871.*

Flegg JA., Guerin PJ., White NJ & Stepniewska K. (2011) Standardizing the measurement of parasite clearance in falciparum malaria: the parasite clearance estimator. *Malar J **10,**339.*

Foote SJ & Kemp DJ. (1989) Chromosomes of malaria parasites. *Trends Genet **5,**337-342.*

Foote SJ., Kyle DE., Martin RK., Oduola AM., Forsyth K., Kemp DJ & Cowman AF. (1990) Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature **345,**255-258.*

Gabriel S., Ziaugra L & Tabbaa D.(2009) SNP genotyping using the Sequenom MassARRAY iPLEX platform. *Curr Protoc Hum Genet Chapter 2:Unit 2 12.*

Gabriel S & Ziaugra L. (2004) SNP genotyping using Sequenom MassARRAY 7K platform. *Curr Protoc Hum Genet Chapter 2:Unit 2 12.*

Gadalla NB., Adam I., Elzaki SE., Bashir S., Mukhtar I., Oguike M., Gadalla A., Mansour F., Warhurst D., El-Sayed BB & Sutherland CJ. (2011) Increased *pfmdr1* copy number and sequence polymorphisms in *Plasmodium falciparum* isolates from Sudanese malaria patients treated with artemether-lumefantrine. *Antimicrob Agents Chemother **55,**5408-5411.*

**Gardner MJ., Hall N., Fung E., White O., Berriman M., Hyman RW., Carlton JM., Pain A., Nelson KE., Bowman S., Paulsen IT., James K., Eisen JA., Rutherford K., Salzberg SL., Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J., Selengut J., Haft D., Mather MW., Vaidya AB., Martin DM., Fairlamb AH., Fraunholz MJ., Roos DS., Ralph SA.,

McFadden GI., Cummings LM., Subramanian GM., Mungall C., Venter JC., Carucci DJ., Hoffman SL., Newbold C., Davis RW., Fraser CM & Barrell B. (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum. Nature **419,**498-511.*

Ginsburg H. (1994) Transport pathways in the malaria-infected erythrocyte. Their characterization and their use as potential targets for chemotherapy. *Biochem Pharmacol **48,**1847-1856.*

Ginzinger DG. (2002) Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Experimental hematology **30,**503-512.*

Hartl DL., Volkman SK., Nielsen KM., Barry AE., Day KP., Wirth DF & Winzeler EA. (2002) The paradoxical population genetics of *Plasmodium falciparum. Trends Parasitol **18,**266-272.*

Jelinek T., Kilian AH., Curtis J., Duraisingh MT., Kabagambe G., von Sonnenburg F & Warhurst DC.(1999) *Plasmodium falciparum*: selection of serine 108 of dihydrofolate reductase during treatment of uncomplicated malaria with co-trimoxazole in Ugandan children. *Am J Trop Med Hyg **61,**125-130.*

Johnson JD., Dennull RA., Gerena L., Lopez-Sanchez M., Roncal NE & Waters NC. (2007) Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. *Antimicrob Agents Chemother **51,**1926-1933.*

Kemp DJ. (1992) Antigenic diversity and variation in blood stages of *Plasmodium falciparum. Immunol Cell Biol **70 (Pt 3),**201-207.*

Kimura E., Mattei D., di Santi SM & Scherf A. (1990) Genetic diversity in the major merozoite surface antigen of *Plasmodium falciparum* high prevalence of a third polymorphic form detected in strains derived from malaria patients. *Gene **91,**57-62.*

Kooij TW., Janse CJ & Waters AP. (2006) Plasmodium post-genomics, better the bug you know? *Nat Rev Microbiol **4,**344-357.*

Liljander A., Wiklund L., Falk N., Kweku M., Martensson A., Felger I & Farnert A. (2009).Optimization and validation of multi-coloured capillary electrophoresis for genotyping of *Plasmodium falciparum* merozoite surface proteins (MSP1 and 2). Malar J **8,**78.

Lim AS & Cowman AF (1996) *Plasmodium falciparum*: chloroquine selection of a cloned line and DNA rearrangements. *Exp Parasitol **83,**283-294.*

Marshall VM., Zhang L., Anders RF & Coppel RL. (1996) Diversity of the vaccine candidate AMA-1 of *Plasmodium falciparum*. *Mol Biochem Parasitol **77,**109-113.*

Miller LH & Su X. (2011) Artemisinin: discovery from the Chinese herbal garden. *Cell **146,**855-858.*

Monis PT., Andrews RH & Saint CP. (2002) Molecular biology techniques in parasite ecology. *International journal for parasitology **32,**551-562.*

Nayyar GM., Breman JG., Newton PN & Herrington J. (2012) Poor-quality antimalarial drugs in southeast Asia and sub-Saharan Africa. *Lancet Infect Dis **12,**488-496.*

Nkhoma SC., Nair S., Al-Saai S., Ashley E., McGready R., Phyo AP., Nosten F & Anderson TJ. (2009) Population genetic correlates of declining transmission in a human pathogen. *Mol Ecol **22,**273-285.*

Phyo AP., Nkhoma S., Stepniewska K., Ashley EA., Nair S., McGready R., ler Moo C., Al-Saai S., Dondorp AM., Lwin KM., Singhasivanon P., Day NP., White NJ., Anderson TJ& Nosten F. (2012) Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. *Lancet **379,**1960-1966.*

Price RN., Uhlemann AC., Brockman A., McGready R., Ashley E., Phaipun L., Patel R., Laing K., Looareesuwan S., White NJ., Nosten F & Krishna S. (2004) Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet **364,**438-447.*

Price RN., Uhlemann AC., van Vugt M., Brockman A., Hutagalung R., Nair S., Nash D., Singhasivanon P., Anderson TJ., Krishna S., White NJ & Nosten F. (2006) Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. *Clin Infect Dis **42,**1570-1577.*

Rason MA., Randriantsoa T., Andrianantenaina H., Ratsimbasoa A & Menard D. (2008) Performance and reliability of the SYBR Green I based assay for the routine monitoring of susceptibility of *Plasmodium falciparum* clinical isolates. *Trans R Soc Trop Med Hyg **102,**346-351.*

Rathod PK., McErlean T & Lee PC. (1997) Variations in frequencies of drug resistance in *Plasmodium falciparum*. *Proc Natl Acad Sci U S A **94,**9389-9393.*

Reed MB., Saliba KJ., Caruana SR., Kirk K & Cowman AF.(2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature **403,**906-909.*

Rosenberg R., Andre RG., Ngampatom S., Hatz C & Burge R. (1990) A stable, oligosymptomatic malaria focus in Thailand. *Trans R Soc Trop Med Hyg **84,**14-21.*

Stepniewska K., Ashley E., Lee SJ., Anstey N., Barnes KI., Binh TQ., D'Alessandro U., Day NP., de Vries PJ., Dorsey G., Guthmann JP., Mayxay M., Newton PN., Olliaro P., Osorio L., Price RN., Rowland M., Smithuis F., Taylor WR., Nosten F & White NJ. (2010) In vivo parasitological measures of artemisinin susceptibility. *J Infect Dis **201,**570-579.*

Su S., Yang S., Ding R & Davidson EA. (1996) Primary structure of the variable region of monoclonal antibody 2B10, capable of inducing anti-idiotypic antibodies that recognize the C-terminal region of MSA-1 of *Plasmodium falciparum*. *Infect Immun **64,**326-331.*

Tang K,, Fu DJ., Julien D,, Braun A., Cantor CR & Koster H. (1999) Chip-based genotyping by mass spectrometry. *Proc Natl Acad Sci U S A **96,**10016-10020.*

Trager W & Jensen JB. (1976) Human malaria parasites in continuous culture. *Science **193,**673-675.*

Tyner SD., Lon C., Se Y., Bethell D., Socheat D., Noedl H., Sea D., Satimai W., Schaecher K., Rutvisuttinunt W., Fukuda MM., Chaorattanakawee S., Yingyuen K., Sundrakes S., Chaichana P., Saingam P., Buathong N., Sriwichai S., Chann S., Timmermans A., Saunders DL & Walsh DS. (2012) Ex vivo drug sensitivity profiles of *Plasmodium falciparum* field isolates from Cambodia and Thailand, 2005 to 2010, determined by a histidine-rich protein-2 assay. *Malar J **11,**198.*

Volkman SK., Barry AE., Lyons EJ., Nielsen KM., Thomas SM., Choi M., Thakore SS., Day KP., Wirth DF & Hartl DL. (2001) Recent origin of *Plasmodium falciparum* from a single progenitor. *Science **293,**482-484.*

Wang P., Sims PF & Hyde JE. (1997) A modified in vitro sulfadoxine susceptibility assay for *Plasmodium falciparum* suitable for investigating Fansidar resistance. *Parasitology **115 (Pt 3),**223-230.*

Wilson CM., Serrano AE., Wasley A., Bogenschutz MP., Shankar AH & Wirth DF. (1989) Amplification of a gene related to mammalian mdr genes in drug-resistant *Plasmodium falciparum*. *Science **244,**1184-1186.*

Witkowski B., Iriart X., Soh PN., Menard S., Alvarez M., Naneix-Laroche V., Marchou B., Magnaval JF., Benoit-Vical F & Berry A. (2010) *pfmdr1* amplification

associated with clinical resistance to mefloquine in West Africa: implications for efficacy of artemisinin combination therapies. *J Clin Microbiol **48,**3797-3799.*

Wong ML & Medrano JF. (2005) Real-time PCR for mRNA quantitation. *BioTechniques **39,**75-85.*

World Health Organization. (2010) Global report on antimalarial efficacy and drug resistance: 2000–2010. *World Health Organization, Geneva,Switzerland.*

APPENDICES

Appendix 1: *Pfmdr1* Copy number distribution for isolates from archives (1995-1999) Kisumu.

Sample Name	Cт SD	2-^^Ct	Sample Name	Cт SD	2-^^Ct	Sample Name	Cт SD	2-^^Ct	2-^^Ct Average
3D7	0.33	1.00	**3D7**	0.43	1.00	**3D7**	0.46	1.00	**1.00**
DD2	0.37	3.54	**DD2**	0.37	2.80	**DD2**	0.28	2.43	**2.92**
KS176	0.76	1.19	**KS176**	0.07	1.03	**KS 176**	0.12	1.14	**1.12**
KS144	0.48	1.13	**KS144**	0.34	0.89	**KS144**	0.37	1.19	**1.07**
KS084	1.03	1.23	**KS084**	1.03	1.23	**KS 084**	0.47	1.21	**1.22**
KS041	0.26	0.96	**KS041**	0.40	1.03	**KS 041**	0.62	1.12	**1.04**
KS017	0.21	0.91	**KS017**	0.48	0.95	**KS 017**	1.02	1.17	**1.01**
KS168	1.18	1.03	**KS168**	0.27	1.21	**KS 168**	0.38	1.04	**1.09**
KS012	0.33	0.77	**KS012**	0.65	1.09	**KS 012**	0.59	1.19	**1.02**
KS140	0.32	1.60	**KS140**	0.33	1.19	**KS 140**	0.38	0.87	**1.22**
KK004	0.25	0.97	**KK004**	0.25	0.97	**KK 004**	0.25	0.97	**0.97**
KS156	0.24	1.36	**KS156**	0.30	1.36	**KS 156**	0.47	1.08	**1.27**
KS157	1.08	1.57	**KS157**	0.32	0.95	**KS 157**	0.65	0.90	**1.14**
KS164	0.97	1.39	**KS164**	0.97	1.39	**KS 164**	0.97	1.39	**1.39**
KS031	0.54	1.16	**KS031**	0.89	0.88	**KS 031**	0.11	1.55	**1.20**
KS260	0.55	1.06	**KS260**	0.36	1.04	**KS 260**	0.11	1.11	**1.07**
KS004	0.27	2.42	**KS004**	0.17	1.57	**KS 004**	0.33	1.37	**1.78**
KS100	0.51	0.97	**KS 100**	0.51	0.97	**KS 100**	0.51	0.97	**0.97**
KS405	0.27	1.28	**KS405**	0.59	0.89	**KS 405**	0.27	1.28	**1.15**
KS196	0.55	1.09	**KS196**	0.31	0.85	**KS 196**	0.55	1.09	**1.01**
KS414	1.28	1.59	**KS414**	1.43	1.06	**KS 414**	1.29	1.09	**1.25**
KS448	0.22	1.21	**KS448**	0.28	1.02	**KS 448**	0.22	1.21	**1.14**
KS504	0.78	0.89	**KS504**	0.34	0.68	**KS 504**	0.75	0.81	**0.79**
KS105	0.44	1.46	**KS105**	0.63	0.99	**KS 105**	1.05	0.82	**1.09**
KS5	0.57	0.67	**KS5**	0.56	0.52	**KS5**	0.59	0.78	**0.66**
KS585	0.24	0.93	**KS585**	0.60	0.79	**KS 585**	0.44	0.76	**0.82**
KB	0.33	0.81	**KB**	0.29	0.69	**KB**	0.70	0.61	**0.70**
M24	0.63	1.11	**M24**	0.59	1.10	**M24**	0.66	0.77	**0.99**

TK05	0.29	1.10	**TK05**	0.26	0.95	**TK 05**	0.37	0.77	**0.94**
KS47	0.47	1.02	**KF47**	0.67	0.74	**KF 47**	0.23	0.91	**0.89**
KS17	0.33	0.80	**KS170**	0.33	0.80	**KS 170**	0.12	0.97	**0.86**
SOMKC	0.54	0.78							**0.78**

Appendix 2: Pfmdr1 copy number distribution for isolates from archives (2000-2003)

Sample Id	C_T SD	2-^^Ct	Repeat 1	C_T SD	2-^^Ct	Repeat 2	C_T SD	2-^^Ct	2-^^Ct Average
3D7.	0.46	1.00	**3D7**	0.06	1.00	**3D7.**	0.09	1.00	**1.00**
DD2.	0.17	2.79	**DD2.**	0.39	2.50	**DD2.**	0.37	3.16	**2.82**
NNY011	0.23	1.23	**NNY011**	0.14	1.30	**NNY011**	0.67	2.09	**1.54**
NNY002	0.24	1.73	**NNY002**	0.40	1.40	**NNY 002**	0.28	1.66	**1.60**
NNY010	0.29	1.34	**NNY010**	0.12	1.40	**NNY 010**	0.40	1.38	**1.37**
NNY09/002	0.29	1.43	**NNY09/002**	0.31	2.03	**NNY 09/02**	0.83	1.71	**1.73**
NNY015	0.37	1.18	**NNY015**	0.34	1.39	**NNY 015**	0.37	1.90	**1.49**
NNY08/11	0.24	1.33	**NNY08/11**	0.09	1.37	**NNY 08/011**	0.37	1.27	**1.33**
NNY05	0.10	1.47	**NNY05**	0.24	1.44	**NNY 05**	0.25	1.13	**1.34**
NNY003	0.06	1.37	**NNY003**	1.21	1.65	**NNY 003**	0.69	1.42	**1.48**
KS 608	0.19	1.82	**KS608**	0.23	1.35	**KS608**	3.47	0.21	**1.13**
ALH001	0.23	1.49	**NNY001**	0.36	1.72	**NNY 001**	0.58	1.37	**1.53**
ALH007	0.09	1.44	**ALH007**	0.41	1.36	**ALH 007**	0.45	1.47	**1.43**
ALH004	0.06	1.37	**ALH004**	0.75	1.56	**ALH 004**	1.49	0.50	**1.14**
KS599	0.29	1.72	**KS599**	0.27	1.56	**KS599**	0.60	1.67	**1.65**
ALH22	0.21	1.31	**ALH22**	0.38	1.22	**ALH 022**	0.42	1.86	**1.46**
KS011	0.43	1.27	**KS011**	0.13	1.17	**KS011**	2.30	1.28	**1.24**
KS603	0.21	1.38	**KS603**	0.21	1.26	**KS 603**	0.44	1.34	**1.33**
NNY008	0.40	1.71	**NNY008**	0.30	1.27	**NNY 008**	0.99	2.12	**1.70**
NNY009	1.66	1.39	**NNY009**	0.46	2.06	**NNY 009**	0.14	1.71	**1.72**
NNY012	0.64	1.05	**NNY012**	0.73	1.19	**NNY012**	0.42	1.23	**1.16**
ALH008			**ALH008**			**ALH 008**	0.42	1.08	**1.08**
MDH026	0.22	1.57	**MDH026**	0.46	1.15	**MDH 026**	0.45	1.03	**1.25**
MDH033	0.32	1.24	**MDH033**	0.64	1.50	**MDH 033**	0.94	1.29	**1.34**
MDH038	0.42	1.17	**MDH038**	0.34	1.66	**MDH 038**	0.60	1.20	**1.34**

MDH005	0.36	1.74	**MDH005**	0.34	1.32	**MDH 005**	0.66	0.90	**1.32**
MDH014	0.40	1.19	**MDH014**	1.37	1.29	**MDH 014**	0.06	0.84	**1.11**
MDH017	0.27	1.36	**MDH017**	0.39	1.16	**MDH 017**	0.93	1.18	**1.23**
MDH001	0.22	0.84	**MDH001**	0.24	1.25	**MDH 001**	0.24	1.17	**1.09**
ENT 29	0.60	1.08	**ENT 29**	0.43	1.17	**ENT 29**	0.43	1.07	**1.10**
ENT 10	0.49	1.23	**ENT10**	0.18	1.16	**ENT10**	0.18	0.99	**1.12**
NNY 18	0.20	1.26	**NNY18**	0.23	1.00	**NNY18**	0.23	1.19	**1.15**
KER 3132	0.68	1.12	**KER3132**	0.18	1.15	**KER3132**	0.18	1.07	**1.11**
KER3798	0.30	1.07	**KER3798**	0.20	1.32	**KER3798**	0.20	1.08	**1.16**
KS189	0.38	1.22	**KS189**	0.38	1.12	**KS189**	0.38	1.35	**1.23**
MAT 030	0.79	1.02	**MAT030**	0.21	1.19	**MAT030**	0.21	1.20	**1.14**
KS 207	0.79	1.36	**KS207**	0.17	1.04	**KS207**	0.17	1.00	**1.13**
MDH 005	0.54	0.88	**MDH005**	0.23	1.14	**MDH005**	0.23	1.26	**1.09**
MDH 004	0.28	1.11	**MDH004**	0.29	1.17	**MDH004**	0.29	0.99	**1.09**
MDH 021	0.50	1.40	**MDH021**	0.28	1.09	**MDH021**	0.28	1.11	**1.20**
KER3255	0.46	1.21	**KER3255**	0.26	0.89	**KER3255**	0.26	1.10	**1.07**
MAT112	1.20	0.85	**MAT112**	0.14	1.16	**MAT112**			**1.00**
MAL 021			**MAL021**	0.20	0.99	**MAL021**	0.20	1.11	**1.05**

Sample Id	**C_T SD**	**2-^^Ct**	**Repeat 1**	**C_T SD**	**2-^^Ct**	**Repeat 2**	**C_T SD**	**2-^^Ct**	**2-^^Ct Average**
3D7.	0.46	1.00	**3D7**	0.06	1.00	**3D7.**	0.09	1.00	**1.00**
DD2.	0.17	2.79	**DD2.**	0.39	2.50	**DD2.**	0.37	3.16	**2.82**
NNY011	0.23	1.23	**NNY011**	0.14	1.30	**NNY011**	0.67	2.09	**1.54**
NNY002	0.24	1.73	**NNY002**	0.40	1.40	**NNY 002**	0.28	1.66	**1.60**
NNY010	0.29	1.34	**NNY010**	0.12	1.40	**NNY 010**	0.40	1.38	**1.37**
NNY09/002	0.29	1.43	**NNY09/002**	0.31	2.03	**NNY 09/02**	0.83	1.71	**1.73**
NNY015	0.37	1.18	**NNY015**	0.34	1.39	**NNY 015**	0.37	1.90	**1.49**
NNY08/11	0.24	1.33	**NNY08/11**	0.09	1.37	**NNY 08/011**	0.37	1.27	**1.33**
NNY05	0.10	1.47	**NNY05**	0.24	1.44	**NNY 05**	0.25	1.13	**1.34**
NNY003	0.06	1.37	**NNY003**	1.21	1.65	**NNY 003**	0.69	1.42	**1.48**
KS 608	0.19	1.82	**KS608**	0.23	1.35	**KS608**	3.47	0.21	**1.13**
ALH001	0.23	1.49	**NNY001**	0.36	1.72	**NNY 001**	0.58	1.37	**1.53**
ALH007	0.09	1.44	**ALH007**	0.41	1.36	**ALH 007**	0.45	1.47	**1.43**
ALH004	0.06	1.37	**ALH004**	0.75	1.56	**ALH 004**	1.49	0.50	**1.14**
KS599	0.29	1.72	**KS599**	0.27	1.56	**KS599**	0.60	1.67	**1.65**

ALH22	0.21	1.31	**ALH22**	0.38	1.22	**ALH 022**	0.42	1.86	**1.46**
KS011	0.43	1.27	**KS011**	0.13	1.17	**KS011**	2.30	1.28	**1.24**
KS603	0.21	1.38	**KS603**	0.21	1.26	**KS 603**	0.44	1.34	**1.33**
NNY008	0.40	1.71	**NNY008**	0.30	1.27	**NNY 008**	0.99	2.12	**1.70**
NNY009	1.66	1.39	**NNY009**	0.46	2.06	**NNY 009**	0.14	1.71	**1.72**
NNY012	0.64	1.05	**NNY012**	0.73	1.19	**NNY012**	0.42	1.23	**1.16**
ALH008			**ALH008**			**ALH 008**	0.42	1.08	**1.08**
MDH026	0.22	1.57	**MDH026**	0.46	1.15	**MDH 026**	0.45	1.03	**1.25**
MDH033	0.32	1.24	**MDH033**	0.64	1.50	**MDH 033**	0.94	1.29	**1.34**
MDH038	0.42	1.17	**MDH038**	0.34	1.66	**MDH 038**	0.60	1.20	**1.34**
MDH005	0.36	1.74	**MDH005**	0.34	1.32	**MDH 005**	0.66	0.90	**1.32**
MDH014	0.40	1.19	**MDH014**	1.37	1.29	**MDH 014**	0.06	0.84	**1.11**
MDH017	0.27	1.36	**MDH017**	0.39	1.16	**MDH 017**	0.93	1.18	**1.23**
MDH001	0.22	0.84	**MDH001**	0.24	1.25	**MDH 001**	0.24	1.17	**1.09**
ENT 29	0.60	1.08	**ENT 29**	0.43	1.17	**ENT 29**	0.43	1.07	**1.10**
ENT 10	0.49	1.23	**ENT10**	0.18	1.16	**ENT10**	0.18	0.99	**1.12**
NNY 18	0.20	1.26	**NNY18**	0.23	1.00	**NNY18**	0.23	1.19	**1.15**
KER 3132	0.68	1.12	**KER3132**	0.18	1.15	**KER3132**	0.18	1.07	**1.11**
KER3798	0.30	1.07	**KER3798**	0.20	1.32	**KER3798**	0.20	1.08	**1.16**
KS189	0.38	1.22	**KS189**	0.38	1.12	**KS189**	0.38	1.35	**1.23**
MAT 030	0.79	1.02	**MAT030**	0.21	1.19	**MAT030**	0.21	1.20	**1.14**
KS 207	0.79	1.36	**KS207**	0.17	1.04	**KS207**	0.17	1.00	**1.13**
MDH 005	0.54	0.88	**MDH005**	0.23	1.14	**MDH005**	0.23	1.26	**1.09**
MDH 004	0.28	1.11	**MDH004**	0.29	1.17	**MDH004**	0.29	0.99	**1.09**
MDH 021	0.50	1.40	**MDH021**	0.28	1.09	**MDH021**	0.28	1.11	**1.20**
KER3255	0.46	1.21	**KER3255**	0.26	0.89	**KER3255**	0.26	1.10	**1.07**
MAT112	1.20	0.85	**MAT112**	0.14	1.16	**MAT112**			**1.00**
MAL 021			**MAL021**	0.20	0.99	**MAL021**	0.20	1.11	**1.05**

Appendix 3: *Pfmdr1* copy number distribution in isolates from efficacy clinical trial 2013-2014

Sample ID	CT SD	2-^^Ct	Sample ID	CT SD	2-^^Ct RPT1	Sample ID	CT SD	2-^^Ct RPT2	2-^^Ct Average
3D7	0.33	1.00	**3D7**	0.12	1.00	**3D7**	0.36	1.00	**1.00**
DD2	0.16	2.65	**DD2**	0.49	2.95	**DD2**	0.24	2.48	**2.69**
M69D0	0.23	1.87	**M069D0**	0.13	1.22	**M069D0**	0.21	1.02	**1.37**
M070D0	0.38	1.76	**M070D0**	0.08	1.56	**M070D0**	0.06	1.30	**1.54**
M072D0	0.51	1.97	**M072D0**	0.51	1.38	**M072D0**	0.26	1.25	**1.54**
M073D0	0.43	2.02	**M073D0**	0.90	1.54	**M073D0**	0.20	1.92	**1.83**
M074D0	0.11	1.99	**M074D0**	0.10	1.35	**M074D0**	0.17	1.01	**1.45**
M076D0	0.51	2.20	**M076D0**	0.18	1.55	**M076D0**	0.04	1.33	**1.69**
M077D0			**M077D0**	0.20	1.58	**M077D0**	0.43	1.37	**1.48**
M079D0	0.72	1.78	**M079D0**	0.65	1.54	**M079D0**	0.59	1.47	**1.60**
M081D0	0.12	1.64	**M081D0**	0.42	1.44	**M081D0**	0.22	1.25	**1.44**
M082D0			**M137D0**	0.88	1.77	**M137D0**	0.29	1.56	**1.66**
M087D0	0.98	1.35	**M128D0**	0.16	1.62	**M128D0**	0.44	1.31	**1.42**
M088D0			**M088D0**	0.79	1.30	**M088D0**	0.26	1.45	**1.37**
M089D0	0.47	1.72	**M089D0**	0.02	1.53	**M089D0**	0.15	1.26	**1.39**
M093D0	0.67	2.42	**M093D0**	0.09	1.63	**M093D0**	0.04	1.67	**1.91**
M094D0	0.43	1.58	**M094D0**	0.21	1.18	**M094D0**	0.53	1.07	**1.27**
M095D0	0.78	2.66	**M095D0**	0.62	1.55	**M095D0**	0.26	1.58	**1.93**
M096D0	0.20	1.55	**M096D0**	0.23	1.68	**M096D0**	0.17	1.59	**1.60**
M099D0	0.54	2.51	**M099D0**	0.30	1.40	**M099D0**	0.23	1.46	**1.79**
M101D0	0.85	1.99	**M101D0**	0.22	1.74	**M101D0**	0.08	1.55	**1.76**
M107D0	0.24	1.59	**M107D0**	0.64	1.38	**M107D0**	0.09	1.47	**1.48**
M108D0			**M108D0**	0.27	1.16	**M108D0**	0.26	1.64	**1.40**
M109D0	0.72	2.31	**M109D0**	0.10	1.49	**M109D0**	0.07	1.53	**1.77**
M127D0			**M133D0**	0.24	1.05	**M133D0**	0.36	1.05	**1.05**
M111D0	0.61	1.20	**M111D0**	0.44	1.17	**M111D0**	0.21	1.06	**1.14**
M115D0	0.60	1.48	**M115D0**	0.19	1.05	**M115D0**	0.12	1.12	**1.21**
M120D0	0.81	1.26	**M120D0**	0.33	1.64	**M120D0**	0.21	1.33	**1.41**
M124D0	0.27	1.69	**M124D0**	0.30	1.44	**M124D0**	0.36	1.27	**1.46**
M125D0	0.32	2.01	**M125D0**	0.55	1.51	**M125D0**	0.20	1.23	**1.58**
M127.D0	0.24	3.36	**M127D0**	0.05	1.46	**M127D0**	0.11	1.29	**2.03**
M002D0	0.02	1.08	**M002D0**	0.40	0.92	**M002D0**	0.66	0.78	**1.00**
M003D0	0.22	1.16	**M003D0**	0.28	1.10	**M003D0**	0.83	1.10	**1.13**
M004D0	0.46	1.27	**M004D0**	0.19	0.84	**M004D0**	1.62	2.30	**1.06**

M007D0	0.63	1.09	**M007D0**	0.28	1.05				**1.07**
M016D0	0.51	1.29	**M016D0**	0.09	1.49	**M016D0**	0.28	1.62	**1.39**
M009D0	0.20	1.33	**M009D0**	0.24	0.90	**M009D0**	0.74	1.19	**1.11**
M011D0	0.18	1.13	**M011D0**	0.25	1.01	**M011D0**	0.49	0.95	**1.07**
M014D0	0.37	1.06	**M014D0**	0.20	0.97	**M014D0**	0.24	1.04	**1.02**
M018D0	0.34	1.18	**M018D0**	0.35	0.95	**M018D0**	0.57	0.81	**1.07**
M026D0	0.28	1.11	**M026D0**	0.69	1.55	**M026D0**	0.68	0.87	**1.33**
M020D0	0.55	0.98	**M020D0**	0.82	1.00	**M020D0**	0.24	0.86	**0.99**
M023D0	0.27	1.05	**M023D0**	0.50	1.00	**M023D0**	0.66	1.00	**1.02**
M025D0	0.15	1.06	**M025D0**	0.19	0.99	**M025D0**	0.08	0.89	**1.02**
M027D0	0.36	0.96	**M027D0**	0.17	0.83	**M027D0**	1.00	1.15	**0.89**
M028D0	0.66	1.12	**M028D0**	0.15	1.02	**M028D0**	0.38	1.19	**1.07**
M036D0	0.14	1.24	**M036D0**	0.02	0.91	**M036D0**	0.19	1.47	**1.08**
M039D0	0.30	1.17	**M039D0**	0.28	0.92	**M039D0**	0.22	0.93	**1.04**
M041D0	0.12	1.22	**M041D0**	0.19	1.85	**M041D0**	0.59	1.14	**1.53**
M048D0	0.97	0.52	**M048D0**	0.42	1.03	**M048D0**	0.92	0.86	**0.78**
M050D0	0.53	1.08	**M050D0**	0.18	0.81	**M050D0**	0.62	1.33	**0.95**
M053D0	0.12	1.08	**M053D0**	0.08	0.76	**M053D0**	0.26	1.08	**0.92**
M061D0	0.67	1.05	**M061D0**	0.19	0.96	**M061D0**	0.59	0.72	**1.01**
M063D0	0.75	0.82	**M063D0**	0.74	1.53	**M063D0**	0.09	0.71	**1.17**
M064D0	0.53	1.11	**M064D0**	0.11	0.88	**M064D0**	1.17	1.10	**1.00**
M066D0	0.21	1.19	**M066D0**	0.69	1.09	**M066D0**	0.31	1.03	**1.14**
M046D0	0.25	1.60	**M046D0**	0.10	1.67	**M046D0**	0.56	1.62	**1.63**
M044D0	0.17	1.14	**M044D0**			**M044D0**	0.09	1.19	**1.14**
M068D0	0.39	1.25	**M068D0**			**M068D0**	0.40	1.04	**1.25**
M141D0	0.15	1.16	**M141D0**	0.75	1.57	**M141D0**	1.35	1.15	**1.36**
M143D0	0.19	1.02	**M143D0**	0.31	1.32	**M143D0**	0.42	0.96	**1.17**
M182D0	0.19	1.09	**M182D0**	0.26	1.42	**M182D0**	0.33	1.03	**1.25**
M184D0	0.33	1.11	**M184D0**	0.20	1.33	**M184D0**	0.06	0.97	**1.22**
M185D0	0.22	0.87	**M185D0**	0.27	1.10	**M185D0**	0.33	0.80	**0.98**
M186D0	0.10	1.07	**M186D0**	0.57	1.48	**M186D0**	1.05	1.07	**1.27**
M191D0	0.18	1.06	**M191D0**	0.33	1.19	**M191D0**	0.48	0.87	**1.13**
M192D0	0.30	1.09	**M192D0**	0.30	1.39	**M192D0**	0.30	1.01	**1.24**
M199D0	0.30	1.25	**M199D0**	0.28	1.27	**M199D0**	0.27	0.92	**1.26**
M201D0	0.25	1.10	**M201D0**	0.21	1.44	**M201D0**	0.17	1.05	**1.27**
M207D0	0.19	1.15	**M207D0**	0.38	1.34	**M207D0**	0.57	0.97	**1.24**
M211D0	0.09	1.25	**M211D0**	0.19	1.49	**M211D0**	0.29	1.09	**1.37**
M212D0	0.13	1.13	**M212D0**	0.16	1.35	**M212D0**	0.20	0.99	**1.24**
M214D0	0.10	1.20	**M214D0**	0.10	1.19	**M214D0**		0.86	**1.19**

Appendix 4: Single nucleotide polymorphism results for 1995-1999 isolates from Kisumu.

Single nucleotide polymorphisms (1995-1999) *P. falciparum* isolates from Kisumu											
ID	**KS 017**	**KS 164**	**KS 448**	**KS 004**	**KS 168**	**KS 031**	**KS 504**	**KB**	**KS 585**	**KS 156**	**KS 196**
CombinedSNP_MAL13_6300	G	G	G	C	G	G	G	G	GC	G	G
CombinedSNP_MAL14_9357	T	T	T	T	T	T	T	AT	T	T	T
CombinedSNP_MAL14_8697	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_2384	G	G	G	G	G	G	G	GC	G	G	G
CombinedSNP_MAL13_6116	C	C	C	C	C	C	C	TC	C	C	C
CombinedSNP_MAL14_9360	T	T	A	T	T	T	A	T	A	AT	AT
CombinedSNP_MAL13_5862	G	G	G	G	G	G	G	GA	G	G	G
CombinedSNP_MAL13_5938	G	G	G	G	G	G	G	GA	G	G	G
CombinedSNP_MAL13_1823	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8449	T	T	T	T	T	T	T	AT	T	T	T
CombinedSNP_MAL14_8769	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_8445	T	T	T	T	T	T	T	TA	T	T	T
CombinedSNP_MAL13_6117	C	C	C	C	C	C	C	GC	C	C	C
CombinedSNP_MAL13_5817	C	C	C	C	C	C	C	TC	C	C	C
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9361	C	C	C	C	C	C	C	TC	C	C	C
CombinedSNP_MAL13_6305	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8702	T	C	T	C	C	C	T	CT	CT	CT	T
CombinedSNP_MAL13_6016	A	A	A	A	A	A	A	GA	A	A	A

CombinedSNP_MAL13_6069	T	T	T	T	T	T	T	TC	T	T	T
CombinedSNP_MAL14_8696	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_3474	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_4043	A	A	A	A	A	A	A	CA	A	A	A
CombinedSNP_MAL14_9356	C	C	C	C	C	C	C	CG	C	C	C
CombinedSNP_MAL14_8744	C	C	C	C	C	C	C	CG	C	C	C

Single nucleotide polymorphisms (1995-1999) P. falciparum isolates from Kisumu

ID	KS 260	KS 005	KF 29	KS 041	KS 105	KS 004	KS 144	KS 140	KS 100A	KS 176	KS 084
CombinedSNP_MAL13_6300	C	G	C	G	GC	C	G	C	G	G	G
CombinedSNP_MAL14_9357	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8697	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_2384	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_6116	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9360	T	AT	A	A	A	T	AT	A	AT	T	T
CombinedSNP_MAL13_5862	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_5938	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_1823	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8449	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8769	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_8445	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL13_6117	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_5817	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C	C	C	C

CombinedSNP_MAL14_9361	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_6305	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8702	T	CT	T	C	CT	C	CT	T	C	T	T
CombinedSNP_MAL13_6016	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL13_6069	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8696	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_3474	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_4043	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_9356	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8744	C	C	C	C	C	C	C	C	C	C	C

Single nucleotide polymorphisms (1995-1999) *P. falciparum* isolates

ID	KS 170	KS 012	KS 157	KS 414	KK 004	KS 405	KS 011	KF 47
CombinedSNP_MAL13_6300	C	GC	C	C	C	G	C	G
CombinedSNP_MAL14_9357	T	AT	T	T	T	T	T	AT
CombinedSNP_MAL14_8697	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_2384	G	GC	G	G	G	G	G	G
CombinedSNP_MAL13_6116	C	TC	C	C	C	C	TC	TC
CombinedSNP_MAL14_9360	T	T	A	T	T	A	T	T
CombinedSNP_MAL13_5862	A	GA	G	G	G	G	A	G
CombinedSNP_MAL13_5938	G	GA	G	G	G	G	G	G
CombinedSNP_MAL13_1823	C	C	C	C	C	C	C	C

CombinedSNP_MAL14_8449	T	AT	T	T	T	T	AT	AT
CombinedSNP_MAL14_8769	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_8445	T	TA	T	T	T	T		TA
CombinedSNP_MAL13_6117	G	GC	C	C	C	C	G	GC
CombinedSNP_MAL13_5817	T	TC	C	C	C	C		TC
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9361	T	TC	C	C	C	C	T	TC
CombinedSNP_MAL13_6305	C	C	C	C	C	C		C
CombinedSNP_MAL14_8702	T	CT	C	C	C	C	C	T
CombinedSNP_MAL13_6016	A	GA	A	A	A	A	A	GA
CombinedSNP_MAL13_6069	C	TC	T	T	T	T	C	TC
CombinedSNP_MAL14_8696	C	GC	G	G	G	G	C	G
CombinedSNP_MAL13_3474	AG	AG	G	G	G	G	AG	G
CombinedSNP_MAL13_4043	C	CA	A	A	A	A	C	CA
CombinedSNP_MAL14_9356	G	CG	C	C	C	C	G	CG
CombinedSNP_MAL14_8744	G	CG	C	C	C	C	G	CG

Appendix5: Single nucleotide polymorphism results for 2000-2003 isolates from Kisumu.

Single nucleotide polymorphism (2000-2003) *P.falciparum* isolate from Kisumu											
	KN 49	**KS 140B**	**KS 036**	**KS 599**	**KS 189**	**KS 084**	**KS 207**	**KS 096**	**KS 041**	**KS 100B**	**KS 126**
CombinedSNP_MAL13_6300	G	G	C	G	G	G	G	G	G	G	G
CombinedSNP_MAL14_9357	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8697	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_2384	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_6116	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9360	A	A	T	A	T	A	T	T	A	A	A
CombinedSNP_MAL13_5862	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_5938	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_1823	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8449	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8769	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_8445	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL13_6117	C	C	C	C	C	C	C	C	C	C	C

CombinedSNP_MAL13_5817	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9361	C	C	C	C	T	C	C	C	C	C	C
CombinedSNP_MAL13_6305	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8702	CT	T	T	C	T	T	T	C	CT	C	T
CombinedSNP_MAL13_6016	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL13_6069	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8696	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_3474	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_4043	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_9356	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8744	C	C	C	C	C	C	C	C	C	C	C

Single nucleotide polymorphism (2000-2003) *P.falciparum* isolate from Kisumu

	KS 603	**KS 011**	**KS 176**	**KS 047**	**KN 006**	KS 144C	KS 144	KS 041C
CombinedSNP_MAL13_6300		C	G	C	G	G	G	G
CombinedSNP_MAL14_9357	T		T			T	T	T
CombinedSNP_MAL14_8697	T		C		C	C	C	C
CombinedSNP_MAL13_2384		G	G	G	G			G
CombinedSNP_MAL13_6116	C	TC	C	C	TC	C	C	C
CombinedSNP_MAL14_9360		T	T	T	T	AT	T	A

CombinedSNP_MAL13_5862		A	G	A	G	G	G	G
CombinedSNP_MAL13_5938			G			G		G
CombinedSNP_MAL13_1823			C			C	C	C
CombinedSNP_MAL14_8449	A	AT	T	A		T	AT	T
CombinedSNP_MAL14_8769	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_8445			T			T	T	T
CombinedSNP_MAL13_6117	G	G	C	G	C	C	C	C
CombinedSNP_MAL13_5817	TC		C			C	TC	C
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9361	T	T	C	T	C	C	C	C
CombinedSNP_MAL13_6305	C		C	C		C	C	C
CombinedSNP_MAL14_8702	C	C	T	C	C	T	C	C
CombinedSNP_MAL13_6016	GA	A	A	A	A	A	A	A
CombinedSNP_MAL13_6069		C				T	T	T
CombinedSNP_MAL14_8696	G	C		GC	G	G	G	G
CombinedSNP_MAL13_3474	G	AG		AG	G	G	G	G
CombinedSNP_MAL13_4043	A	C				A	A	A
CombinedSNP_MAL14_9356		G			C	C	C	C
CombinedSNP_MAL14_8744		G		G	C	C	C	C

Appendix 6: Single nucleotide polymorphism results for 2005-2010 isolates from Kisumu

Single nucleotide polymorphism(2005-2010) *P.falciparum* isolates

	NN 018	NN 003	NN 005	NN 012	NN0 11A	NN 009	NN 008	NN 15	NN 002	NN 010	Wild	Mutant
CombinedSNP_MAL13_6300	C	G	G	GC	G	G	GC	G	G	G	**G**	**C**
CombinedSNP_MAL14_9357	T	T	T	T	T	T	T	T	T	T	**T**	**A**
CombinedSNP_MAL14_8697	C	C	C	C	C	C	C	C	C	C	**C**	**T**
CombinedSNP_MAL13_2384	G	G	G	G	G	G	G	G	G		**G**	**C**
CombinedSNP_MAL13_6116	C	C	C	C	C	C	C	C	C	C	**C**	**T**
CombinedSNP_MAL14_9360	T	A	AT	AT	T	AT	AT	AT	AT	T	**A**	**T**
CombinedSNP_MAL13_5862	G	G	G	G	G	G	G	G	G	G	**G**	**A**
CombinedSNP_MAL13_5938	G	G	G	G	G	G	G	G	G		**G**	**A**
CombinedSNP_MAL13_1823	C	C	C	C	C	C	C	C	C	C	**C**	**T**
CombinedSNP_MAL14_8449	T	T	T	T	T	T	T	T	T	AT	**T**	**A**
CombinedSNP_MAL14_8769	A	A	A	A	A	A	A	A	A	A	**A**	**G**
CombinedSNP_MAL14_8445	T	T	T	T	T	T	T	T	T	T	**T**	**A**
CombinedSNP_MAL13_6117	C	C	C	C	C	C	C	C	C	C	**C**	**G**
CombinedSNP_MAL13_5817	C	C	C	C	C	C	C	C	C		**C**	**T**
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C	C	C	**C**	**T**
CombinedSNP_MAL14_9361	C	C	C	C	C	C	C	C	C	C	**C**	**T**
CombinedSNP_MAL13_6305	C	C	C	C	C	C	C	C	C		**C**	**A**
CombinedSNP_MAL14_8702	T	C	CT	C	CT	CT	CT	C	CT	C	**T**	**C**

CombinedSNP_MAL13_6016	A	A	A	A	A	A	A	A	A	A	**A**	**G**
CombinedSNP_MAL13_6069	T	T	T	T	T	T	T	T	T		**T**	**C**
CombinedSNP_MAL14_8696	G	G	G	G	G	G	G	G	G	G	**G**	**C**
CombinedSNP_MAL13_3474	G	G	G	G	G	G	G	G	G	G	**G**	**A**
CombinedSNP_MAL13_4043	A	A	A	A	A	A	A	A	A		**A**	**C**
CombinedSNP_MAL14_9356	C	C	C	C	C	C	C	C	C	C	**C**	**G**
CombinedSNP_MAL14_8744	C	C	C	C	C	C	C	C	C	C	**C**	**G**

Appendix 7: Single nucleotide polymorphism results for 2013-2014 isolates from Kisumu

Single nucleotide polymorphism(2013-2014) *P.falciparum* isolates from Kombewa- Kisumu

Sample ID	M02	M36	M50	M72	M87	M101	M124	M141	M199	M8	M46
CombinedSNP_MAL13_6300	C	G	C	G	GC	G	G	G	GC	G	G
CombinedSNP_MAL14_9357	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8697	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_2384	G	G	G	G					G	G	G
CombinedSNP_MAL13_6116	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9360	T	T	A	AT	AT	A	A	T	T	A	A
CombinedSNP_MAL13_5862	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_5938	G		G	G	G		G		G		G
CombinedSNP_MAL13_1823	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8449	T	T	T	T	T	T	T	T	T	AT	T
CombinedSNP_MAL14_8769	A	A	A	A	A	A	A	A	A		A
CombinedSNP_MAL14_8445	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL13_6117	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_5817	C	C	C	C	C	C	C	C	C	TC	C
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9361	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_6305	C	C	C	C	C	C	C	C	C		C
CombinedSNP_MAL14_8702		C	C	CT	CT	CT	CT		CT	C	CT
CombinedSNP_MAL13_6016	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL13_6069	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8696	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_3474	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_4043	A	A	A	A	A	A	A	A	A	A	A

CombinedSNP_MAL14_9356	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8744	C	C	C	C	C	C	C	C	C	C	C

Single nucleotide polymorphism(2013-2014) *P.falciparum* isolates

Sample ID	M107.	M125.	M143.	M204M.	M046D21	M004.	M064.	M063.	M074.	M089.	M108.
CombinedSNP_MAL13_6300	G	G	G	G	G	G	G	G	G	G	GC
CombinedSNP_MAL14_9357	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8697	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_2384			G	G	G				G		
CombinedSNP_MAL13_6116	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9360	A	A	A	T	A	T	T	T	A	T	AT
CombinedSNP_MAL13_5862	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_5938	G	G	G	G		G	G		G	G	
CombinedSNP_MAL13_1823	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8449	T	T	T	T	AT	T	T	AT	T	T	AT
CombinedSNP_MAL14_8769	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_8445	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL13_6117	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_5817	C	C	C	C	TC	C	C	C	C	C	C
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9361	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_6305	C	C	C	C	C	CA	C	C	C	C	C
CombinedSNP_MAL14_8702	CT	T	T	CT	CT	C	C	CT	T	CT	CT
CombinedSNP_MAL13_6016	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL13_6069	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8696	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_3474	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_4043	A	A	A	A	A	A	A	A	A	A	A

CombinedSNP_MAL14_9356	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8744	C	C	C	C	C	C	C	C	C	C	C

Single nucleotide polymorphism(2013-2014) *P.falciparum* isolates

Sample ID	M48	M09	M26	M046	M068	M79	M95	M111	M137	M186F	M214M
CombinedSNP_MAL13_6300	G	GC	G	G	G	C	G	G	G	G	G
CombinedSNP_MAL14_9357	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8697	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_2384		G	G	G	G			G		G	G
CombinedSNP_MAL13_6116	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9360	A	AT	T	AT	T	T	T	A	A	T	T
CombinedSNP_MAL13_5862	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_5938	G	G		G	G	G				G	G
CombinedSNP_MAL13_1823	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8449	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8769	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_8445	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL13_6117	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_5817	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9361	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_6305	C	CA		CA	C	C	C	C	C	C	C
CombinedSNP_MAL14_8702	T	CT	CT	C	C	C	CT	C	CT		CT
CombinedSNP_MAL13_6016	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL13_6069	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8696	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_3474	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_4043	A	A	A	A	A	A	A	A	A	A	A

CombinedSNP_MAL14_9356	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8744	C	C	C	C	C	C	C	C	C	C	C

Single nucleotide polymorphism(2013-2014) *P.falciparum* isolates

Sample ID	M079	M076.	M109.	M128.	M184.	M211.	M061	M003.	M024	M077	M005
CombinedSNP_MAL13_6300	G	G	G	G	G	G	C	G	C	GC	
CombinedSNP_MAL14_9357	T	T	T	T	T	T	T	T	T		
CombinedSNP_MAL14_8697	C	C	C	C	C	C	C	C			TC
CombinedSNP_MAL13_2384		G	G		G			G			G
CombinedSNP_MAL13_6116	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9360	AT	A	A	AT	T	A	AT	A			
CombinedSNP_MAL13_5862	G	G	G	G	G	G	G	G	G		
CombinedSNP_MAL13_5938		G	G	G	G						
CombinedSNP_MAL13_1823		C	C	C	C	C	CT	C			
CombinedSNP_MAL14_8449		T	T	T	T	AT	AT	T	A	A	AT
CombinedSNP_MAL14_8769	A	A	A	A	A		A	A	A		A
CombinedSNP_MAL14_8445	T	T	T	T	T	T		T			
CombinedSNP_MAL13_6117	C	C	C	C	C	C		C			
CombinedSNP_MAL13_5817	C	C	C	C	C	TC		C	TC	T	
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9361	C	C	C	C	C	C	TC	C	TC	C	T
CombinedSNP_MAL13_6305		C	C	C	C		C	CA			C
CombinedSNP_MAL14_8702	C		CT		CT	C	C			C	C
CombinedSNP_MAL13_6016	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL13_6069	T	T	T	T	T	T	T			T	
CombinedSNP_MAL14_8696	G	G	G	G	G	G	GC		G	G	G
CombinedSNP_MAL13_3474	G	G	G	G	G	G	G		G	AG	G
CombinedSNP_MAL13_4043	A	A	A	A	A	A	A		A	A	

CombinedSNP_MAL14_9356	C	C	C	C	C	C	C		C	C		
CombinedSNP_MAL14_8744	CG	C	C	C	C	C	C					

Single nucleotide polymorphism(2013-2014) P.falciparum isolates

Sample ID	M039.	M061.	M073.	M088.	M127.	M182.	M207.	M018	M048.	M069.	**M140 .**	M081.
CombinedSNP_MAL13_6300	G	C	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL14_9357	T	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8697	C	C	C	C	C	C	C		C	C	C	C
CombinedSNP_MAL13_2384	G	G	G				G		G		G	
CombinedSNP_MAL13_6116	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9360	A	A	AT	A	A	A	T	A	A	A	AT	T
CombinedSNP_MAL13_5862	G	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_5938		G	G	G	G	G		G	G	G	G	G
CombinedSNP_MAL13_1823	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8449	T	T	T	T	T	T	T	AT	T	T	T	T
CombinedSNP_MAL14_8769	A	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_8445	T	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL13_6117	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_5817	C	C	C	C	C	C	C	TC	C	C	C	C
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9361	C	C	C	C	C	C	C	C	TC	C	C	C
CombinedSNP_MAL13_6305	C	C	C	C	C	C	C		C	C	C	C
CombinedSNP_MAL14_8702	CT		CT	C		CT	CT	C	CT	T	CT	CT
CombinedSNP_MAL13_6016	A	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL13_6069	T	T	T	T	T	T	T	T	T	T	T	T

CombinedSNP_MAL14_8696	G	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_3474	G	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_4043	A	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_9356	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8744	C	C	CG	C	C	C	C	C	C	C	C	C

Single nucleotide polymorphism(2013-2014) *P.falciparum* isolates

Sample ID	M120.	M192.	M007	M045	M011.	M115.	M138.	M191.	M002	M028.	M053.	M096.
CombinedSNP_MAL13_6300	G	G	GC	C	G	GC	G	G	G	G	C	G
CombinedSNP_MAL14_9357	T	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8697	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_2384	G	G	G	G	G	G	G	G	G	G	G	
CombinedSNP_MAL13_6116	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9360	T	T	A	A	AT	T	A	AT	A	AT	T	A
CombinedSNP_MAL13_5862	G	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_5938	G		GA		G	G		G	G	G	G	
CombinedSNP_MAL13_1823	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8449	T	T	T	T	T	T		T	T	T	T	T
CombinedSNP_MAL14_8769	A	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_8445	T	T	T	TA	T	T	T	T	T	T	T	T
CombinedSNP_MAL13_6117	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_5817	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL13_4041	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_9361	C	C	C	C	C	C	C	C	C	C	C	C

CombinedSNP_MAL13_6305	C	C	C		C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8702	T	CT	C	C		CT	C			T	C	
CombinedSNP_MAL13_6016	A	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL13_6069	T	T	T	T	T	T	T	T	T	T	T	T
CombinedSNP_MAL14_8696	G	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_3474	G	G	G	G	G	G	G	G	G	G	G	G
CombinedSNP_MAL13_4043	A	A	A	A	A	A	A	A	A	A	A	A
CombinedSNP_MAL14_9356	C	C	C	C	C	C	C	C	C	C	C	C
CombinedSNP_MAL14_8744	C	C	C	C		C	C	C	C	C	C	C

Appendix 8 ethical clearance letters

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **March 20, 2013**

TO: **DR. BEN ANDAGALU,**
PRINCIPAL INVESTIGATOR

THRO': **DR. RASHID JUMA,**
THE DIRECTOR, CCR,
NAIROBI

Forwarded 26/3/2013

Dear Sir,

RE: **SSC PROTOCOL NO. 2518 (*INITIAL SUBMISSION*): IN VIVO AND IN VITRO EFFICACY OF ARTEMISININ COMBINATION THERAPY IN KISUMU COUNTY, WESTERN KENYA.**

This is to inform you that during the 213th meeting of the KEMRI/ERC held on 19th March 2013, the above referenced study was reviewed.

The Committee notes that the above referenced study seeks to assess the degree of artemisinin resistance in subjects presenting with uncomplicated *P. Falciparum* malaria.

The Committee concluded that due consideration has been given to the ethical issues that may arise from the conduct of the study and granted approval for implementation effective **19th March 2013**.

Please note that authorization to conduct this study will automatically expire on **18 March 2014.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **05 February 2014**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study

Sincerely,

EAB

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

USA MEDICAL RESEARCH UNIT

KENYA MEDICAL RESEARCH INSTITUTE

Jomo Kenyatta Highway
Kisumu, Kenya

P.O. Box 54, 40100 Kisumu, Kenya Tel: +254 731 925 682 +254 706 131 186 E-mail: kisumu.admin@usamru-k.org

Dr. Ben Andagalu,
Principle Investigator, USAMRU-K
POB 54, Kisumu.
18TH September 2013

TO, **Secretary/CEO,**
National Commission for Science, Technology and Innovation (NACOSTI),
P.O. Box 30623-00100,
NAIROBI

Dear Sir/Madam,

RE: MSC.PROJECT SSC/ERC CLEARANCE (BIDII S. NGALAH-TM-305-0872/2012)

This is to confirm that the above named student is doing a project entitled "**Major genome region underlying artemisinin resistance in malaria parasite isolates from Kisumu county, western Kenya"**. This project is nested under the *in vivo* efficacy clinical trial which is ongoing. The ongoing study is cleared under the SSC Protocol NO.2518 and WRAIR Protocol NO.1935. Mr. Bidii Stephen Ngalah is the Principal Investigator in the nested project addressing the following objectives:

General objective

To determine the genetic polymorphism in *P. falciparum* parasite associated with artemisinin resistance in western Kenya.

Specific objectives

1. To analyze the genetic polymorphism of *P. falciparum* in chromosome 10, 13 and 14 of sample isolates collected before and after the introduction of ACTs in Kenya.
2. To determine the association of slow clearance rate with polymorphism in *P. falciparum* in chromosome 10, 13 and 14 of samples collected from an on-going in vivo efficacy clinical trial study.
3. To assess the *pfmdr1* copy number of sample isolates collected before and after the introduction of ACTS in Kenya.

Yours Sincerely,

Dr. Ben Andagalu

The Walter Reed Project

8. The PI submitted the point-by-point response and revised protocol (Version 3.3, dated 21 November 2012) to address the WRAIR IRB stipulations on 6 February 2013. The HSPB reviewed the response and determined that some supporting documentation was still pending. The KEMRI ERC approval was received on 29 March 2013 and the pending supporting documentation was received on 1 April 2013. The protocol was submitted back to the WRAIR IRB for their review on 10 April 2013.

The protocol (Version 3.3, dated 21 November 2012) was reviewed at the 10 April 2013 fully convened WRAIR IRB meeting. The WRAIR IRB voted to approve the protocol as written. **The WRAIR IRB determined that this protocol met the criteria outlined in 32 CFR 219.111, 10 USC 980, and 45 CFR 46.405, as this is a greater than minimal risk study involving children that presents the prospect of direct benefit to the individual subjects.**

9. As this is a greater than minimal risk study, this protocol was submitted to the U.S. Army Medical and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protections Office (HRPO) for headquarters level review on 13 July 2012. The USAMRMC ORP HRPO provided their pre-review comments on 31 August 2012. Since the initial submission of this protocol to the USAMRMC ORP HRPO, a new USAMRMC policy, 2013-75 has been issued. In accordance with this new policy, this protocol still requires headquarters level review by the USAMRMC ORP HRPO. The USAMRMC ORP HRPO approval is currently pending for this protocol and will need to be submitted to the HSPB prior to the issuance of the WRAIR Commander Approval Authorization.

10. The following supporting documentation should be submitted to the WRAIR HSPB prior to the issuance of the WRAIR Commander Approval Authorization: USAMRMC ORP HRPO approval.

11. The following documents are included as part of this approval recommendation:
   a. Protocol, Version 3.3 dated 21 November 2012;
   b. Recruitment Script (English, Luo, and Kiswahili), Version 2, dated 1 August 2012;
   c. Adult Consent Form (English, Luo, and Kiswahili), Version 3.2, dated 21 November 2012;
   d. Parent/Guardian Consent Form (English, Luo, and Kiswahili), Version 3.2, dated 21 November 2012;
   e. Assent Form (English, Luo, and Kiswahili), Version 3.2, dated 21 November 2012; and
   f. Translation Verification Certificates for both the Luo and Kiswahili translations of all documents outlined in 11.b-e.

12. As this is a greater than minimal risk protocol, I recommend that Volunteer Registry Data Forms be required. The WRAIR expiration date is **10 April 2014**. The PI is responsible for submitting a continuing review report to the KEMRI ERC and the WRAIR IRB in time for the report to be reviewed and accepted/approved prior to the respective

CORRECTION

MCMR-UWZ-C 29 April 2013

MEMORANDUM FOR Director, Human Subjects Protection Branch (HSPB), Walter Reed Army Institute of Research (WRAIR), 503 Robert Grant Ave., Silver Spring, MD 20910-7500

SUBJECT: Recommendation of Approval for the Greater than Minimal Risk Human Subjects Research Protocol **WRAIR #1935**, HRPO Log #A-17502, SSC# 2518

1. I recommend approval of the protocol, **WRAIR #1935**, HRPO Log #A-17502, SSC# 2518, entitled "*In vivo* and *In vitro* Efficacy of Artemisinin Combination Therapy in Kisumu County, Western Kenya," (Protocol Version 3.3, dated 21 November 2012), submitted by Ben Andagalu, M.D., MSc, Kenya Medical Research Institute (KEMRI)/Walter Reed Project (WRP).

2. This study is funded by the Department of Emerging Infectious Diseases of the United States Army Medical Research Unit. Regulatory approval is being sought from the Kenya Pharmacy and Poisons Board (PPB) for use of the study drugs in Kenyan citizens.

3. This will be a two-arm randomized open-label trial conducted in Kisumu County, Western Kenya at the Kisumu West District Hospital, the Kisumu East District Hospital, and the Obama Children's Hospital at the Nyanza Provincial General Hospital. 118 patients (adult/child) aged between 6 months and 65 years inclusive residing within the study area, presenting with uncomplicated malaria to one of the study sites will be recruited for the study. The general objective of this study is to assess the degree of artemisinin resistance in subjects presenting with uncomplicated *P. falciparum* malaria in Western Kenya. The primary objective is to determine parasitological clearance rates by microscopy and quantitative polymerase chain reaction (PCR) assay for the first 72 hour period after first Artemisinin Combination Therapy (ACT) dose in patients with uncomplicated *P. falciparum* malaria.

Data generated by this study will provide a snapshot of the current situation regarding *P. falciparum* sensitivity to ACTs in Western Kenya. By having subjects in one of the study arms receive artesunate and then the partner drug after completion of the artemisinin phase will enable the accurate evaluation of the artemeisinin derivative without the confounding influence of the partner drug. Sequential administration of the components of an ACT drug is recognized by the World Health Organization (WHO) as one of the ways in which ACTs can be administered. There will be close follow-up of the subjects throughout the duration of the study, and as such, subjects who fail to respond adequately will receive prompt rescue treatment. Since it is largely expected that most subjects in Western Kenya will have satisfactory responses to ACTs, data from this study will provide baseline information regarding parasite characteristics when compared to data from Thailand, an area that has reported resistance to ACTs. This, in turn, will potentially enable the identification of key markers, both in the host and the parasite, that may assist in the early detection of resistance, and also to better understand the development of resistance to ACTs.

As such, the data generated from this study, both on its own and when compared to and pooled with data from similar studies that will be conducted in Ghana, Peru and Thailand, will potentially inform both local and international policy regarding ACT use for the treatment of uncomplicated *P. falciparum* malaria.

4. The WRAIR Scientific Review Committee (SRC) approved the protocol (Version 2, dated 30 May 2012) on 13 June 2012. There were no scientific changes made to the protocol between Version 2, dated 30 May 2012 and Version 3.3, dated 21 November 2012.

5. The HSPB conducted a review of the protocol (Version 2, dated 30 May 2012) and sent the Protocol Evaluation Form (PEF), dated 11 July 2012, to the Principal Investigator (PI). The PI responded to the HSPB PEF on 15 August 2012, providing both a point-by-point response and a revised protocol (Version 3, dated 1 August 2012).

The HSPB conducted a review of the updated protocol (Version 3, dated 1 August 2012) and determined that additional items needed to be addressed. The HSPB outlined additional comments in the HSPB Follow-up #1, dated 6 September 2012, and sent it to the PI. The PI responded to HSPB Follow-up #1 on 12 September 2012, providing both a point-by-point response and a revised protocol (Version 3.1, dated 12 September 2012).

The HSPB conducted a review of the updated protocol (Version 3.1, dated 12 September 2012) and determined that the protocol was ready for submission to the 10 October 2012 Institutional Review Board (IRB) meeting. The protocol was not reviewed at the 10 October 2012 IRB meeting due to time constraints, however, comments from the IRB reviewers was sent to the PI on 12 October 2012.

The PI responded to the IRB reviewer comments on 2 November 2012, providing both a point-by-point response and a revised protocol (Version 3.2, dated 26 October 2012). The HSPB point of contact (POC) reviewed the response and determined that the protocol was ready for submission to the 14 November 2012 IRB Meeting.

6. The protocol (Version 3.2, dated 26 October 2012) was reviewed at the 14 November 2012 fully convened WRAIR IRB meeting. The WRAIR IRB voted to table the protocol with stipulations. The WRAIR IRB also recommended that the protocol be submitted to the Kenya Medical Research Institute (KEMRI) Ethical Review Committee (ERC) once the stipulations have been addressed, but prior to resubmitting the protocol for review by the fully convened WRAIR IRB.

7. The KEMRI Scientific Steering Committee (SSC) approved the protocol (Version 3.3, dated 21 November 2013) on 4 February 2013. The KEMRI ERC approved the protocol (Version 3.3, dated 21 November 2012) on 19 March 2013.

expiration dates to avoid an interruption in work. A closeout report will be due to the WRAIR HSPB on **10 April 2018**. **No changes, amendments, or addenda may be made to the protocol without prior review and approval by the KEMRI ERC, the WRAIR IRB, and the USAMRMC ORP HRPO, as applicable.**

13. The point of contact for this action is Teresa R. Soderberg, M.A., RAC, CIM, at (301) 319-9438 or Teresa.R.Soderberg.civ@mail.mil.

KENT E. KESTER
COL, MC
Chair, Institutional Review Board
Walter Reed Army Institute of Research

CF:
Ben Andagalu, M.D., MSc
Jessica Cowden, MAJ, MC
Regulatory Affairs, Kenya
Thomas Logan, COL, MS
MCMR-RP

Appendix 9: List of Publications

1. **Bidii S. N,** Luiser A. I, Agnes C. C, Lorna J. C, Dennis W. J, Peninah M, Irene O, Jack O, Redemptah A. Y, Jelagat C, Emmanuel M, Grace M, Angela O. A, Zipporah N, Ben A, Hoseah M. A[1], Edwin K. (2015) Analysis of Major Genome Loci Underlying Artemisinin Resistance and pfmdr1 Copy Number in pre- and post-ACTs in Western Kenya. Sci. Rep. 5, 8308; DOI: 10.1038/srep08308.

2. Cheruiyot, J, Dennis W. J, Angela A. O, Luiser I, Benjamin O, Agnes C, Redemptah Y, Charles O, Jelagat C, Penninah M, **Bidii S. N,** Lorna J.C, Fredrick E, Jacob J, Wallace D. B, Hoseah M.A, Ben A and Edwin K. (2014) Polymorphisms in Pfmdr1, Pfcrt, and Pfnhe1 genes are associated with reduced in vitro activities of quinine in *Plasmodium falciparum* isolates from western Kenya. *Antimicrob Agents Chemother* **58**, 3737-43.

3. Dennis W. J, Angela A. O, Luiser I, Benjamin O, Agnes C, Redemptah Y, Charles O, Jelagat C, Penninah M, **Bidii S. N,** Lorna J.C, Fredrick E, Jacob J, Wallace D. B, Hoseah M.A, Ben A and Edwin K. (2014) Trends in drug resistance codons in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase genes in Kenyan parasites from 2008 to 2012.*Malaria Journal 13:250*.