SURVEILLANCE FOR MOLECULAR MARKERS OF *PLASMODIUM FALCIPARUM* RESISTANCE TO ARTEMISININ-BASED COMBINATION THERAPIES IN WESTERN KENYA

BY

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DECLARATION

I declare that this thesis is my original work and has not been presented to any other university or institution for a degree or any other award.

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DEDICATION

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ABSTRACT

Recent decreases in the global malaria burden are partly due to the deployment of artemisinin-based combination therapies (ACTs) for treatment of uncomplicated *Plasmodium falciparum* malaria. However, these significant gains are threatened by emergence of drug-resistant malaria parasites. In Africa, *P. falciparum* remains susceptible to ACTs. However, due to the emergence of parasites that are resistant to ACTs in Southeast Asia and the recent report of declined responsiveness of *P. falciparum* infections to ACTs at the Kenyan coast, there is need for continuous monitoring of ACT resistance in high malaria transmission areas such as western Kenya. Currently, therapeutic efficacy studies (TES) are considered the gold standard for determining antimalarial drug efficacy. However, the World Health Organization recommends that TES be complemented with surveillance for molecular markers associated with parasite resistance to monitor the emergence of resistance before it leads to widespread clinical resistance. Therefore, surveillance for mutations in *P. falciparum kelch 13* (*Pfk13*) propeller domain and *P. falciparum multi-drug resistance protein 1* (*Pfmdr1*) gene, which have been implicated in parasite resistance to ACTs, is critical in informing the current status of resistance to ACTs. This study characterized the mutation profile of *Pfk13* propeller region and *Pfmdr1* gene in a malaria endemic area of western Kenya. The specific objectives were to determine; the mutations in *Pfk13* propeller region (codons 458, 493, 539, 543, 561 and 580); the mutations in *Pfmdr1* gene (codons 86, 184, 1034, 1042 and 1246) and association with treatment outcome. This laboratory based experimental study used archived blood samples from a recently completed TES (2016-2017) which assessed the efficacy of artemether-lumefantrine (AL) and dihydroartemisinin-piperaquine (DP) in children aged 6-59 months in western Kenya. A total of 423 samples which included 323 samples collected pre-treatment (day 0) and 110 samples collected on the day of recurrent parasitaemia (up to day 42) were analyzed. Parasite genomic DNA was extracted from dried blood spots using QIAamp DNA Mini Kit as described by the manufacturer. Single nucleotide polymorphisms was determined by nested Polymerase Chain Reaction and Sanger sequencing. Sequencing of the *Pfk13* gene was successful for 93.8% samples. For all the samples tested, none of the *Pfk13* mutations that have been associated with artemisinin resistance was detected. However, other non-synonymous mutations which have not been associated with resistance were detected.; for example; Out of 317 day 0 samples, 2 (0.6%) had S522**C**, 5 (1.6%) had A578**S,** 1 (0.3%) E596**D** mutations and 309 (97.5%) were wild type. Out of 95 recurrent infection samples, 2 (2.3%) had A578**S,** 1 (1.1%) had C580**F** and 85 (96.6%) samples were wild type. For multidrug resistant marker *Pfmdr1*, 95.8% samples were successfully sequenced. Out of 320 day 0 samples, 1 (0.3%) had N86**Y**, 192 (59.7%) had Y184**F**, 30 (9.4%) had D1246**Y** mutations and 117 (36.6%) samples were wild type (NYSND). Out of 95 recurrent infection samples, 1 (1.1%) had N86**Y**, 59 (62.1%) had Y184**F**, 5 (5.3%) had D1246**Y** mutations and 33 (34.7%) samples were wild type (NYSND). There was no statistically significant association between any of the observed *Pfk13* mutations with treatment outcome for both AL treatment arms (S522**C** and C580**F**; Fisher statistic; $p = 1$ and $p = 0.16$ respectively) and DP treatment arm (S522**C** and A578**S**; Fisher statistic; $p = 1$ and $p = 0.22$ respectively). Although a high frequency of *Pfmdr1* Y184**F** mutations was detected, there was no statistically significant association between these mutations with treatment failure (recrudescence) in both treatment arms (Fisher statistic; $p = 1$ and $p = 0.735$, for AL and DP treatment arms respectively). These results indicate absence of mutations associated with parasite resistance to ACTs in western Kenya. However, continued monitoring for molecular

markers of ACT resistance is needed for providing timely evidence-based malaria treatment policies in western Kenya and other malaria endemic regions.

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LIST OF ACRONYMS AND ABBREVIATIONS

ACPR	Adequate clinical and parasitological response
ACT	Artemisinin-based combination therapy
AE	Elution buffer
AL	Artemether-lumefantrine
AL1	Lysis buffer
AQ	Amodiaquine
ATL	Tissue lysis buffer
AW1	Wash buffer 1
AW2	Wash buffer 2
CDC	Centers for disease Control and Prevention
CGHR	Centre for Global Health Research
DBS	Dried Blood Spot
DNA	Deoxyribonucleic acid
dNTPs	Dioxynucleoside triphosphates

DP	Dihydroartemisinin-piperaquine
EDTA	Ethylenediaminetetraacetic acid
ETF	Early Treatment Failure
GA	Georgia
GCP	Good Clinical Practice
GLURP	Glutamate Rich Protein
IPTp	Intermittent preventive treatment in pregnancy
IRS	Indoor residual spraying
ITN	Insecticide-treated nets
KEMRI	Kenya Medical Research Institute
LCF	Late Clinical Failure
LLINs	Long-lasting insecticidal nets
LPF	Late Parasitological Failure
LTF	Late Treatment Failure
MSP	Merozoite surface protein
NMCP	National Malaria Control Programme

PCR	Polymerase Chain Reaction
Pfcrt	*P. falciparum chloroquine resistance transporter* gene
Pfk13	*Plasmodium falciparum kelch 13*
Pfmdr1	*Plasmodium falciparum multi-drug resistance protein 1*
RDT	Rapid diagnostic test
RR	Relative Risk
SCRH	Siaya County Referral Hospital
SGS	School of Graduate Studies
SOP	Standard Operating Procedure
SP	Sulfadoxine-pyrimethamine
USA	United States of America
WHO	World Health Organisation

DEFINITION OF TERMS

Adequate clinical and parasitological response (ACPR): Absence of parasitemia on day 42 irrespective of axillary temperature without previously meeting any of the criteria of Early Treatment Failure or Late Clinical Failure or Late Parasitological Failure.

Artemisinin-based combination therapy (ACT): Refers to the current recommended drugs for the treatment of *Plasmodium falciparum* malaria. It is a combination of a fast acting artemisinin-based compound with a drug from a different class. Companion drugs include lumefantrine, mefloquine, amodiaquine, sulfadoxine/pyrimethamine, piperaquine and chlorproguanil/dapsone. Artemisinin derivatives include dihydroartemisinin, artesunate and artemether. A co-formulated drug is one in which two different drugs are combined in one tablet.

Drug resistance: The World Health Organization (WHO) defines resistance to antimalarials as the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a medicine given in doses equal to or higher than those usually recommended but within the tolerance of the subject, provided drug exposure at the site of action is adequate. Resistance to antimalarials arises because of the selection of parasites with genetic mutations or gene amplifications that confer reduced susceptibility to the antimalarial drug.

Late Treatment Failure: Late treatment failure (LTF) is defined as meeting criteria of either late clinical failure (LCF) or late parasitological failure (LPF). Late clinical failure is defined as either development of danger signs or severe malaria after day 3 in the presence of parasitemia, or presence of parasitemia and or history of fever on any day from day 4 to day 42 while LPF is

defined as the presence of parasitemia on any day from day 7 to day 42 and axillary temperature <37.5 ºC, without previously meeting any of the criteria of early treatment failure or LCF.

***Plasmodium falciparum kelch 13* (*Pfk13*):** Is an exon gene located at chromosome 13. It codes for a putative kelch protein and has three domains: a plasmodium-specific domain, a BTB/POZ, and a C-terminal six-blade propeller. Mutations in the propeller region have been linked to antimalarial drug resistance.

***Plasmodium falciparum multi-drug resistance protein 1* (*Pfmdr1*):** Is a protein located at chromosome 5 encoding a P-glycoprotein homologue 1. It is an adenosine triphosphate-binding cassette protein located on the parasite's food vacuole.

Recurrent parasitaemia: The recurrence of asexual parasitaemia following treatment. This can be caused by a recrudescence, a relapse (in *P. vivax* and *P. ovale* infections only) or a new infection.

Therapeutic efficacy studies (TES): Are studies that monitor therapeutic efficacy of drugs, using a standardized WHO protocol. In falciparum malaria, it involves assessing clinical and parasitological outcomes of treatment for at least 28 days after the start of adequate treatment and monitoring for the reappearance of parasites in blood. A follow-up of 42 days is recommended in high transmission areas.

Uncomplicated malaria: Symptomatic infection with malaria parasitaemia without signs of severity and/or evidence of vital organ dysfunction.

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CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria remains a global health problem and the cause of an estimated 216 million new infections and 445,000 deaths annually (WHO, 2017a). In Kenya, malaria remains a major cause of morbidity and mortality with an estimated 3.5 million new clinical cases and 10,780 deaths each year (WHO, 2017a). The areas around Lake Victoria and the Coastal region present the highest risk (KMIS, 2015). The uncomplicated form of *Plasmodium falciparum* infection can be easily and successfully treated with an artemisinin-based combination therapy (ACT) (Otienoburu *et al.*, 2016). However, the emergence and spread of *P. falciparum* resistance to antimalarial drugs is now one of the greatest challenges facing the global effort to control malaria (Otienoburu *et al.*, 2016). In Africa, *P. falciparum* remains susceptible to ACTs (Ogutu *et al.*, 2014; Venkatesan *et al.*, 2014). However, due to the emergence of parasites that are resistant to ACTs in Southeast Asia (Ariey *et al.*, 2014) and the recent report of declined responsiveness of *P. falciparum* infections to ACTs at the Kenyan coast (Borrmann *et al.*, 2011), there is need for continuous monitoring of ACT resistance in high malaria transmission areas such as western Kenya. The World Health Organization (WHO) recommends routine monitoring of antimalarial drug efficacy for effective case management and detection of resistance (WHO, 2016b). While *in vitro*, *ex vivo* drug testing and therapeutic efficacy studies (TES) are important for assessing the effectiveness of antimalarial drugs (Talundzic *et al.*, 2018), molecular surveillance using genetic markers associated with resistance provides a valuable tool for

tracking resistance, guiding treatment policy as well as providing an in-depth understanding of the development and spread of resistance.

Plasmodium falciparum kelch 13 (*Pfk13*) is an exon gene located at chromosome 13 (Mohon *et al.*, 2014). It codes for a putative kelch protein and has three domains: a plasmodium-specific domain, a BTB/POZ, and a C-terminal six-blade propeller (Mohon *et al.*, 2014). The *Pfk13* is well conserved across *Plasmodium* species and is thought to mediate protein-protein interactions (Ariey *et al.*, 2014). Polymorphisms in the *Pfk13* propeller region have been identified as a key causal determinant of artemisinin resistance in Southeast Asia, (Ghorbal *et al.*, 2014; Ariey *et al.*, 2014; Straimer *et al.*, 2015), and have served as valuable molecular markers for tracking and monitoring artemisinin-resistant parasites. Mutations in the *Pfk13* propeller gene that have been associated with delayed parasite clearance and validated by *in vivo* and *in vitro* data are; N458**Y**, Y493**H**, R539**T**, I543**T**, R561**H**, and C580**Y** (WHO, 2017b). These mutations and delayed parasite clearance are common in parts of Cambodia, Thailand, Myanmar, and Vietnam (Ashley *et al.*, 2014; Bosman *et al.*, 2014; Tun *et al.*, 2015). Mutations in the *Pfk13* propeller region that have been correlated with artemisinin resistance have not been reported in Kenya (Kamau *et al.*, 2015; Muwanguzi *et al.*, 2016). However, there is need for continued surveillance for these mutations in high malaria transmission zones like western Kenya where drug resistance can emerge due to widespread use and possible misuse of antimalarial drugs which can create extraordinary selective pressure on the parasite, or spread of drug resistance parasites due to imported malaria.

The *P. falciparum multi-drug resistance protein 1* (*Pfmdr1*) gene, located at chromosome 5, encode a P-glycoprotein homologue 1 (Veiga *et al.,* 2016). In *P. falciparum,* the function of the *Pfmdr1* product is unknown, but the protein localizes to the membrane of the food vacuole, the site of action of a number of drugs, suggesting that it is a drug transporter (Cowman *et al.*, 1991). Polymorphisms within the *Pfmdr1* gene that encodes a trans-membrane homologue of the P-glycoprotein 1 protein have been implicated to impact on sensitivity to multiple antimalarial drugs (Foote *et al.*, 1990; Valderramos *et al.*, 2006; Sanchez *et al.*, 2010), with individual polymorphisms leading to opposite effects on different drugs (Koenderink *et al.*, 2010). The main implicated single nucleotide polymorphisms (SNPs) in the *Pfmdr1* include N86**Y**, Y184**F**, S1034**C**, N1042**D** and D1246**Y** (Foote *et al.*, 1990). However, the possible role of the *Pfmdr1* 86**Y**, 184**F**, 1034**C**, 1042**D** and 1246**Y** alleles as molecular markers of ACT resistance remains to be investigated in western Kenya.

In TES studies, the patient clinical and parasitological response (treatment outcome) is classified into adequate clinical and parasitological response (ACPR), early treatment failure (ETF) or late treatment failure (LTF) (WHO, 2009a) (Appendix 4). Understanding the relationship between molecular markers, parasite resistance and treatment failure is important for prolonging the lifespan of ACTs (Laufer *et al.*, 2007). The *Pfk13* propeller region mutations have been associated with delayed parasite clearance (Imwong *et al.*, 2015) which has been shown to lead to more treatment failures after ACT treatment (White, 2008). Clinical trials performed in Africa have also provided evidence for the selection of particular *Pfmdr1* alleles in patients with newly acquired infections or recurrent *P. falciparum* infections within 28 or 42 days after ACT treatment (Sisowath *et al.*, 2005; Dokomajilar *et al.*, 2006b; Humphreys *et al.*, 2007; Zongo *et*

al., 2007; Happi *et al.*, 2009; Ljolje *et al.*, 2018). Previous studies done in western Kenya have compared the prevalence of *Pfmdr1* and *Pfk13* molecular markers in pre- and post-ACT samples (Achieng *et al.*, 2015; Kamau *et al.*, 2015) and association with parasite clearance rates (Achieng *et al.*, 2015). However, the association between these mutations in the *Pfmdr1* gene and *Pfk13* propeller region with TES treatment outcomes is yet to be investigated in western Kenya.

Currently, there are very limited drugs that can replace ACTs and the emergence and spread of ACT-resistant parasites in endemic areas will be a significant setback in the fight against malaria. Molecular markers of drug resistance are potentially useful tools for monitoring the emergence and spread of parasites resistant to ACTs. The current study determined the prevalence of mutations at *Pfmdr1* and *Pfk13* propeller region in *P. falciparum* isolates collected in western Kenya and the association between these mutations with treatment outcome. The *Pfmdr1* gene codons tested were N86**Y** (i.e., denoting an encoded amino acid change from asparagine (N) to tyrosine (**Y**) at codon 86), F184**Y**, S1034**C**, D1042**N**, D1246**Y**, and in *Pfk13* propeller region the codons tested were N458**Y**, Y493**H**, R539**T**, I543**T**, R561**H**, and C580**Y.**

1.2 Statement of the Problem

Current strategies for malaria treatment rely on the use of ACTs. Outside Southeast Asia, *P. falciparum* parasites remain susceptible to ACTs. However, due to the emergence of parasites resistance to ACTs in Southeast Asia and the recent report of declined responsiveness of *P. falciparum* infections to ACTs at the Kenyan coast, the need for monitoring ACT resistance in western Kenya, a high transmission zone, cannot be over emphasized. *In vivo* studies are the source of crucial data on the efficacy of antimalarial drugs. However, the WHO recommends that these studies be complemented with molecular marker studies to monitor the emergence of

resistant parasites before it leads to widespread clinical resistance. Some of the molecular markers that have been implicated in parasite resistance to ACTs are mutations in the *Pfk13* propeller domain and *Pfmdr1* gene. Periodic surveillance for these molecular markers of resistance is critical in endemic areas in order to detect early signs of emerging parasite resistance and effectively track and contain the development and spread of resistance.

1.3 Objectives of the Study

1.3.1 Main objective

To characterise the mutation profile of *Pfk13* propeller region and *Pfmdr1* gene in *P. falciparum* parasite and their association with treatment outcomes in a malaria endemic area of western Kenya

1.3.2 Specific objectives

1. To determine gene mutations in the *Pfk13* propeller region (codons 458, 493, 539, 543, 561 and 580) in *P. falciparum* parasite isolates from western Kenya
2. To determine *Pfmdr1* gene mutations (codons 86, 184, 1034, 1042 and 1246) in *P. falciparum* parasite isolates from western Kenya.
3. To determine the association between the mutations in the *Pfk13* propeller region and *Pfmdr1* gene with treatment outcome.

1.4 Research Questions

1. What is the prevalence of gene mutations in the *Pfk13* propeller region (codons 458, 493, 539, 543, 561 and 580) in *P. falciparum* isolates from western Kenya?
2. What is the prevalence of *Pfmdr1* gene mutations (codons 86, 184, 1034, 1042 and 1246) in *P. falciparum* isolates from western Kenya?

1.5 Null Hypothesis

3. There is no association between gene mutations in the *Pfk13* propeller region and *Pfmdr1* gene with treatment outcome.

1.6 Significance of the Study

The results indicated absence of molecular markers for ACT resistance in western Kenya. The results can provide evidence to the Kenyan National Malaria Control Programme (NMCP) for the sustained use of ACTs in western Kenya. However, continued monitoring for molecular markers of ACT resistance is needed for providing timely evidence-based malaria treatment policies in western Kenya and other malaria endemic regions

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria Epidemiology

Malaria remains a major public health problem, especially in sub-Saharan Africa (WHO, 2017a). In 2016, there were an estimated 216 million new cases and 445,000 deaths globally, with sub-Saharan African contributing 90% of all malaria deaths (WHO, 2017a). In Kenya, malaria remains a major cause of morbidity and mortality with an estimated 3.5 million new clinical cases and 10,780 deaths each year (WHO, 2017a). The areas around Lake Victoria and on the Coast present the highest risk (KMIS, 2015). In the past 15 years, while malaria transmission at Kenya's coast has declined substantially (O'Meara WP, 2008), malaria transmission has remained relatively high in western Kenya. This area of Kenya remains the most important focus of malaria transmission nationally (DOMC, 2011).

Children under the age of 5 years and pregnant women are the most vulnerable groups (KMIS, 2015). Malaria infection is frequently more fatal in children than in adults, resulting in respiratory distress, neurological problems and severe anaemia, and leading to death in 5-35% of severe infections (Halliday *et al.*, 2014; NMCP, 2016). A cross-sectional study conducted by Kenya Medical Research Institute/Centers for Disease Control and Prevention (KEMRI/CDC) collaboration in July-August of 2013 in Siaya County found a malaria prevalence of 64.9% by rapid diagnostic test (RDT) in children <5 years of age (KEMRI/CDC unpublished data).

2.2 Malaria Causative Agents and Vectors

Five species of malaria parasites infect humans; *P. falciparum, P. vivax, P. ovale*, *P. malariae* and *P. knowlesi* (Sabbatani *et al.*, 2010). Among the human malaria parasites, *P. falciparum* is the most dangerous species and associated with high morbidity and mortality (Snow *et al.*, 2005; Cox *et al.*, 2007). Female mosquitos belonging to the genus *Anopheles* transmit malaria. The main vectors of malaria parasites in Africa are *Anopheles gambiae sensu lato (sl)* and *A. funestus* complex (WHO, 2016a). In Kenya, the *P. falciparum* accounts for more than 95% of infections and the main vectors being *A. gambiae sensu stricto (ss), A. arabiensies* and *A. funestus* (WHO, 2016a).

2.3 Clinical Presentations of Malaria

Malaria symptoms due to *P. falciparum* generally start approximately two weeks after the bite of an infected mosquito (Greenwood *et al*., 2008). Initial symptoms may include fever, chills, headache, joint and muscle pain, sweating and vomiting (Greenwood *et al*., 2008). Acute complications may result from haemolysis leading to anaemia and the propensity of infected red blood cells (RBCs) to become adhesive (Milner *et al*., 2008). This leads to the sequestration of adhesive parasite in capillaries causing local inflammatory reactions and damage to vital organs (Milner *et al*., 2008). This leads to cerebral, hepatic, renal or pulmonary malaria (Maitland *et al.*, 2004; Milner *et al.*, 2008).

The majority of malaria related morbidity and mortality in African children presents by severe manifestations like hyperparasitaemia, hypoglycaemia, cerebral malaria, malarial anaemia, respiratory distress and prostrations (Breman, 2001). Uncomplicated *P. falciparum* malaria is characterized by fever or history of fever within 24 hours, parasitaemia, without signs of severity

or evidence (clinical or laboratory) of vital organ dysfunction (WHO, 2010b). Severe malaria is defined as having fever or history of fever within 24 hours with *P. falciparum* parasitaemia, with signs of severity and/or evidence (clinical or laboratory) of vital organ dysfunction (WHO, 2010b). Severe complications of malaria are associated with cerebral malaria characterized by impaired consciousness, followed by severe headache. These manifestations are due to the sequestration of the infected erythrocytes in the cerebral microvasculature (Rosenberg *et al.*, 1990).

2.4 Malaria Prevention and Control Strategies

Malaria is preventable, treatable and curable (Williams *et al.*, 2009). Its epidemiology is highly variable, and control strategies are rolled out based on local environmental, biological, social and health system factors (WHO, 2013). Despite this complexity, in most countries, especially those in Africa with the highest burden, three approaches to reduce mortality and morbidity based on effective and low-cost interventions can be applied to give full coverage of all populations at risk (WHO, 2002). These are; vector control, prompt access to treatment, especially for young children, prevention and control in pregnant women, and prompt access to diagnosis and effective treatment (WHO, 2002).

The goals of malaria vector control include protection of individual people against infective malaria mosquito bites and reduction of the intensity of local malaria transmission at the community level (Teklehaimanot *et al.*, 2007). Vector control includes use of LLINs, IRS, and other integrated vector management strategies (WHO, 2013). Compliance to vector control has however, proven difficult in developing countries with limited funds, governmental corruption and isolated rural communities (Teklehaimanot *et al.*, 2007).

Prompt parasitological confirmation by microscopy or RDTs is recommended before treatment is started (WHO, 2010a). In settings with limited health facility access, diagnosis and treatment should be provided at community level through a programme of community case management (WHO, 2010a). To effectively reduce malaria burden, treatment must be with effective antimalarial drugs.

2.5 Antimalarial Drugs

The treatment policy for malaria has changed in the last 2 decades due to failing therapeutic efficacy of antimalarial drugs (Amin *et al.*, 2007). Until 1998, chloroquine was the drug of choice due to its simple dosing schedule, high efficacy, low cost and good safety profile (Mbaisi *et al.*, 2004). However, resistance developed against chloroquine leading to replacement with the sulfadoxine-pyrimethamine (SP) combination. Soon thereafter, resistance also developed against SP in 2004 (Mbaisi *et al.*, 2004). In 2005, WHO recommended that ACTs be used as first-line treatments for falciparum malaria in all countries where malaria was endemic (Nosten *et al.*, 2007).

To increase clinical efficacy and slow the emergence of parasite resistance, ACT depends on the combined action of two independent drugs (Ljolje *et al.*, 2018). The fast-acting artemisinin derivative, with a half-life on the order of hours, which not only achieve rapid clearance of the asexual parasites that cause symptomatic blood-stage infection, but also reduce the numbers of sexual-stage parasites (gametocytes) responsible for transmission and a second, longer acting partner drug, which eliminates the remaining parasites and limits selection of artemisinin resistance (Nosten *et al.*, 2007).

There are currently five ACTs recommended by WHO; artesunate-amodiaquine, artesunate-mefloquine, artesunate sulfadoxine-pyrimethamine (AS+SP), artemether-lumefantrine (AL) and dihydroartemisinin-piperaquine (DP) (Nosten *et al.*, 2007). In Kenya, the ACTs advocated for treatment of uncomplicated malaria are AL and DP. They were adopted as first and second-line antimalarial therapy in 2006 and 2010 respectively. Sulfadoxine-pyrimethamine is still recommended for intermittent preventive therapy (IPT) during pregnancy (Amin *et al.*, 2007). In Siaya county, western Kenya, 76.5% use AL for treatment of malaria and 23.5% use other antimalarial drugs for treatment of malaria (Onyango *et al.*, 2012). Adherence to ACT prescription in this county is 42.1% and 57.9% among individuals above 13 and less than 13 years, respectively (Onyango *et al.*, 2012).

2.5.1 Artemether-lumefantrine (AL)

Artemether-lumefantrine was the first fixed dose combination of an artemisinin derivative with a second antimalarial compound with different mode of action which became available in 2004 (Nosten *et al.*, 2007). Lumefantrine is active against all the human malaria parasites, including multi-drug resistant *P. falciparum* and has shorter elimination half-life (4-5 days) and, importantly, has never been available as monotherapy. Artemether-lumefantrine is the most procured ACT for public and private sectors globally (WHO, 2013), with 56 countries using it as first-line or second-line treatment (WHO, 2010b).

Previous studies in Africa (including Kenya) and Southeast Asia showed excellent efficacy > 90% with AL (Mayxay *et al.*, 2012; Agarwal *et al.*, 2013; WWARN, 2015) except in 3 studies from Cambodia and Thailand (WWARN, 2015). One study from Cambodia later confirmed as

having artemisinin resistance, and the treatment failures from the two studies in Thailand were associated with slow parasite clearance (WWARN, 2015). In a recent study where individual patient data from 31 clinical trials was analysed, the overall clinical efficacy for patients treated with AL was shown to be 94.8%; 93.8% efficacy in East Africa and 96.2% in West Africa (Venkatesan *et al.*, 2014). In Uganda, remarkable changes in parasite polymorphisms were seen after increasing AL usage (Tumwebaze *et al.*, 2016). Within 4 years, there was a reverse trend in the parasites populations from high-level chloroquine-resistant and lumefantrine-sensitive parasites to chloroquine-sensitive parasites with decreased sensitivity to lumefantrine (Tumwebaze *et al.*, 2016).

In a study conducted in Mbita Point, western Kenya, residual sub-microscopic parasitaemia at day 3 was detected in 31.8% patients despite high cure rates and rapid parasite clearance times after treatment with AL or DP (Beshir *et al.*, 2013). Day 3 positivity was associated with higher risk of reappearance of asexual parasite on day 28 or 42 detected by microscopy (Beshir *et al.*, 2013, 2017). Although AL remains highly efficacious and *Pfk13* polymorphisms are not associated with reduced susceptibility to ACTs in sub-Saharan Africa, AL is associated with selection of SNPs in *P. falciparum chloroquine resistance transporter* gene (*Pfcrt*) and *Pfmdr1* in parasite reinfections (Sisowath *et al.*, 2005; Sisowath *et al.*, 2007; Achieng *et al.*, 2015).

2.5.2 Dihydroartemisinin-piperaquine (DP)

Dihydroartemisinin is the active metabolite of all artemisinin compounds (artemisinin, artesunate, artemether, etc.) and is available as a drug in itself. It is sold commercially in combination with piperaquine and has been shown in East Africa as an alternative to AL (Arinaitwe *et al.*, 2009; Bassat *et al.*, 2009). Piperaquine is a bisquinoline compound related to

chloroquine and other 4-aminoquinolines (Nosten *et al.*, 2007). Its advantages over AL include once-daily dosing and a longer half-life (4-5 weeks) of the partner drug, which may prevent re-infection in areas of intense malaria transmission (Agarwal *et al.*, 2013).

Dihydroartemisinin-piperaquine has been used since 1978, mainly in China (Nosten *et al.*, 2007). The fixed dose combination formulated in tablets containing dihydroartemisinin (40 mg) and PQ (320 mg) is commercially available in many countries; Asia as Artekin (Holleykin Pharmaceutical Co. Ltd., Guangzhou, China) and Duocotecxin (Beijing Holley-Cotec Pharmaceuticals Co. Ltd., Beijing, China) and also more recently in Africa. Clinical trials have shown that the fixed combination given once daily for 3 days was effective and well tolerated (Mayxay *et al.*, 2006; Jenkins *et al.*, 2015). The main determinant of the parasitological efficacy is the slow elimination of PQ (Nosten *et al.*, 2007). Previous studies in western Kenya showed excellent efficacy > 90% with DP (Agarwal *et al.*, 2013; Ogutu *et al.*, 2014).

2.6 Resistance to Artemisinin Combination Therapies (ACTs)

Resistance to ACT can involve artemisinin resistance, partner drug resistance, or both (WHO, 2016b). Artemisinin resistance manifests in delayed parasite clearance in the first 3 days following anti-malarial therapy and survival of ring stage parasites (Dondorp *et al.*, 2009; Ariey *et al.*, 2014). As a result, fewer malaria parasites are killed during the 3 days of ACT treatment, leaving more for the partner drug to clear (Imwong *et al.*, 2015). This action increases the risk of treatment failure and facilitates selection of partner drug resistance (White, 2016). In contrast, partner drug resistance manifests as a late treatment failure, where a patient who initially cleared parasites develops recurrent parasitaemia within 4-6 weeks following anti-malarial therapy

(Ljolje *et al.*, 2018). Resistance to both components of the ACT results in substantial reductions in cure rates (White, 2016).

Resistance to ACTs has been observed in western Cambodia, Thailand, Vietnam, and Myanmar (Noedl *et al.*, 2008; Phyo *et al.*, 2012; Tun *et al.*, 2015; Imwong *et al.*, 2015). A declining efficacy of the artesunate/mefloquine combination has been noted in the Cambodia-Thailand border region of Southeast Asia, an epicenter of antimalarial drug resistance (Wongsrichanalai *et al.*, 2008). In addition, clinical resistance to artesunate, manifested as delayed clearance of parasitemia after therapy was documented in 2008 (Noedl *et al.*, 2008; Dondorp *et al.*, 2009; Phyo *et al.*, 2012). Other reports from Cambodia have shown recrudescent infections after treatment with DP, raising the concern that resistance to artemisinin partner drugs has been facilitated by the spread of artemisinin resistance (Saunders *et al.*, 2014).

In Africa, ACT remains efficacious and safe (Agarwal *et al.*, 2013; Ashley *et al.*, 2014). However, resistance to previous generations of anti-malarial drugs such as chloroquine and SP emerged in the 1970s in Southeast Asia and eventually spread to the Indian sub-continent and then to Africa (Roper *et al.*, 2014). It is, therefore, critical that continuous monitoring of the therapeutic response of ACT is carried out in endemic areas in order to detect early warning signs and effectively track and contain the development and spread of artemisinin resistance.

2.7 Methods of Assessing Parasite Resistance to Antimalarial Drugs

Surveillance of drug resistance can be done using three complementary methods; TES / *in vivo* efficacy studies for the detection of treatment failures (also referred to as therapeutic efficacy

studies), *in vitro* assessment of parasite sensitivity to drugs, and the analysis of molecular markers associated with drug resistance (Nsanzabana *et al.*, 2018).

2.7.1 Therapeutic efficacy studies (TES) / *In vivo* tests

These tests involve the treatment of a group of symptomatic and parasitemic individuals with known doses of the drug and subsequent monitoring of the parasitological and/or clinical response over time (Nsanzabana *et al.*, 2018). Having received appropriate treatment, the patients are followed up by parasitological and clinical assessments for a specified number of days (from 28 to 63 days, depending on the half-life of the medicine assessed and malaria endemicity), after which the treatment outcome is determined as successful or not (WHO, 2009a). The patient's clinical and parasitological response is classified into ACPR, ETF or LTF (LTF is classified as late parasitological failure (LPF) and late clinical failure (LCF)) (WHO, 2009a). These classifications are described in Appendix 4.

Currently, this assessment is done in routine surveillance and has become the gold standard to guide treatment policy in malaria endemic countries (WHO, 2009a). The advantage of the *in vivo* tests over the *in vitro* assays is that they can be conducted in the field with little equipment and personnel and the results are easy to interpret (Nsanzabana *et al.*, 2018). They also reflect the true biological nature of treatment response, which involves a complex interaction between the parasites, the drugs, and the host response, compared to *in vitro* tests which measure only the interaction between the parasites and the drugs (Basco *et al.*, 2000). The major limitation with this test for evaluation is that resistance may not always be detected, due to pharmacokinetic variation, re-infections, multiple infections, non-compliance or interference with the acquired immune response (Basco *et al.*, 2000).

2.7.2 *In vitro* tests

The assessment of *P. falciparum* parasites susceptibility to anti-malarial drugs can be performed phenotypically, using parasite strains collected from patients (*ex vivo*) or with culture-adapted isolates (*in vitro*) (Trager *et al.*, 1976). The assessment can be done by culturing parasites in the presence of anti-malarial drugs at varying concentrations to determine the growth inhibitory effect of the drugs or by exposing parasites to a specific high concentration for a relatively short period (Witkowski *et al.*, 2013). The parasite growth is then measured using various techniques, and results used to determine either the concentration that inhibits parasite growth by 50% (50% inhibitory concentration; IC50) (Nsanzabana *et al.*, 2018) or the survival rate (Witkowski *et al.*, 2013).

In vitro tests avoid many of the confounding factors which influence *in vivo* tests by removing parasites from the host and placing them into a controlled experimental environment (Rieckmann *et al.*, 1978). However, the test has certain significant disadvantages. The correlation of *in vitro* response with clinical response in patients is neither clear nor consistent, and the correlation appears to depend on the level of acquired immunity within the population being tested (Nsanzabana *et al.*, 2018).

2.7.3 Surveillance for molecular markers

Surveillance of molecular markers associated with drug resistance is another method of estimating efficacy of a drug (Nsanzabana *et al.*, 2018). Genetic markers from a sub-set of the parasite population are expected to reflect the prevalence of these SNPs in the total parasite population (Nsanzabana *et al.*, 2018). If molecular makers that accurately predict treatment failure are available these can be used for molecular surveillance and further guide in decisions

regarding drug policies (WHO, 2017b). In the last two decades, the mechanisms of resistance to the most widely used anti-malarial drugs have been elucidated in part using molecular techniques, and anti-malarial resistance is often associated with SNPs or amplifications of the genes coding for drug target proteins or transporters (Picot *et al.*, 2009). Molecular surveillance for anti-malarial resistance could provide useful real-time information on spatial and temporal trends for anti-malarial drug resistance to monitor the appearance and spread of anti-malarial resistance.

2.8 Molecular Markers for *P. falciparum* Resistance to Antimalarial Drugs

Characterization of molecular markers of drug resistance is an important aspect of understanding resistance to antimalarial treatment (Ariey *et al.*, 2014). A number of genes involved or potentially involved in *P. falciparum* antimalarial drug resistance have been identified. These are the genes encoding dihydrofolate reductase (*Pfdhfr*), dihydropteroate synthase (*Pfdhps*), the *Pfcrt*, the *Pfmdr1*, Na+/H+ exchanger (*Pfnhe-1*) and cytochrome *b* (Cui *et al.*, 2015) and propeller domain of the *Pfk13* gene (Ariey *et al.*, 2014).

2.8.1 *P. falciparum kelch 13 (Pfk13)*

Plasmodium falciparum kelch 13 is an exon gene located at chromosome 13 (Mohon *et al.*, 2014). It codes for a putative kelch protein and has three domains: a plasmodium-specific domain, a BTB/POZ, and a C-terminal six-blade propeller (Mohon *et al.*, 2014). The *Pfk13* is well conserved across *Plasmodium* species and is thought to mediate protein-protein interactions (Ariey *et al.*, 2014). The *Pfk13* molecular marker for artemisinin resistance was recently discovered following whole-genome sequencing of an artemisinin-resistant parasite line from Africa and clinical parasite isolates from Cambodia (Cheeseman *et al.*, 2012; Takala-Harrison *et al.*, 2014). Using a combined resistance selection and genomic approach, mutations in the

propeller domain of the *Pfk13* gene (PF3D7_1343700) associated with delayed parasite clearance after artemisinin therapy in Southeast Asia was identified (Ariey *et al.*, 2014). The authors found that when a *Pfk13* mutant parasite was repaired to wild type, the artemisinin resistance was lost; and that conversely, sensitive parasites become more resistant to artemisinin once the wild-type *Pfk13* gene is converted to mutant (Straimer *et al.*, 2015).

The frequently reported *Pfk13* mutations are C580**Y**, R539**T**, Y493**H**, I543**T** in Cambodia, Lao PDR and VietNam and F446**L**, N458**Y**, P574**L**, R561**H** in the China, Myanmar and Thailand (Ashley *et al.*, 2014; Bosman *et al.*, 2014; Tun *et al.*, 2015; WHO, 2017b). Mutations, correlated with delayed parasite clearance and which have been validated by *in vivo* and *in vitro* data are; N458**Y**, Y493**H**, R539**T**, I543**T**, R561**H**, and C580**Y** (WHO, 2017b). The candidate mutations correlated with delayed parasite clearance are P441**L**, F446I, G449**A**, G538**V**, P553**L**, V568**G**, P574**L** and A675**V** (WHO, 2017b). Mutations, which have been not been associated with artemisinin resistance, are E252**Q** and A578**S** (WHO, 2017b).

In Africa, the most frequent *Pfk13* mutation observed is A578**S** (WHO, 2017b). However, this allele has not been associated with clinical or *in vitro* resistance to artemisinin (WHO, 2017b). A study investigating polymorphism in *Pfk13* propeller region across 12 countries in sub-Saharan Africa including Kenya identified more than 20 unique mutations (Kamau *et al.*, 2015). However, this study did not identify any of the mutations related to artemisinin resistance reported in Southeast Asia (Kamau *et al.*, 2015). A study in Dakar, Senegal did not report any mutation between the six blades of *Pfk13* (Torrentino-Madamet *et al.*, 2014). In 2013, a non-synonymous mutation at *Pfk13* position 579 (M579**I**) was isolated from a Chinese worker who

had malaria 8 weeks after returning from Equatorial Guinea; however, the circumstances of the case and the parasite origin are not well documented (Lu *et al.*, 2017). In a study conducted in western Kenya, using samples obtained in 2009 from Kenyan children treated with AL and DP, no mutation associated with artemisinin resistance was observed (Muwanguzi *et al.*, 2016). The *Pfk13* mutations that have been associated with artemisinin resistance have not been reported in Kenya, however, with the recent emergence and spread of these mutations in the Southeast Asia indicates the need of continuous monitoring of these molecular markers especially in high malaria transmission zones like western Kenya.

2.8.2 *P. falciparum multidrug resistance protein 1 (Pfmdr1)*

Plasmodium falciparum multi-drug resistance protein 1 gene, located at chromosome 5, encode a P-glycoprotein homologue 1 (Veiga *et al.*, 2016). In *P. falciparum*, the function of the *Pfmdr1* product is unknown, but the protein localizes to the membrane of the food vacuole, the site of action of a number of drugs, suggesting that it is a drug transporter (Cowman *et al.*, 1991). Polymorphisms in transport proteins can mediate resistance via enhancing efflux of the drugs from cells (Borges-Walmsley *et al.*, 2003; Picot *et al.*, 2009). In humans, P-glycoprotein polymorphisms are associated with resistance to cancer drugs (Sharom, 2011). In *P. falciparum*, the function of the *Pfmdr1* product is unknown, but the protein localizes to the membrane of the food vacuole, the site of action of a number of drugs, suggesting that it is a drug transporter (Cowman *et al.*, 1991).

Polymorphisms within the *Pfmdr1* gene that encodes a trans-membrane homologue of the P-glycoprotein 1 protein have been implicated to impact on sensitivity to multiple antimalarial drugs (Foote *et al.*, 1990; Valderramos *et al.*, 2006; Sanchez *et al.*, 2010), with individual polymorphisms leading to opposite effects on different drugs (Koenderink *et al.*, 2010). The

main implicated *Pfmdr1* polymorphisms include N86**Y**, Y184**F**, S1034**C**, N1042**D** and D1246**Y** (Foote *et al.*, 1990). The *Pfmdr1* polymorphisms have been associated with decreased susceptibility to amodiaquine, lumefantrine, mefloquine and artesunate (Price *et al.*, 2004; Eyase *et al.*, 2013). Increased copy number of the *Pfmdr1* gene, which is prevalent in Southeast Asia, have been shown to reduce susceptibility to mefloquine (Price *et al.*, 2004; Sidhu *et al.*, 2006). Experimental evidence has also shown that *Pfmdr1* amplification also led to decreased sensitivity to quinine, lumefantrine and artemisinin (Price *et al.*, 2004; Sidhu *et al.*, 2006; Alker *et al.*, 2007). Mutations at *Pfmdr1* N86**Y** and D1246**Y** which are common in Africa, have been linked to decreased sensitivity to chloroquine and amodiaquine, but increased sensitivity to lumefantrine, mefloquine and artemisinin (Duraisingh *et al.*, 2000b; Reed *et al.*, 2000; Duraisingh *et al.*, 2000a; Mwai *et al.*, 2009; Tumwebaze *et al.*, 2015). Other polymorphisms primarily seen outside Africa (including 1034**C** and 1042**D**) are associated with altered sensitivity to lumefantrine, mefloquine and artemisinin (Reed *et al.*, 2000; Pickard *et al.*, 2003; Sidhu *et al.*, 2005; Sidhu *et al.*, 2006). Previous study in western Kenya that compared the prevalence of *Pfmdr1* molecular marker in pre- and post-ACT samples revealed a significant increase in *Pfmdr1* N86-184**F**-D1246 haplotype; the polymorphism that is associated with reduced susceptibility to AL (Achieng *et al.*, 2015). Though the study did not find any association between parasite clearance rates and these *Pfmdr1* polymorphisms (Achieng *et al.*, 2015), there is need for close monitoring of the *Pfmdr1* 86**Y**, 184**F**, 1034**C**, 1042**D** and 1246**Y** molecular markers in response to continued use of ACTs in western Kenya

2.9 Association between Mutations in the *Pfk13* Propeller Region or *Pfmdr1* Gene with Treatment Outcome

In TES studies the patient's clinical and parasitological response (treatment outcome) is classified into ACPR, and LTF and ETF (WHO, 2009a) (Appendix 4). The ETF and LTF are recurrent infections. To differentiate between recrudescence and re-infection in recurrent infections, a genotypic analysis based on merozoite surface protein-2 (MSP-2), glutamate-rich protein (GLURP), and merozoite surface protein-1 (MSP-1) is performed (WHO, 2008) (Appendix 3). An assessment of the correlation between anti-malarial treatment outcome and molecular markers would improve the early detection and monitoring of drug resistance by *P. falciparum.*

The *Pfk13* propeller gene mutations have been associated with delayed parasite clearance (Imwong *et al.*, 2015). Delayed parasite clearance is usually associated with the reduced activity of artemisinins and recrudescence is associated with the less efficacy of the long acting drugs (piperaquine/lumefantrine) (Imwong *et al.*, 2015). Artemisinin resistance is characterized by slow parasite clearance in Thailand and Cambodia (Noedl *et al.*, 2008; Dondorp *et al.*, 2009). Clearance (assessed by microscopy) of sensitive *P. falciparum* parasite is achieved within 2 days in 95% of patients whereas artemisinin-resistant infections remain slide positive for 3 or more days; treatment failure is more common in such infections after ACT treatment (White, 2008). In western Kenya, the presence of residual sub-microscopic *P. falciparum* parasites in children on day 3 after ACT treatment was reported in a previous study and this was associated with subsequent recrudescence and transmission (Beshir *et al.*, 2013). However this was not associated with any mutations in the *Pfk13* propeller region. While the *Pfk13* propeller gene

mutations related to artemisinin resistance have not been reported in western Kenya there is need for continued surveillance of these mutations and determine whether the mutations, if any, affect treatment outcome.

Clinical trials performed in Africa (Sisowath *et al.*, 2005; Dokomajilar *et al.*, 2006b; Humphreys *et al.*, 2007; Zongo *et al.*, 2007; Happi *et al.*, 2009; Ljolje *et al.*, 2018) have provided evidence for the selection of particular *Pfmdr1* alleles in patients with newly acquired infections or recurrent *P. falciparum* infections within 28 or 42 days after ACT treatment. In Zanzibar, the post treatment prevalence of *Pfmdr1* was significantly higher than pre-treatment levels in parasites that re-infected patients within the 42-day follow-up period (Sisowath *et al.*, 2005). This finding is supported by evidence from Uganda (Dokomajilar *et al.*, 2006a), where AL treatment for uncomplicated malaria selected newly infecting parasites carrying the N86-184**F**-D1246 haplotype. In Nigeria, the *Pfmdr1* haplotype N86-184**F**-D1246 was significantly associated with treatment failures and was selected for among post treatment samples obtained from patients with newly acquired or recrudescing infections after treatment with AL (Happi *et al.*, 2009). In Angola, N86-Y184-D1246 haplotype was significantly more likely to be found in late treatment samples (Ljolje *et al.*, 2018). The *Pfmdr1* 86**Y** allele was also overrepresented in recrudescence plus reinfection samples from participants treated with AL (Ljolje *et al.*, 2018). In a study from Tanzania, treatment with AL was associated with selection of newly infecting parasites containing the mutant *Pfmdr1* N86 allele (Sisowath *et al.*, 2005). While this association has been observed in other countries in Africa, it remains to be investigated in western Kenya.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

Archived blood samples collected as part of a TES conducted at the Siaya County Referral Hospital (SCRH) and two nearby health facilities (Mulaha and Bar Agulu dispensaries) in Siaya County, western Kenya were used in this study. Siaya County (Figure 1) is located in northeast of Lake Victoria and lies between Latitude 0^{o} to 26' to 0^{o} to 18' North and Longitude 35^{o} to 58' East and 34^{o} to 33' West (Odhiambo *et al.*, 2012).

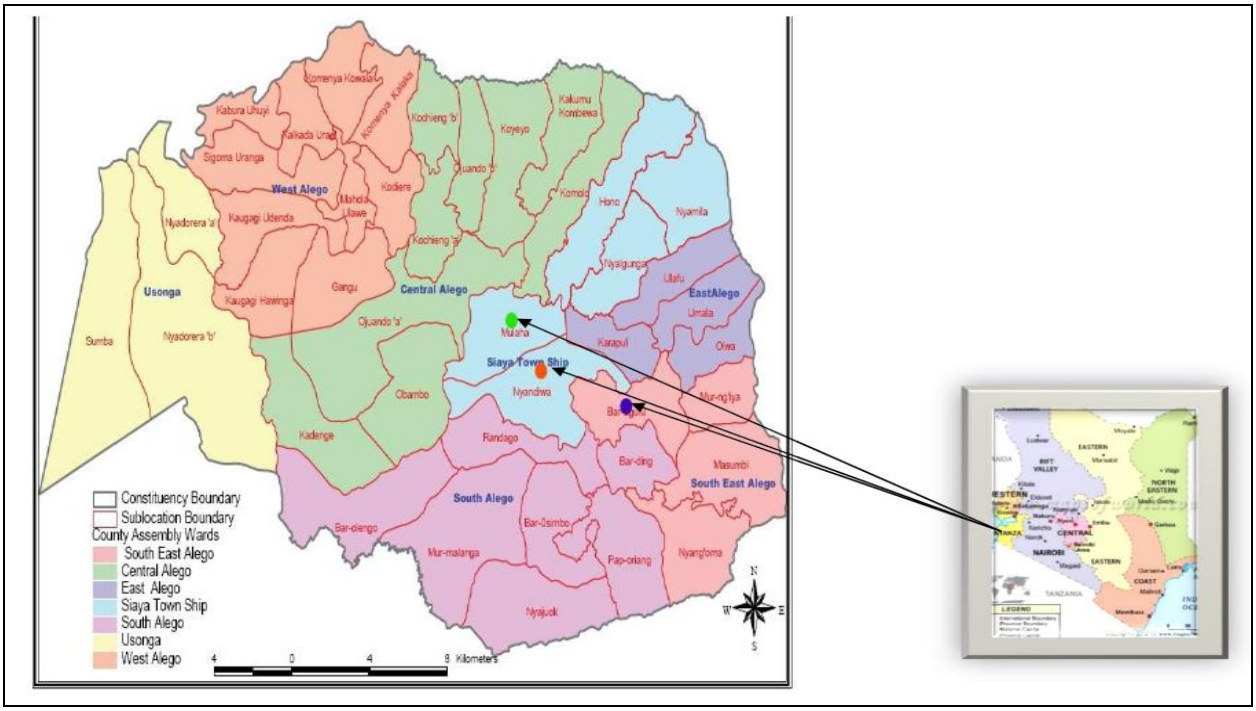


Figure 1: Map of study site

Figure showing the map of the study sites; SCRH, Mulaha and Bar Agulu which are located in Siaya County. Siaya County lies between Latitude 0^{o} 26' to 0^{o} 18' North and Longitude 35^{o} 58' East and 34^{o} 33' West (Odhiambo *et al.*, 2012).

Rainfall is seasonal in Siaya county with the 'long' rains usually occurring from March through July and 'short' rains between October and December with bimodal peaks in April-May and November-December (Odhiambo *et al.*, 2012). Average temperature ranges between 17.8^{0}C and 35.8^{0}C at a mean altitude of approximately 1070 meters above sea level (Odhiambo *et al.*, 2012). Malaria transmission is holoendemic in this area with two major seasonal peaks in July and November (Odhiambo *et al.*, 2012).

Siaya County was selected as the study site because it is one of the regions in Kenya with the highest prevalence of malaria (KMIS, 2015). A cross-sectional study conducted by KEMRI/CDC collaborative programme in July-August of 2013 in this area found a malaria prevalence of 64.9% by RDT in children <5 years of age (KEMRI/CDC unpublished data). In this county, 76.5% use AL and 23.5% used other antimalarial drugs for treatment of malaria (Onyango *et al.*, 2012). Adherence to ACT prescription in this county is 42.1% and 57.9% among individuals above 13 and less than 13 years, respectively (Onyango *et al.*, 2012).

3.3 Study Design

This laboratory-based experimental study used archived blood samples collected as part a TES carried out by the KEMRI-CDC (KEMRI SSC Protocol: 2857). Samples collected on pre-treatment (day 0) and on day of recurrent parasitaemia (either days 7, 14, 21, 28, 35, 42 or any sick visit) after treatment with AL or DP were used in this study. Since the samples available from the recently completed TES was within the sample size calculated for this study, all malaria positive samples by microscopy collected on day 0 and day of recurrent parasitaemia were analysed for molecular markers associated with parasite resistance to AL or DP.

3.2 Study Population

The current study used archived blood samples collected from children who were recruited in the TES study conducted in 2016-2017 to monitor the efficacy of AL and DP for treatment of uncomplicated *P. falciparum* malaria in western Kenya. The TES study enrolled participants attending the outpatient departments of SCRH, Mulaha and Bar Agulu dispensaries. The children aged 6-59 months were recruited and treated with either AL or DP. All patients had cleared their parasites by day 3. Of the 330 participants, 111 returned with a recurrent malaria infection. Genotyping of the MSP-1, MSP-2, and GLURP identified 24 recrudescent infections (15 and 9 in the AL and DP arms respectively). Archived samples, 323 pre-treatment samples and 110 recurrent infections, were used in this current study. Seven (7) day 0 samples and 1 recurrent infection sample were not analysed due to either insufficiency or they were missing.

Children were selected as the study population because malaria infection is frequently more fatal in children than in adults (Halliday *et al.*, 2014). In addition, WHO recommends that efficacy studies be conducted in children aged 6-59 months in areas of high malaria transmission like western Kenya as treatment failure manifests easily in this age group because of low immunity (WHO, 2009a, 2010b). The TES that was recently conducted in the study area also enabled comparison between the treatment outcomes with the molecular markers of drug resistance (KEMRI/CDC unpublished data).

3.4 Inclusion Criteria

The patients were recruited into the TES study as described by the WHO protocol on methods for surveillance of antimalarial drug efficacy (WHO, 2009a). Patients were eligible to participate in the study if they were aged 6-59 months, had symptoms compatible with uncomplicated

malaria and parasite densities of pure *P. falciparum* parasitaemia of >2,000 asexual stages/μL, body (axillary) temperature of >37.4°C or a history of fever in the 24 to 48 hours preceding presentation to the health facility, the absence of other concomitant illnesses, no history of antimalarial use in the 2 weeks preceding presentation and if parent/guardian provided written informed consent. Malaria positive samples by microscopy (slide-confirmed mono-infection with *P. falciparum)* collected on day 0 and on the day of recurrent parasitaemia were included in the current study.

3.5 Exclusion Criteria

Patients with severe malaria, severe malnutrition, serious underlying diseases (renal, cardiac, or hepatic diseases), and known allergies to the study drugs were excluded from the study. The samples were excluded from the current study if the parent/guardian did not provide written informed consent for long-term storage and testing of samples.

3.6 Sample Size Determination

Sample size was estimated using the Cochran's formula (Cochran, 1963). The formula was used because it is suitable for large populations and the equation yields a representative sample for proportions.

$\mathbf{n_o = (z^2pq)/e^2}$

$\mathbf{n_o}$ = sample size.

z = z value at 95% confidence interval (1.96).

p = estimated proportion in the population based on previous studies (64.9%).

q = 1-**p**.

e = level of precision (margin of error = 5%).

Hence $\mathbf{n_o} = (1.96)^2 \times 0.649\ (1-0.649)/\ (0.05)^2 = 314.14$

5% of the samples added to cater for inadequacy = 314.14*105% = 330. Based on the above formula, the sample size was 330.

3.7 Laboratory Procedures

3.7.1 Sample collection

A trained phlebotomist collected finger-prick blood sample using aseptic technique. The finger was prepared by cleaning with 70% alcohol swabs and allowed to air dry. A sterile lancet was pressed firmly against the finger to make a puncture. The first drop that contains excess tissue fluid was wiped away. The next drops of blood (300µL) were collected into an Ethylenediaminetetraacetic acid (EDTA) tube by gently massaging the finger. The tube was capped and then gently inverted eight times to mix the blood. A gauze pad was held over the puncture site for approximately 2 minutes to stop bleeding. The contaminated materials and lancets were disposed in biohazard waste containers and transported to a central laboratory for incineration. The specimens were appropriately labelled with study codes and collection dates. The sample collected was used for malaria microscopy and preparation of dried blood spots (DBS) for molecular genotyping of the parasite.

3.7.2 Malaria microscopy

Malaria microscopy was carried out according to WHO basic malaria microscopy guidelines, 2010. Thick (for parasite density determination) and thin (for *Plasmodium* species identification) smears was prepared from 6µL and 2µL of whole blood sample respectively. The smears were allowed to air dry and then stained with 3% Giemsa stain for 1 hour. The slides were read at 100x objective lens under oil immersion using a compound microscope for determination of both asexual and sexual stage of parasites. A blood smear was considered negative if 200 microscopic high-powered fields showed no parasites. If a blood smear was positive, malaria parasites were

counted in 40 microscopic high-powered fields. *Plasmodium falciparum* positive samples were genotyped for drug resistance markers.

3.7.3 Preparation of Dried Blood Spots

The DBS was prepared using 50µL of whole blood measured and spotted in Whatman 903 protein saver Cards (Pittsburgh, PA). The card was labelled with unique patient identifiers and left to air dry overnight at room temperature. Once the card dried, the DBS was placed into individual Ziploc bags with desiccants awaiting deoxyribonucleic acid (DNA) extraction.

3.7.4 Deoxyribonucleic acid (DNA) extraction

Genomic DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) as described by the manufacturer in the Qiagen DNA extraction manual. Whole blood spot was cut from the DBS and placed into 1.5mL micro-centrifuge tube. One hundred and eighty microliters (180µL) of tissue lysis buffer (ATL) was added and incubated at 85°C for 10 minutes. Proteinase K (20µL) solution was added, mixed by vortexing and incubated at 56°C for 1 hour. The mixture was centrifuged at 6,000 x g for 1 minute to remove drops from inside the lid. Two hundred microliters (200µL) of lysis buffer (AL1) was added. The mixture was mixed thoroughly by vortexing and incubated at 70°C for 10 minutes. The mixture was centrifuged at 6,000 x g for 1 minute to remove drops from inside the lid, 200µL of absolute ethanol (96-100%) added and mixed thoroughly by vortexing. The mixture was centrifuged at 6,000 x g for 1 minute to remove drops from inside the lid and applied to the QIAamp Mini spin column in a 2mL collection tube without wetting the rim. The cap was closed and centrifuged at 6,000 x g for 1 minute. The QIAamp Mini spin column was placed into a clean 2mL collection tube and the tube containing the filtrate discarded. The QIAamp Mini spin column was carefully opened and 500µL of wash buffer 1 (AW1) was added without wetting the rim. This was followed by

centrifugation at 6,000 x g for 1 minute. The QIAamp Mini spin column was placed in a clean 2mL collection tube and the collection tube containing the filtrate discarded. The QIAamp Mini spin column was carefully opened and 500µL of wash buffer 2 (AW2) added without wetting the rim. This was followed by centrifugation at 20,000 x g for 3 minutes. The QIAamp Mini spin column was placed in a sterile 1.5mL micro-centrifuge and the collection tube containing the filtrate discarded. The QIAamp Mini spin column was carefully opened and 100µL of elution buffer (AE) added without wetting the rim. Incubation at room temperature for 5 minutes was done followed by centrifugation at 6,000 x g for 1 minute to collect the DNA. The DNA was stored at -20°C for parasite genotyping for MSP-1 and MSP-2 and GLURP alleles and drug-resistance testing.

3.7.5 *Plasmodium falciparum* genotyping for MSP-1 and MSP-2 and GLURP alleles

Comparison of pre- and post-antimalarial drug therapy for *P. falciparum* isolates based on band sizes to determine as to whether the parasites are clonally a persistent infection (recrudescent) or new infection (re-infection) was done by genotyping the MSP-1, MSP-2 and GLURP alleles. The decision tree for genotyping *P. falciparum* using 'serial' testing method starting with MSP-2, followed by GLURP and finally MSP-1 was used (WHO, 2008) (Appendix 3).

Block 2 of MSP-l and block 3 of MSP-2 were amplified by nested Polymerase Chain Reaction (PCR) using Gene Amp PCR System 9700 (PE Biosystems, USA) following a modified protocol of that described by Snounou (Snounou, 2002). The first (primary) PCR was a multiplex reaction containing sets of primers for both blocks in one master mix reaction. Products of the first PCR were re-analyzed by allelic-family-specific primers in five separate master mix preparations corresponding to Kl, Mad20 and RO33 allelic families of MSP-l, and 3D7 as well as FC27 allelic

families of MSP-2. Multiplex primary PCR followed by nested PCR for MSP-1 and MSP-2 alleles was performed using 50-250ng of genomic DNA. Similar to the MSP blocks, primary PCR was done followed by nested PCR for GLURP allele using 50-250ng of genomic DNA. In all reactions, 25µL total PCR reaction volumes was used containing final concentrations of; 2mM $MgCl_2$ (Roche, Germany), 200µL deoxynucleoside triphosphates (dNTPs) (Applied Biosystems, USA), 1.25Units/µL Hotstart Taq polymerase (Takara Bio. Inc. Japan) and PCR water (Mediatech, inc. VA, USA). Every PCR run included positive controls for every MSP-l and MSP-2 allele *(P. falciparum* culture DNA) and a negative control (PCR water) (Mediatech, Inc., VA, USA). The summary of the primer details and thermoprofile for each reaction are in appendices 1 and 2 respectively.

The nested PCR products were run on agarose gels (3% for MSP-l and 2% for MSP-2 and GLURP alleles) (Sigma Chemical Co. MO, USA) pre-stained with 2µL ethidium bromide. From each of the nested PCR amplicons, 50-250ng of DNA was mixed with 1µL of 6× loading dye (New England Biolabs Ltd., Ontario, Canada) before loading into the gel wells. To separate the DNA amplicons into distinct bands, electrophoresis was carried out for 1 hour 30 minutes at 120 volts after which gels were observed under UV Trans illuminator at 312nm to visualize the bands. Enumeration of DNA bands and their molecular size analysis was done using Lab Works software, V.4.0 against 100bp standard molecular marker (New England Biolabs Ltd., Ontario, Canada).

Parasite isolates were classified as recrudescent or re-infection based on the genetic polymorphism in MSP-1, MSP-2 and GLURP. If the genotype pattern for the two paired samples

of a study participant were the same or had a difference of ≤20bp then the interpretation was recrudescence. A new infection was indicated when the difference in the band sizes was >20bp (WHO, 2008)(Appendix 3).

3.7.6 *Plasmodium falciparum kelch 13 (Pfk13)* propeller domain PCR amplification

Pfk13 amplification PCRs and sequencing were performed according to described method by Talundzic *et al.*, (2015).

3.7.6.1 Primary PCR amplification for *Pfk13* propeller domain

Primary PCR amplification for *Pfk13* propeller domain was performed using 50-250ng of DNA sample in a 25µL PCR mix containing 0.5µM (each) oligonucleotides with a sense sequence 5'-GCAAATAGTATCTCGAAT-3' and antisense 5'-CTGGGAACTAATAAAGAT-3'. Amplification was carried out in a 96-well format GeneAmp PCR system 9700 (Applied Biosystems, Foster City, USA), in reaction mixes containing final concentrations of 0.2µM dNTPs, 1.0 U HF Phusion DNA Polymerase and 1× Phusion HF Buffer supplied by the manufacturer. Cycling conditions were as follows: initial denaturation at 98°C for 2 minutes, followed 40 cycles each of denaturation at 98°C for 10 seconds, annealing at 48°C for 30 seconds and elongation at 68°C for 2 minutes and 30 seconds. A final extension of 68°C for 10 minutes was included, before cooling the products to 4°C.

3.7.6.2 Secondary PCR amplification for *Pfk13* propeller domain

Secondary PCR amplification for *Pfk13* propeller domain was performed using 50-250ng of primary PCR products in a 25µL PCR mix containing 0.5µM (each) oligonucleotides with a sense sequence 5'- GATAAACAAGGAAGAATATTCT -3' and antisense 5'-CGGAATCTAATATGTTATGTTCA -3' to obtain a 784 -bp fragment. Amplification was

carried out in a 96-well format GeneAmp PCR system 9700 (Applied Biosystems, Foster City, USA), in reaction mixes containing final concentrations of 0.2µM dNTPs, 1.0 U HF Phusion DNA Polymerase, and 1× Phusion HF Buffer supplied by the manufacturer. Cycling conditions were as follows: initial denaturation at 98°C for 2 minutes, followed 40 cycles each of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds, and elongation at 68°C for 1 minute. A final extension of 68°C for 10 minutes was included, before cooling the products to 4°C.

3.7.7 *Plasmodium falciparum multi-drug resistance protein 1 (Pfmdr1)* gene PCR amplification

Gene amplification and sequencing of the *Pfmdr1* gene was performed based on the Standard Operating Procedure (SOP) from CDC Atlanta Malaria laboratory.

3.7.7.1 Primary PCR amplification of *Pfmdr1* gene fragment for codon 86 and 184

Primary PCR amplification for *Pfmdr1* gene fragment for codon 86 and 184 was performed using 50-250ng of genomic DNA in a 25µL PCR mix containing 0.2µM (each) oligonucleotides with a sense sequence 5'-CCGTTTAAATGTTTACCTGCAC-3' and antisense 5'-TGGGGTATTGATTCGTTGCAC -3'. Amplification was carried out in a 96-well format GeneAmp PCR system 9700 (Applied Biosystems, Foster City, USA), in reaction mixes containing final concentration of 1× Promega Master Mix supplied by the manufacturer. Cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed 40 cycles each of denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute, and elongation at 72°C for 1 minute. A final extension of 72°C for 10 minutes was included, before cooling the products to 4°C.

3.7.7.2 Secondary PCR amplification of *Pfmdr1* gene fragment for codon 86 and 184

Secondary PCR amplification for *Pfmdr1* gene fragment for codon 86 and 184 was performed using 50-250ng of primary PCR products in a 25µL PCR mix containing 0.2µM (each) oligonucleotides with a sense sequence 5'-GTATGTGCTGTATTATCAGGAG -3' and antisense 5'- AGCCTCTTCTATAATGGACATG -3'. Amplification was carried out in a 96-well format GeneAmp PCR system 9700 (Applied Biosystems, Foster City, USA), in reaction mixes containing final concentration of 1× Promega master mix supplied by the manufacturer. Cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed 40 cycles each of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 45 seconds. A final extension of 72°C for 10 minutes was included, before cooling the products to 4°C.

3.7.7.3 Primary PCR amplification of *Pfmdr1* gene fragment for codon 1034-1246

Primary PCR amplification for *Pfmdr1* gene fragment for codon 1034-1246 was performed using 50-250ng of genomic DNA in a 25µL PCR mix containing 0.2µM (each) oligonucleotides with a sense sequence 5'- GCATTTAGTTCAGATGATGAAATG -3' and antisense 5'- CCATATGGTCCAACATTTGTATC -3'. Amplification was carried out in a 96-well format GeneAmp PCR system 9700 (Applied Biosystems, Foster City, USA), in reaction mixes containing final concentration of 1× Promega Master Mix supplied by the manufacturer. Cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed 40 cycles each of denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute, and elongation at 65°C for 1 minute. A final extension of 72°C for 10 minutes was included, before cooling the products to 4°C.

3.7.7.4 Secondary PCR amplification of *Pfmdr1* gene fragment for codon 1034 and 1042

Secondary PCR amplification for *Pfmdr1* gene fragment for codon 1034 and 1042 was performed using 50-250ng of primary PCR products in a 25µL PCR mix containing 0.2µM (each) oligonucleotides with a sense sequence 5'-GTATGTGCTGTATTATCAGGAG-3' and antisense 5'-TTTTGCATTTTCTGAATCTCCTT -3'. Amplification was carried out in a 96-well format GeneAmp PCR system 9700 (Applied Biosystems, Foster City, USA), in reaction mixes containing final concentration of 1× Promega Master Mix supplied by the manufacturer. Cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed 40 cycles each of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, and elongation at 72°C for 45 seconds. A final extension of 72°C for 10 minutes was included, before cooling the products to 4°C.

3.7.7.5 Secondary PCR amplification of *Pfmdr1* gene fragment for codon 1246

Secondary PCR amplification for *Pfmdr1* gene fragment for codon 1246 was performed using 50-250ng of primary PCR products in a 25µL PCR mix containing 0.2µM (each) oligonucleotides with a sense sequence 5'- GCAATCGTTGGAGAAACAGG -3' and antisense 5'- TTCGATAAATTCATCTATAGCAG -3'. Amplification was carried out in a 96-well format GeneAmp PCR system 9700 (Applied Biosystems, Foster City, USA), in reaction mixes containing final concentration of 1× Promega Master Mix supplied by the manufacturer to obtain a 414-bp product. Cycling conditions were follows: initial denaturation at 95°C for 5 minutes, followed 40 cycles each of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C for 45 seconds. A final extension of 72°C for 10 minutes was included, before cooling the products to 4°C.

3.7.8 Staining and visualization

To ensure that the PCR was successful (appearance of bands with the correct base pairs) and the obtained products were the correct base pair before sequencing, the resulting products were resolved on a 1% agarose gel, stained with 10µL GelRed nucleic acid stain, and visualized on an UV Trans illuminator (Spectroline Corporation, Westbury, NY).

3.7.9 Sanger sequencing by capillary electrophoresis

Sanger sequencing by capillary electrophoresis was performed using the following procedure:

3.7.9.1 PCR product purification

The PCR products were purified by use of Exonuclease I - Shrimp Alkaline Phosphatase (USB, Affymetrix, USA) reagent. The reagent was prepared by mixing 0.025µL Exonuclease I, 0.25µL Shrimp Alkaline Phosphatase and 4.725µL of water to make a total volume of 5µL. The mixture was mixed well by vortexing and 4µL was added per well. A total of 10µL of amplified PCR products was added to each well. The reaction plate was sealed with an adhesive film and centrifuged briefly. Amplification was carried out in a 96-well format GeneAmp PCR system 9700 (Applied Biosystems, Foster City, USA). Cycling conditions were as follows: 37°C for 25 minutes, followed by 80°C for 20 minutes and the products cooled to 4°C.

3.7.9.2 Cycle sequencing

Cycle sequencing experiments were performed using 1µL of purified PCR products in a 10µL mix in two separate reaction plates with 10µM oligonucleotides of a sense and antisense primers. Amplification was carried out in a 96-well format GeneAmp PCR system 9700 (Applied Biosystems, Foster City, USA), in reaction mixes containing final concentrations of 5.9µL water, 2µL of 5x buffer and 0.3µL of big dye supplied by the manufacturer (BigDye terminator cycle

sequencing kit; Applied Biosystems, Foster City, USA). Cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed 39 cycles each of denaturation at 95°C for 30 seconds, annealing at 55°C for 35 seconds, and elongation at 72°C for 45 seconds. A final extension of 72°C for 5 minutes was included, before cooling the products to 4°C.

3.7.9.3 Sequencing reaction purification

Ethanol (100% and 70%) was pre-chilled at 4^0 C fridge before use. Two microliters of 125mM EDTA (pH 8.0) was added per well of 10μL sequencing reaction. Then, 2μL of 3M Sodium Acetate (pH 5.2) was added per well. Then, 30μL of cold 100% ethanol was added per well and mixed well by pipetting. The plate was sealed and incubated at room temperature for 15 minutes then centrifuged at 3200rpm for 30 minutes at 15^0C with acceleration set at 9 and deceleration set at 5. Immediately, the plate seal was removed, it was wrapped with paper towel, inverted and centrifuged at 1000 x g for 5 minutes at 15^0C with acceleration set at 9 and deceleration set at 5. Thirty microliters of cold 70% ethanol was added per well and mixed well by vortexing. The plate seal was removed immediately, the plate wrapped with paper towel, inverted and centrifuged at 1000 x g for 5 minutes at 15^0C with acceleration set at 9 and deceleration set at 5. The plate was air dried for 1 hour to remove ethanol. Hi-Di formamide (12μL) was added per well and mixed well, plate sealed with gray septa and centrifuged briefly. Sequencing was carried out in ABI 3130XL DNA sequencer (Applied Biosystems, Foster City, USA).

3.8 Data Management and Statistical Analysis

3.8.1 Prevalence of *Pfk13* SNPs, *Pfmdr1* haplotypes and *Pfmdr1* SNPs

Geneious R10 software (Biomatters, San Francisco, CA, USA) was used to identify specific SNPs by assembling the sequences with annotated reference sequences of the gene of interest.

The reference sequences obtained from http://plasmodb.org. The SNPs at *Pfk13* propeller region were identified by comparing with reference 3D7 strain (PF3D7_1343700). The SNPs at *Pfmdr1* gene were identified by comparing with reference 3D7 strain (PF3D7_0523000). The count of samples with wild type and mutant alleles was used to generate the prevalence of the *Pfk13* SNPs, *Pfmdr1* haplotypes, and *Pfmdr1* SNPs. Samples with mixed alleles were considered mutant.

3.8.2 Association between *Pfmdr1* gene or *Pfk13* propeller region polymorphisms with treatment outcome

Statistical analysis was performed using MedCalc software (Acacialaan 22, Oostend, Belgium). The association between treatment outcome and either *Pfmdr1* gene or *Pfk13* propeller region polymorphisms was analyzed using two methods as decribed by (Ljolje *et al.*, 2018). In the first method, the prevalence of the polymorphisms in pretreatment samples was compared between patients who experienced ACPR and patients who experienced recrudescence. The relative risk of recrudescence associated with a given polymorphism was calculated as the ratio of the probability of recrudescence in patients carrying the polymorphism prior to treatment versus the probability of recrudescence in patients with wild type pretreatment parasites. In the second method, the prevalence of polymorphisms was compared between pretreatment samples from cases of ACPR and day of failure samples from all cases of recurrent infections (recrudescence and reinfection). Statistical significance of association between any observed mutations and the treatment outcome (ACPR versus recrudescent infections or ACPR versus recurrent infections) was tested using a Fisher's exact test. Statistical significance was defined by a p value of <0.05 for all analyses.

3.9 Ethical Considerations

Approval for the study, which included testing of molecular markers for drug resistance, was obtained from the Kenya Medical Research Institute-Scientific and Ethics Review Unit (KEMRI/SERU); KEMRI SSC Protocol: 2857 (Appendix 6). Proposal approval for this study was obtained from Maseno University School of Graduate Studies (SGS) (Appendix 5). This current study only used samples which parent/guardian provided written informed consent for long-term storage of samples and future testings (Appendix 7). The current study was conducted in compliance with the protocol, good clinical practice (GCP) guidelines, and all applicable regulatory requirements. Participant's confidentiality was ensured by coding and omitting information that identifies them.

CHAPTER FOUR

RESULTS

4.1 Clinical and Laboratory Characteristics of the Study Group

Three hundred and twenty three (323) pre-treatment samples collected at enrolment and the 110 samples collected on any other day when enrolled participants returned with a recurrent malaria infection were analysed for drug resistance markers.

A summary of clinical and laboratory characteristics of the study population (pre-treatment samples) is shown in Table 1. The gender, age and baseline parasite density were comparable among the two treatment arms (gender: Chi-square test, $p = 0.960$; age: Mann-Whitney U test, $p = 0.129$ and baseline parasite density: Mann-Whitney U test, $p = 0.857$). There was statistically significant association in treatment outcomes with AL arm having significantly higher number of recurrent infections than the DP arm (Chi-square test, $p <$ **0.001**).

Table 1: Clinical and laboratory characteristics of the study group (pre-treatment)

Characteristic	Treatment arm AL (n=160)	DP (n=163)	*p value*
Gender N (%)			
Male	82 (51.2)	85 (52.1)	0.960[b]
Female	78 (48.8)	78 (47.2)	
Age, months[a]	31	33	0.129[c]
Baseline parasite density, parasites/µL[a]	76,315	77,444	0.857[c]
Treatment outcome			
ACPR, N (%)	8 (52.5)	126 (78.8)	**<0.001**[b]
Total recurrent infections, N (%)	76 (47.5)	34 (21.2)	

Table showing the summary of clinical and laboratory characteristics of the pre-treatment samples. n is the total number of samples. N is the total number of samples with the characteristic. [a] Data are presented as medians. [b]Statistical significance determined by the Chi-square test; [c] Statistical significance determined by Mann-Whitney U test.

A summary of clinical and laboratory characteristics of recurrent infection samples is shown in Table 2. The gender, age, parasite density and recurrent infections were comparable among the two treatment arms (gender: Chi-square test, p = 0.825; age: Mann-Whitney U test, p = 0.490: parasite density: Mann-Whitney U test, p = 0.453 and recurrent infections: Chi-square test, p = 0. 648).

Table 2: Clinical and laboratory characteristics of the study group (recurrent infections)

	Treatment arm		
Characteristic	**AL (n=67)**	**DP (n=43)**	***p value***
Gender N (%)			
Male	30 (44.8)	21 (48.8)	0.825[b]
Female	37 (55.2)	22 (51.2)	
Age, months[a]	35	31	0.490[c]
Parasite density, parasites/µL[a]	16,092	26,981	0.453[c]
Total recurrent infections			
Recrudescence, N (%)	15 (23.1)	9 (27.3)	0. 648[b]
New-infection, N (%)	50 (76.9)	24 (72.7)	

Table showing the summary of clinical and laboratory characteristics of the recurrent infections. n is the total number of samples. N is the total number of samples with the characteristic. [a] Data are presented as medians. [b]Statistical significance determined by the Chi-square test; [c] Statistical significance determined by Mann-Whitney U test.

4.2 Samples Successfully Sequenced for *Pfmdr1* gene and *Pfk13* Propeller Region

For the pre-treatment samples (day 0), sequencing of the *Pfk13* propeller region gene was successful for 317/323 (98.1%), and sequencing of the *Pfmdr1* gene was successful for 320/323 (99.1%). For the recurrent infection samples, sequencing of the *Pfk13* propeller region was successful for 88/110 (80.0%) and sequencing of the *Pfmdr1* gene was successful for 95/110 (86.4%), (Table 3).

Table 3: Total number of samples successfully sequenced for *Pfmdr1* gene and *Pfk13* propeller region

Samples	***Pfk13* propeller region**	***Pfmdr1* gene**
Pre-treatment (Day 0)	317/323 (98.1%)	320/323 (99.1%)
Recurrent infections	88/110 (80.0%)	95/110 (86.4%)
Total	405/433 (93.5%)	415/433 (95.8%)

Table showing the total number of samples successfully sequenced for *Pfmdr1* gene and *Pfk13* propeller region.

4.3 Prevalence of *Pfk13* Polymorphisms from Pre-treatment and Recurrent Infection Samples

For all the samples tested, no mutations were detected in codons that have been previously validated to be correlated with artemisinin resistance. These codons are; 458 (N458**Y**), 493 (Y493**H**), 539 (R539**T**), 543 (I543**T**), 561 (R561**H**), and 580 (C580**Y**). However, other non-synonymous mutations which have not been associated with resistance were detected. For example: For day 0 samples, 5/317 (1.6%) had the A578**S** *Pfk13* mutation, 2/317 (0.6%) had S522**C** *Pfk13* mutation, 1/317 (0.3%) had E596**D** *Pfk13* mutation, and the remaining samples 309/317 (97.5%) were wild type for *Pfk13* (Table 3). For recurrent infection samples, 2/88 (2.3%) had the A578**S** *Pfk13* mutation, 1/88 (0.6%) had C580**F** *Pfk13* mutation, and the remaining samples, 85/88 (96.6%) were wild type for *Pfk13* (Table 4).

Table 4: Prevalence of *Pfk13* polymorphisms in pre-treatment and recurrent infection samples

Molecular marker	**Pre-treatment (day 0) samples N/n (%)**	**Recurrent infection samples N/n (%)**
Pfk13	n=317	n=88
Wild type	309/317 (97.5%)	85/88 (96.6%)
S522**C**	2/317 (0.6%)	0
A578**S**	5/317 (1.6%)	2/88 (2.3%)
C580**F**	0	1/88 (1.1%)
E596**D**	1/317 (0.3%)	0

Table showing the prevalence of *Pfk13* polymorphisms in pre-treatment and recurrent infection samples. n is the total number of samples. N is the total number of samples with the characteristic.

4.4 Prevalence of *Pfmdr1* Haplotypes from Pre-treatment and Recurrent Infection Samples

The results for *Pfmdr1* haplotypes are shown in Figure 2. The haplotype **YF**SND was identified in 0.3% of the day 0 isolates and 1.1% of the recurrent infection isolates. The haplotype N**F**SND was identified in 53.6% of the day 0 isolates and 58.9% of the recurrent infection isolates. The haplotype N**F**SN**Y** was identified in 5.6% of the day 0 isolates and 2.1% of the recurrent infection isolates. The haplotype NYSN**Y** was identified in 3.7% of the day 0 isolates and 3.2% of the recurrent infection isolates. No isolates, both day 0 and recurrent infection had haplotypes **YF**SN**Y**, **Y**YSN**Y** and **Y**YSND. The wild type haplotype NYSND was identified in 36.8% of the day 0 samples and 34.7% of the recurrent infection samples.

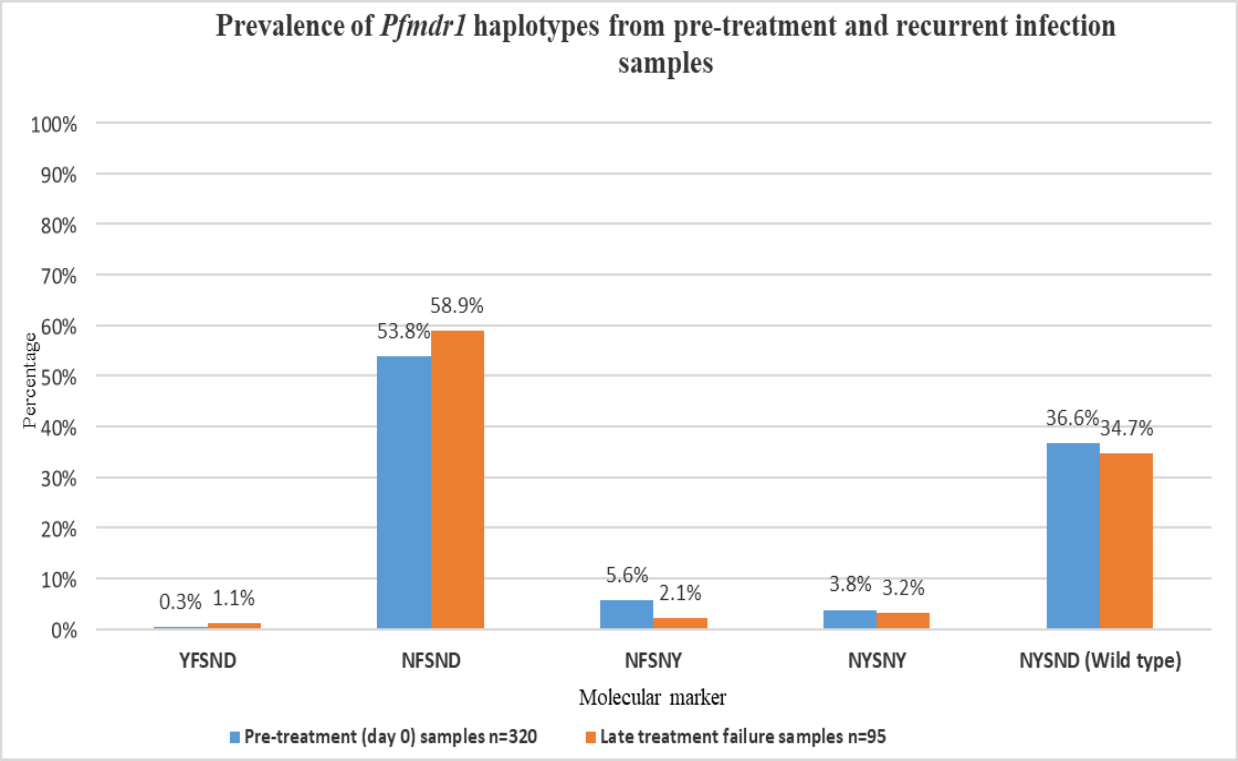


Figure 2: Prevalence of *Pfmdr1* haplotypes from pre-treatment and recurrent infection samples

Bar graph showing the prevalence *Pfmdr1* haplotypes for pre-treatment and recurrent infection samples.

4.5 Prevalence of *Pfmdr1* SNPs from Pre-treatment and Recurrent Infection Samples

The results for *Pfmdr1* polymorphisms are shown in Figure 3. Mutation at codon 86 (N86**Y**) was identified in 0.3% of day 0 samples and 1.1% of the recurrent infection samples. Mutation at codon 184 (Y184**F**) was identified in 59.7% of the day 0 samples and 62.1% of the recurrent infection samples. Mutation in codon 1246 (D1246**Y**) was identified in 9.4% of the tested day 0 samples and 5.3% of the recurrent infection samples. The prevalence of wild type samples was 36.6% for tested day 0 samples and 34.7% day of recurrent infection samples. No mutation was detected at codon 1034 (S1034**C**) and 1042 (N1042**D**) for both day 0 and recurrent infection samples.

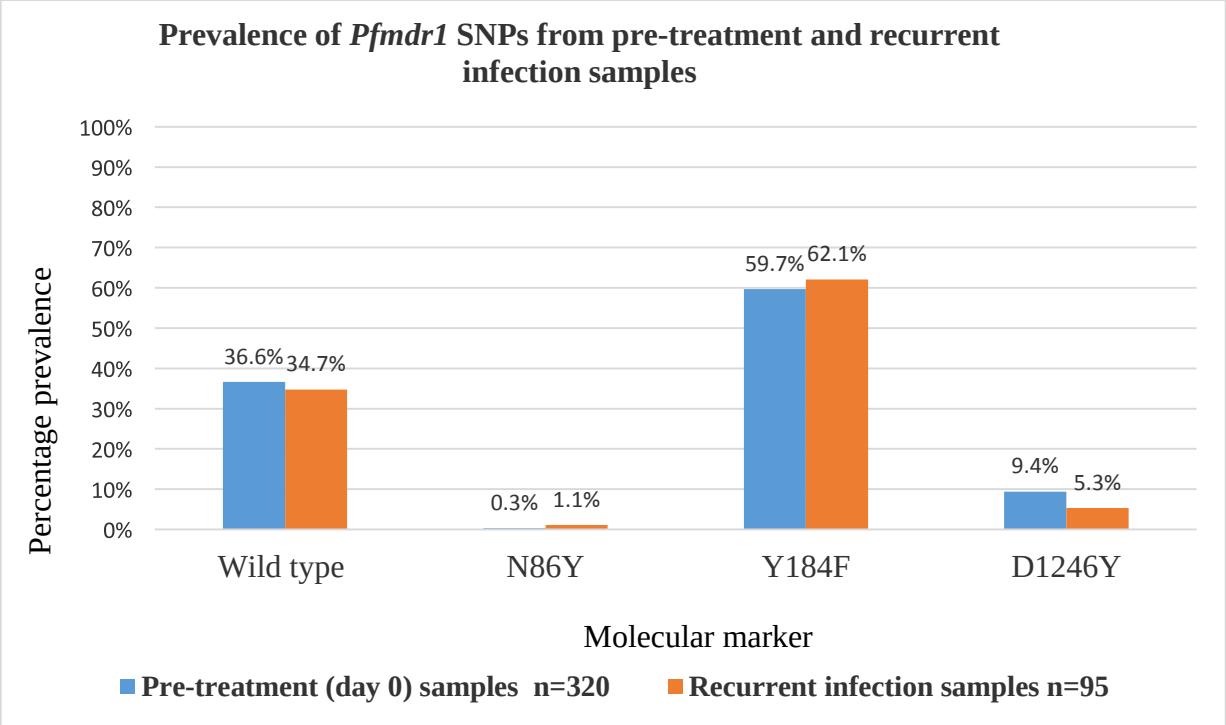


Figure 3: Prevalence of *Pfmdr1* SNPs from pre-treatment and recurrent infection samples

Bar graph showing the prevalence *Pfmdr1* SNPs for pre-treatment and recurrent infection samples.

4.6 Association between *Pfk13* and *Pfmdr1* Polymorphisms with Recurrent Infections in AL Treatment Arm

The association between gene polymorphisms and recurrent infection samples in AL treatment arm is as shown in Table 5. Statistical significance of association between any observed mutations and the treatment outcome was tested using a Fisher's exact test. For *Pfk13* propeller region, there was no statistically significant difference in risk of treatment failure (recrudescence) (S522**C**: relative risk (RR) = 0, p = 1 and C580**F**: RR = 0, p = 0.16). Additionally, there was no statistically significant difference in risk of treatment failure (recrudescence or reinfection) (C580**F**: p = 0.417).

For *Pfmdr1* SNPs, there was no statistically significant difference in risk of recrudescence (86**Y:** RR = 0**,** p **=** 1**;** 184**F:** RR = 0.031**,** p **=**1; and 1246**Y**: RR = 0.032, p = 1). Additionally, there was no statistically significant difference in risk of recrudescence or reinfection (184**F**: p = 0.730 and 1246**Y**: p = 0.428).

For *Pfmdr1* haplotypes, there was no statistically significant difference in risk of recrudescence (**YF**SND: RR = 0, p = 1; N**F**SND: RR= 0.097, p = 1; N**F**SN**Y**: RR = 0.023, p = 1; and NYSN**Y:** RR = 0.040, p = 0.573). Additionally there was no statistically significant difference in risk of recrudescence or reinfection (**YF**SND: p = 1; N**F**SND: p = 1: N**F**SN**Y**: p = 0.283, and NYSN**Y**: p = 1).

Table 5: Association between *Pfk13* and *Pfmdr1* polymorphisms and recurrent infections in AL treatment arm

Molecular marker	Artemether-lumefantrine (AL) treatment arm					
	ACPR N/n (%)	**Recrudescent (DF) N/n (%)**	**Relative Risk**[a]	***p***[b]	**Recrudescence +Reinfection (DF) N/n (%)**	***p***[c]
***Pfk13* SNPs**						
Wild type	**84/85 (98.8%)**	**14/15 (93.3%)**	**Ref**	**Ref**	**59/60 (98.3%)**	**Ref**
S522**C**	1/85 (1.2%)	0	0	1	0	1
A578**S**	0	0	0	1	0	1
C580F	0	1/15 (6.7%)	0	0.16	1/60 (1.7%)	0.417
E596D	0	0	0	1	0	1
***Pfmdr1* SNP**						
N86 (wild type)	**84/85 (98.8%)**	**15/15 (100.0%)**	**Ref**	**Ref**	**62/62 (100.0%)**	**Ref**
86**Y**	1/85 (1.2%)	0	0	1	0	1
Y184 (wild type)	**29/85 (34.1)%**	**5/15 (33.3%)**	**Ref**	**Ref**	**23/62 (37.1%)**	**Ref**
184**F**	56/85 (65.9%)	10/15 (66.7%)	0.031	1	39/62 (62.9%)	0.730
D1246 (wild type)	**74/85 (87.1%)**	**13/15 (86.7%)**	**Ref**	**Ref**	**57/62 (91.9%)**	**Ref**
1246**Y**	11/85 (12.9%)	2/15 9 (13.3%)	0.032	1	5/62 (8.1%)	0.428
***Pfmdr1* haplotype**						
NYSND (wild type)	**25/85 (2.4%)**	**4/15 (26.7%)**	**Ref**	**Ref**	**20/62 (32.3%)**	**Ref**
YFSND	1/85 (1.2%)	0	0	1	0	1
N**F**SND	48/85 (56.5%)	9/15 (60.0%)	0.097	1	37/62 (59.7%)	1
N**F**SN**Y**	7/85 (8.2%)	1/15 (6.7%)	0.023	1	2/62 (3.2%)	0.283
NYSN**Y**	4/85(4.7%)	1/15 (6.7%)	0.040	0.573	3/62 (4.8%)	1

Association between *Pfk13* and *Pfmdr1* polymorphisms and recurrent infections in AL treatment arm. SNP: single nucleotide polymorphism; ACPR: adequate clinical and parasitological response; DF: day of failure; Ref: reference; [a] Relative risk of treatment failure (recrudescence); [b] Statistical significance of difference in risk of treatment failure (recrudescence) determined by fishers exact test; [c] Statistical significance of difference in risk of treatment failure (recrudescence or reinfection) determined by fishers exact test.

4.7 Association between *Pfk13* and *Pfmdr1* Polymorphisms with Recurrent Infections in DP Treatment Arm

The association between gene polymorphisms and recurrent infections in DP treatment arm is as shown in Table 6. Statistical significance of association between any observed mutations and the treatment outcome was tested using a Fisher's exact test. For *Pfk13* propeller region, there was no statistically significant difference in risk of treatment failure (recrudescence) (S522C: RR =0, p = 1 and A578S: RR = 0.106, p = 0.222). Additionally, there was no statistically significant difference in risk of treatment failure (recrudescence or reinfection) (A578S: p = 0.227).

For *Pfmdr1* SNPs, there was no statistically significant difference in risk of recrudescence (86Y: RR = 0 p = 0.067; 184F: RR = 0.005, p = 0.735 and 1246Y: RR = 0, p = 1). Additionally, there was no statistically significant difference in risk of recrudescence or reinfection (86Y: p = 0.208; 184F: p = 1 and 1246Y: p = 0.207).

For *Pfmdr1* haplotypes, there was no statistically significant difference in risk of recrudescence (YFSND: RR = 0, p = 0. 074; NFSND: RR = 0.004, p = 1, NFSNY: RR = 0, p = 1 and NYSNY: RR = 0, p = 0.573). Additionally there was statistically significant difference in risk of recrudescence or reinfection (YFSND: p = 0.219, NFSND: p = 1, NFSNY: p = 0.575, and NYSNY: p = 1).

Table 6: Association between *Pfk13* and *Pfmdr1* polymorphisms and recurrent infections in DP treatment arm

Molecular marker	Dihydroartemisinin-piperaquine (DP) treatment arm					
	ACPR	Recrudescent (DF)	Relative Risk[a]	*p*[b]	Recrudescence +Reinfection (DF)	*p*[c]
***PfK13* SNP**						
Wild type	**122/126 (96.8%)**	**7/8 (87.5%)**	**Ref**	**Ref**	**26/28 (92.6%)**	**Ref**
S522**C**	1/126 (0.8%)	0	0	1	0	1
A578**S**	3/126 (2.4%)	1/8 (12.5%)	0.106	0.222	2/28 (7.1%)	0.227
C580**F**	0	0	0	1	0	1
E596D	0	0	0	1	0	1
***Pfmdr1* SNP**						
N86	**126/126 (100.0)%**	**8/9 (88.9%)**	**Ref**	**Ref**	**32/33 (96.7)%**	**Ref**
86**Y**	0	1/9 (11.1%)	0	0.067	1/33 (3.0%)	0.208
Y184	**53/126 (42.1%)**	**3/9 (33.3%)**	**Ref**	**Ref**	**13/33 (39.4%)**	**Ref**
184**F**	73/126 (57.9%)	6/9 (66.7%)	0.005	0.735	20/33 (60.6%)	1
D1246	**118/126 (93.7%)**	**9/9 (100%)**	**Ref**	**Ref**	**33/33 (100%)**	**Ref**
1246**Y**	8/126 (6.3%)	0	0	1	0	0.207
***Pfmdr1* haplotype**						
NYSND (Wild Type)	**50/126 (39.7%)**	**3/9 (33.3%)**	**Ref**	**Ref**	**13/33 (39.4%)**	**Ref**
YFSND	0	1/9 (11.1%)	0	0.074	1/33 (3%)	0.219
N**F**SND	68/126 (54.0%)	5/9 (55.6%)	0.004	1	19/33 (57.6%)	1
N**F**SN**Y**	5/126 (4.0%)	0	0	1	0	0.575
NYSN**Y**	3/126 (2.4%)	0	0	1	0	1

Association between *Pfk13* and *Pfmdr1* polymorphisms and recurrent infections in DP treatment arm. SNP: single nucleotide polymorphism; ACPR: adequate clinical and parasitological response; DF: day of failure; Ref: reference; [a] Relative risk of treatment failure (recrudescence); [b] Statistical significance of difference in risk of treatment failure (recrudescence) determined by fishers exact test; [c] Statistical significance of difference in risk of treatment failure (recrudescence or reinfection) determined by Fishers exact test.

CHAPTER FIVE

DISCUSSION

5.1 Introduction

The emergence of drug resistance in *P. falciparum* has been a major contributor to the global burden of malaria (Berzosa *et al.*, 2017). Drug resistance complicates treatment, and is one of the most important problems in malaria control (Berzosa *et al.*, 2017). The use of ACTs was recommended by WHO in 2005 as first-line treatments for falciparum malaria in all countries where malaria was endemic (Nosten *et al.*, 2007). This was due to emergence and rapid spread parasite resistance to previously recommended drugs, chloroquine or SP (Happi *et al.*, 2009). Artemisinin combination therapies consists of an artemisinin component, which rapidly clears most parasites, and a longer acting partner drug, which eliminates remaining parasites and limits selection of drug resistance (Nosten *et al.*, 2007). With the on-going challenges related to emergence of artemisinin resistance in Southeast Asia and threat of resistance in Africa (Ljolje *et al.*, 2018), continued surveillance is important for monitoring treatment efficacy and genetic markers associated with anti-malarial drug resistance in Kenya and the rest of malaria-endemic Africa.

5.2 Prevalence of Gene Mutations in the *Pfk13* Propeller Region

For all the samples tested, no *Pfk13* propeller region mutations that have been previously validated to be correlated with artemisinin resistance were detected (N458**Y**, Y493**H**, R539**T**, I543**T**, R561**H**, and C580**Y)**. These findings are consistent with previous studies conducted in western Kenya which did not report any *Pfk13* propeller region mutations that have been

associated with artemisinin resistance in Southeast Asia (Kamau *et al.*, 2015; Muwanguzi *et al.*, 2016). The absence of these *Pfk13* mutations associated with artemisinin resistance in this study in western Kenya as well as in other African countries (Menard *et al.*, 2016) might suggest that artemisinin resistance has not yet emerged on the continent or spread from the Southeast Asia. The recent emergence and spread of multidrug resistance in the Southeast Asia, however, indicates the importance of continuous monitoring of the molecular markers for resistance to artemisinin and partner drugs.

Other non-synonymous mutations which have not been associated with resistance were detected (Table 4) in the *Pfk13* propeller region. The prevalence of the A578**S** mutant allele was highest in recurrent samples, at 2.3%, compared with 1.6% in day 0 samples. The changes were seen without any impact on clinical resistance to artemisinin as observed by absence of parasites on day 3. The A578**S** mutation in propeller region of *Pfk13* has previously been observed in parasites from western Kenya with a prevalence of 1.4% to 2.7% (Kamau *et al.*, 2015; Muwanguzi *et al.*, 2016). However, the presence of mutant A578**S** in 2.3% late treatment failure parasites in DP treatment arm, could suggest that there is selection pressure by DP and needs further investigation as drug pressure increases with increased use of DP.

The current study is the first to report S522**C**, E596**D** and C580**F** *Pfk13* mutations in western Kenya. The presence of these mutations could be an indication that *Pfk13* SNPs are emerging in western Kenya. Of particular concern is the C580**F** mutation which was detected in one recrudescent sample in AL arm. The mutation is in the same amino acid position as the C580**Y** mutation, which was found to be the most critical genetic determinant of artemisinin resistance

in Southeast Asia. Additional molecular and biochemical studies should investigate whether this mutation alter the functions of this protein, resulting in altered artemisinin sensitivity.

5.3 Association between *Pfk13* Propeller Region Mutations with Treatment Outcomes

In this study only one DP and one AL-treated patients with recrudescent parasites harbored a non-synonymous mutation at codon 578 (A578**S**) and 580 (C580**F)** of *Pfk13* propeller region respectively in the day of failure samples (Table 5). However, no statistically significant association was observed (Fisher statistic; $p = 0.222$ and $p = 0.160$ respectively).

The lack of association of A578**S** polymorphism with phenotype is consistent with previous studies conducted in western Kenya, and in four other African countries (Kamau *et al.*, 2015; Muwanguzi *et al.*, 2016) which also showed no association with phenotype. These findings are also consistent with multi-site studies in Southeast Asia and Africa that showed mutation at codon 578 (A578**S**) in *Pfk13* and its lack of association with drug resistance in patients treated with ACT (Ashley *et al.*, 2014). This mutation has commonly been observed in Africa and has been confirmed as not being associated with artemisinin resistance *in vitro* or *in vivo* (Menard *et al.*, 2016).

This data shows that western Kenya remains free of the *Pfk13* propeller region mutations linked to delayed parasite clearance. Despite its lack of association with phenotype, the need for further studies in a large population to assess whether *Pfk13* propeller region mutations are relevant in determining artemisinin resistance in parasite isolates in sub-Saharan Africa. It is possible that the actual SNP(s) that confer resistance are different from one location to another depending on several factors including parasite genetics, malaria transmission intensity, treatment seeking

behavior and adherence to treatment guidelines. In Southeast Asia where ACT resistance emerged, use of artemisinin monotherapy tablets and substandard drugs is rampant which could explain why ACT resistance has emerged in Southeast Asia but has not emerged in other areas (Kaur *et al.*, 2016). In addition, due to limited number of observed mutations in *Pfk13* propeller region, the correlation of observed mutations in the samples from patients who responded to the ACT therapy to those who failed the treatment remains inconclusive. Further phenotyping and genotyping studies will be needed to determine whether artemisinin resistance has spread to or emerged in western Kenya.

5.4 Prevalence of Gene Mutations in the *Pfmdr1* Gene

The present study revealed the high prevalence of Y184**F** mutation in *Pfmdr1* gene. Mutation at codon 184 (Y184**F**) was identified in 59.7% of the day 0 samples and 62.1% of the recurrent infection samples. Mutation in codon 1246 (D1246**Y**) was identified in 9.4% of the tested day 0 samples and 5.3% of the recurrent infection samples. Mutation at codon 86 (N86**Y**) was identified in 0.3% of the day 0 samples and 1.1% of the recurrent infection samples. No mutation was detected at codon 1034 (S1034**C**) and 1042 (N1042**D**) for both day 0 and recurrent infection samples.

The overall prevalence of mutant alleles in *Pfmdr1* N86**Y** decreased significantly over time in comparison to a previous study conducted in Siaya county, western Kenya which reported a prevalence of 69% in samples collected in 2010 (Shah *et al.*, 2015) compared to 0.3% and 1.1% of the day 0 and recurrent infection samples respectively in the current study. Another study conducted in Kisumu County, western Kenya reported a prevalence of 7.6% mutant alleles in *Pfmdr1* N86**Y** in samples collected in 2014 which is consistent with the current findings

(Achieng *et al.*, 2015). The selection of mutant alleles is likely due to the withdrawal of chloroquine and the widespread use of AL as first-line antimalarial treatment in the Kenya, which promoted the selection of the wild-type sequences at this allele as observed in another study in western Kenya (Achieng *et al.*, 2015) in other countries in Africa (Dokomajilar *et al.*, 2006a; Some *et al.*, 2014; Straimer *et al.*, 2015; Achieng *et al.*, 2015).

For *Pfmdr1* Y184**F**, the prevalence of mutant alleles increased over time in comparison to a previous study in Siaya county, western Kenya which reported a prevalence of 23.3 % (Shah *et al.*, 2015) compared to 59.7% and 62.1% in day 0 samples and recurrent infection samples respectively in the current study. The results are consistent with another study conducted in Kisumu County, western Kenya which reported a prevalence of 65% in post ACTs parasites. The data suggests that 184**F** mutant is being is being selected for by the ACTs.

For *Pfmdr1* D1246**Y**, the prevalence of mutant alleles decreased over time in comparison to a previous study in Siaya county and Kisumu county, western Kenya which reported a prevalence of 40% and 16.5% respectively (Achieng *et al.*, 2015; Shah *et al.*, 2015) compared to 9.4% in day 0 samples and 5.3% in recurrent infection samples in the current study. This is likely due to the withdrawal of chloroquine as and could suggest decreased sensitivity to lumefantrine and artemisinin.

5.5 Association between *Pfmdr1* Gene Mutations with Treatment Outcomes

In the present study, no statistically significant association was observed between the *Pfmdr1* 86, 184 and 1246 gene polymorphisms with either recrudescent infections or recurrent infections (recrudescent plus reinfections) after either AL or DP treatment (Table 5 and 6).

In the AL arm, there was no statistically significant difference in risk of recrudescence (Fisher statistic; 184**F**: p = 1 and 1246**Y**: p = 1) for *Pfmdr1* SNPs. Additionally, there was no statistically significant difference in risk of recrudescence or reinfection (Fisher statistic; 184**F**: p = 0.730 and 1246**Y**: p = 0.428). For *Pfmdr1* haplotypes, there was no statistically significant difference in risk of recrudescence (Fisher statistic; **YF**SND: p = 1, N**F**SND: p = 1, N**F**SN**Y**: p = 1, and NYSN**Y**: p = 0.573). Additionally there was no statistically significant difference in risk of recrudescence or reinfection (Fisher statistic; **YF**SND; p = 1, N**F**SND: p = 1, N**F**SN**Y**: p = 0.283, and NYSN**Y**: p = 1).

In the DP arm, there was no statistically significant difference in risk of recrudescence (Fisher statistic; 86**Y**: p = 0.074; 184**F**: p = 0.735 and 1246**Y**: p = 1) for *Pfmdr1* SNPs. Additionally, there was no statistically significant difference in risk of recrudescence or reinfection (86**Y**: p = 0.208; 184**F**: p = 1 and 1246**Y**: p = 0.207). For *Pfmdr1* haplotypes, there was no statistically significant difference in risk of recrudescence (**YF**SND: p = 0. 208; N**F**SND: p = 1, N**F**SN**Y**: p = 1 and NYSN**Y**: p = 0.573). Additionally there was no the statistically significant difference in risk of recrudescence or reinfection (**YF**SND: p = 0.219, N**F**SND: p = 1, N**F**SN**Y**: p = 0.575, and NYSN**Y**: p = 1).

The changes in lumefantrine sensitivity have been associated with polymorphisms in the *Pfmdr1* gene (Sisowath *et al.*, 2007; Venkatesan *et al.*, 2014). For example, Tanzanian parasites having the *Pfmdr1* N**F**D (N86, 184**F**, D1246) haplotype were able to withstand lumefantrine blood concentrations 15-fold higher than parasites with the **YY**Y (86**Y**, Y184, 1246**Y**) haplotype

(Malmberg *et al.*, 2012). In addition, in Uganda, AL was demonstrated to select for haplotypes with N86 in combination with 184**F** and D1246, or both (Taylor *et al.*, 2017). However this association was not observed in the current study. Although a high frequency of *Pfmdr1* Y184**F** SNPs and N**F**SND haplotype was detected, there was no statistically significant association between these mutations with treatment failure (recrudescence) in both treatment arms (Y184F: Fisher statistic; $p = 1$ and $p = 0.735$, for AL and DP treatment arms respectively; N**F**SND: Fisher statistic; $p = 1$ for both treatment arms; Table 5 and Table 6). This is consistent with another study which did not find any association between *Pfmdr1* Y184**F** mutants with susceptibility to various antimalarial drugs (Wurtz *et al.*, 2014). Other studies have, however, reported mutant *Pfmdr1* Y184**F** to be associated with the reduced susceptibility to lumefantrine (Malmberg *et al.*, 2012; Mbaye *et al.*, 2016). The role of *Pfmdr1* N**F**SND haplotype and Y184**F** mutations as a markers of resistance in the current study could be unclear given that both N**F**SND haplotype and Y184**F** mutations were also present in the majority of successfully treated patients in both treatment arms. Therefore, there is need for additional studies to assess the effect of this haplotype/mutant allele on ACT susceptibility given the increasing prevalence of these haplotype/mutant allele over time in western Kenya.

Pfmdr1 N86**Y** mutation has been found to be associated with increased susceptibility to artemisinin (Duraisingh *et al.*, 2000a; Duraisingh *et al.*, 2000b; Price *et al.*, 2004). However, this association was not observed in the present study in both treatment arms. This could be due to a smaller number of observed mutations in *Pfmdr1* N86**Y**, making correlation of observed mutations in the samples from patients who responded to the ACT therapy to those who failed the treatment inconclusive. Additional studies are needed to assess the effect of this mutant allele

on ACT susceptibility given the decreasing prevalence of this mutant allele over time in western Kenya.

CHAPTER SIX

SUMMARY OF THE FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of the Study Findings

The current study determined the prevalence of mutations at *Pfmdr1* gene and *Pfk13* propeller region in *P. falciparum* isolates collected in western Kenya and the association between these mutations with treatment outcome. Results indicated absence of any of the known *Pfk13* propeller region mutations that have been associated with artemisinin resistance. There was high prevalence of mutations in the *Pfmdr1* Y184**F** followed by D1246**Y** and N86**Y** had the lowest prevalence. Results did not indicate any statistically significant association between observed *Pfk13* propeller region mutation, *Pfmdr1* haplotypes or SNPs with treatment outcomes. Despite its lack of association with phenotype, there need for further studies in a large population to assess whether these SNPs are relevant in predicting drug resistance in Africa. It is possible that the actual SNP(s) that confer resistance are different from one location to another depending on several factors including parasite genetics, malaria transmission intensity, treatment seeking behavior and adherence to treatment guidelines. In Southeast Asia where ACT resistance emerged, use of artemisinin monotherapy tablets and substandard drugs is rampant which could explain why ACT resistance has emerged in Southeast Asia but has not emerged in other areas.

6.2 Conclusions

1. The study did not find any of the known *Pfk13* propeller region mutations that have been associated with artemisinin resistance (N458**Y**, Y493**H**, R539**T**, I543**T**, R561**H**, and

C580**Y)**, however, other non-synonymous mutations in the propeller region of *Pfk13* gene were detected.

2. There was high prevalence of *Pfmdr1* Y184**F** mutations in both day 0 and recurrent infections. Although this mutation has been associated with lumefantrine resistance, the implication of these findings is unclear given that the mutation was present in both day 0 and day of failure samples.
3. There was no statistically significant association between observed *Pfk13* propeller region mutation *Pfmdr1* haplotypes or SNPs with treatment outcomes. This provides evidence to the Kenyan NMCPfor the sustained use of ACTs in western Kenya.

6.3 Recommendations from this Study

1. Further *in vivo* clinical studies are required to validate the effect of the other *Pfk13* propeller mutations which were detected.
2. There prevalence of *Pfmdr1* Y184**F** mutations was higher than reported in the previous studies which might suggest that it is being selected for by the ACTs. Further studies are needed to studies to determine the effect of these mutations to partner drugs.
3. Maximizing the efficacy and longevity of ACT as a tool to control malaria will critically depend on pursuing intensive surveillance for molecular markers of parasite resistance as well as implementing *in vitro* and *in vivo* surveillance programs to detect any emergence of resistance and its containment before it becomes widespread.

6.4 Suggestions for Future Studies

1. There is need to conduct detailed assessment of parasite clearance dynamics after treatment with ACT using 6 hourly parasite measurements and correlation of parasite clearance times with whole *Kelch* 13 gene sequences in western Kenya.

2. There is need to conduct similar studies in different malaria transmission zones in Kenya, which have different levels of drug pressure, to compare the prevalence of mutations in the *Pfk13* propeller region and *Pfmdr1* genes.

REFERENCES

Achieng, A. O., Muiruri, P., Ingasia, L. A., Opot, B. H., Juma, D. W., Yeda, R., et al. (2015). Temporal trends in prevalence of Plasmodium falciparum molecular markers selected for by artemether-lumefantrine treatment in pre-ACT and post-ACT parasites in western Kenya. *Int J Parasitol Drugs Drug Resist, 5*(3), 92-99.

Agarwal, A., McMorrow, M., Onyango, P., Otieno, K., Odero, C., Williamson, J., et al. (2013). A randomized trial of artemether-lumefantrine and dihydroartemisinin-piperaquine in the treatment of uncomplicated malaria among children in western Kenya. *Malar J, 12*, 254.

Alker, A. P., Lim, P., Sem, R., Shah, N. K., Yi, P., Bouth, D. M., et al. (2007). Pfmdr1 and in vivo resistance to artesunate-mefloquine in falciparum malaria on the Cambodian-Thai border. *Am J Trop Med Hyg, 76*(4), 641-647.

Amin, A. A., Zurovac, D., Kangwana, B. B., Greenfield, J., Otieno, D. N., Akhwale, W. S., et al. (2007). The challenges of changing national malaria drug policy to artemisinin-based combinations in Kenya. *Malar J, 6*, 72.

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A. C., Khim, N., et al. (2014). A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. *Nature, 505*(7481), 50-55.

Arinaitwe, E., Sandison, T. G., Wanzira, H., Kakuru, A., Homsy, J., Kalamya, J., et al. (2009). Artemether-lumefantrine versus dihydroartemisinin-piperaquine for falciparum malaria: a longitudinal, randomized trial in young Ugandan children. *Clin Infect Dis, 49*(11), 1629-1637.

Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amaratunga, C., Lim, P., Suon, S., et al. (2014). Spread of artemisinin resistance in Plasmodium falciparum malaria. *N Engl J Med, 371*(5), 411-423.

Basco, L., & Ringwald, P. (2000). [Drug-resistant malaria: problems with its definition and technical approaches]. *Sante, 10*(1), 47-50.

Bassat, Q., Mulenga, M., Tinto, H., Piola, P., Borrmann, S., Menendez, C., et al. (2009). Dihydroartemisinin-piperaquine and artemether-lumefantrine for treating uncomplicated malaria in African children: a randomised, non-inferiority trial. *PLoS One, 4*(11), e7871.

Berzosa, P., Esteban-Cantos, A., Garcia, L., Gonzalez, V., Navarro, M., Fernandez, T., et al. (2017). Profile of molecular mutations in pfdhfr, pfdhps, pfmdr1, and pfcrt genes of Plasmodium falciparum related to resistance to different anti-malarial drugs in the Bata District (Equatorial Guinea). *Malar J, 16*(1), 28.

Beshir, K. B., Sutherland, C. J., Sawa, P., Drakeley, C. J., Okell, L., Mweresa, C. K., et al. (2013). Residual Plasmodium falciparum parasitemia in Kenyan children after artemisinin-

combination therapy is associated with increased transmission to mosquitoes and parasite recurrence. *J Infect Dis, 208*(12), 2017-2024.

Beshir, K. B., Sutherland, C. J., Sawa, P., Drakeley, C. J., Okell, L., Mweresa, C. K., et al. (2017). Residual Plasmodium falciparum parasitemia in Kenyan children after artemisinin-combination therapy is associated with increased transmission to mosquitoes and parasite recurrence. *J Infect Dis, 208*(12), 2017-2024.

Borges-Walmsley, M. I., McKeegan, K. S., & Walmsley, A. R. (2003). Structure and function of efflux pumps that confer resistance to drugs. *Biochem J, 376*(Pt 2), 313-338.

Borrmann, S., Sasi, P., Mwai, L., Bashraheil, M., Abdallah, A., Muriithi, S., et al. (2011). Declining responsiveness of Plasmodium falciparum infections to artemisinin-based combination treatments on the Kenyan coast. *PLoS One, 6*(11), e26005.

Bosman, P., Stassijns, J., Nackers, F., Canier, L., Kim, N., Khim, S., et al. (2014). Plasmodium prevalence and artemisinin-resistant falciparum malaria in Preah Vihear Province, Cambodia: a cross-sectional population-based study. *Malar J, 13*, 394.

Breman, J. G. (2001). The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *Am J Trop Med Hyg, 64*(1-2 Suppl), 1-11.

Cheeseman, I. H., Miller, B. A., Nair, S., Nkhoma, S., Tan, A., Tan, J. C., et al. (2012). A major genome region underlying artemisinin resistance in malaria. *Science, 336*(6077), 79-82.

Cochran, W. G. (1963). Methodological problems in the study of human populations. *Ann N Y Acad Sci, 107*, 476-489.

Cowman, A. F., Karcz, S., Galatis, D., & Culvenor, J. G. (1991). A P-glycoprotein homologue of Plasmodium falciparum is localized on the digestive vacuole. *J Cell Biol, 113*(5), 1033-1042.

Cox, J., Hay, S. I., Abeku, T. A., Checchi, F., & Snow, R. W. (2007). The uncertain burden of Plasmodium falciparum epidemics in Africa. *Trends Parasitol, 23*(4), 142-148.

Cui, L., Mharakurwa, S., Ndiaye, D., Rathod, P. K., & Rosenthal, P. J. (2015). Antimalarial Drug Resistance: Literature Review and Activities and Findings of the ICEMR Network. *Am J Trop Med Hyg, 93*(3 Suppl), 57-68.

Dokomajilar, C., Lankoande, Z. M., Dorsey, G., Zongo, I., Ouedraogo, J. B., & Rosenthal, P. J. (2006a). Roles of specific Plasmodium falciparum mutations in resistance to amodiaquine and sulfadoxine-pyrimethamine in Burkina Faso. *Am J Trop Med Hyg, 75*(1), 162-165.

Dokomajilar, C., Nsobya, S. L., Greenhouse, B., Rosenthal, P. J., & Dorsey, G. (2006b). Selection of Plasmodium falciparum pfmdr1 alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic. *Antimicrob Agents Chemother, 50*(5), 1893-1895.

DOMC. (2011). *Kenya Malaria Indicator Survey 2010*. Nairobi, Kenya: Division of Malaria Control, Ministry of Public Health and Sanitation, Kenya National Bureau of Statistics, and ICF Macro.

Dondorp, A. M., Nosten, F., Yi, P., Das, D., Phyo, A. P., Tarning, J., et al. (2009). Artemisinin resistance in Plasmodium falciparum malaria. *N Engl J Med, 361*(5), 455-467.

Duraisingh, M. T., Jones, P., Sambou, I., von Seidlein, L., Pinder, M., & Warhurst, D. C. (2000a). The tyrosine-86 allele of the pfmdr1 gene of Plasmodium falciparum is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. *Mol Biochem Parasitol, 108*(1), 13-23.

Duraisingh, M. T., Roper, C., Walliker, D., & Warhurst, D. C. (2000b). Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the pfmdr1 gene of Plasmodium falciparum. *Mol Microbiol, 36*(4), 955-961.

Eyase, F. L., Akala, H. M., Ingasia, L., Cheruiyot, A., Omondi, A., Okudo, C., et al. (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*(5), e64299.

Foote, S. J., Kyle, D. E., Martin, R. K., Oduola, A. M., Forsyth, K., Kemp, D. J., et al. (1990). Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in Plasmodium falciparum. *Nature, 345*(6272), 255-258.

Ghorbal, M., Gorman, M., Macpherson, C. R., Martins, R. M., Scherf, A., & Lopez-Rubio, J. J. (2014). Genome editing in the human malaria parasite Plasmodium falciparum using the CRISPR-Cas9 system. *Nat Biotechnol, 32*(8), 819-821.

Greenwood, B. M., Fidock, D. A., Kyle, D. E., Kappe, S. H., Alonso, P. L., Collins, F. H., et al. (2008). Malaria: progress, perils, and prospects for eradication. *J Clin Invest, 118*(4), 1266-1276.

Halliday, K. E., Okello, G., Turner, E. L., Njagi, K., McHaro, C., Kengo, J., et al. (2014). Impact of intermittent screening and treatment for malaria among school children in Kenya: a cluster randomised trial. *PLoS Med, 11*(1), e1001594.

Happi, C. T., Gbotosho, G. O., Folarin, O. A., Sowunmi, A., Hudson, T., O'Neil, M., et al. (2009). Selection of Plasmodium falciparum multidrug resistance gene 1 alleles in asexual stages and gametocytes by artemether-lumefantrine in Nigerian children with uncomplicated falciparum malaria. *Antimicrob Agents Chemother, 53*(3), 888-895.

Humphreys, G. S., Merinopoulos, I., Ahmed, J., Whitty, C. J., Mutabingwa, T. K., Sutherland, C. J., et al. (2007). Amodiaquine and artemether-lumefantrine select distinct alleles of the Plasmodium falciparum mdr1 gene in Tanzanian children treated for uncomplicated malaria. *Antimicrob Agents Chemother, 51*(3), 991-997.

Imwong, M., Jindakhad, T., Kunasol, C., Sutawong, K., Vejakama, P., & Dondorp, A. M. (2015). An outbreak of artemisinin resistant falciparum malaria in Eastern Thailand. *Sci Rep, 5*, 17412.

Jenkins, R., Omollo, R., Ongecha, M., Sifuna, P., Othieno, C., Ongeri, L., et al. (2015). Prevalence of malaria parasites in adults and its determinants in malaria endemic area of Kisumu County, Kenya. *Malar J, 14*, 263.

Kamau, E., Campino, S., Amenga-Etego, L., Drury, E., Ishengoma, D., Johnson, K., et al. (2015). K13-propeller polymorphisms in Plasmodium falciparum parasites from sub-Saharan Africa. *J Infect Dis, 211*(8), 1352-1355.

Kaur, H., Clarke, S., Lalani, M., Phanouvong, S., Guerin, P., McLoughlin, A., et al. (2016). Fake anti-malarials: start with the facts. *Malar J, 15*, 86.

KMIS. (2015). Kenya Malaria Indicator Survey.

Koenderink, J. B., Kavishe, R. A., Rijpma, S. R., & Russel, F. G. (2010). The ABCs of multidrug resistance in malaria. *Trends Parasitol, 26*(9), 440-446.

Laufer, M. K., Djimde, A. A., & Plowe, C. V. (2007). Monitoring and deterring drug-resistant malaria in the era of combination therapy. *Am J Trop Med Hyg, 77*(6 Suppl), 160-169.

Ljolje, D., Dimbu, P. R., Kelley, J., Goldman, I., Nace, D., Macaia, A., et al. (2018). Prevalence of molecular markers of artemisinin and lumefantrine resistance among patients with uncomplicated Plasmodium falciparum malaria in three provinces in Angola, 2015. *Malar J, 17*(1), 84.

Lu, F., Culleton, R., Zhang, M., Ramaprasad, A., von Seidlein, L., Zhou, H., et al. (2017). Emergence of Indigenous Artemisinin-Resistant Plasmodium falciparum in Africa. *N Engl J Med, 376*(10), 991-993.

Maitland, K., & Marsh, K. (2004). Pathophysiology of severe malaria in children. *Acta Trop, 90*(2), 131-140.

Malmberg, M., Ferreira, P. E., Tarning, J., Ursing, J., Ngasala, B., Bjorkman, A., et al. (2012). Plasmodium falciparum drug resistance phenotype as assessed by patient antimalarial drug levels and its association with pfmdr1 polymorphisms. *J Infect Dis, 207*(5), 842-847.

Mayxay, M., Thongpraseuth, V., Khanthavong, M., Lindegardh, N., Barends, M., Keola, S., et al. (2006). An open, randomized comparison of artesunate plus mefloquine vs. dihydroartemisinin-piperaquine for the treatment of uncomplicated Plasmodium falciparum malaria in the Lao People's Democratic Republic (Laos). *Trop Med Int Health, 11*(8), 1157-1165.

Mayxay, M., Khanthavong, M., Chanthongthip, O., Imwong, M., Pongvongsa, T., Hongvanthong, B., et al. (2012). Efficacy of artemether-lumefantrine, the nationally-

recommended artemisinin combination for the treatment of uncomplicated falciparum malaria, in southern Laos. *Malar J, 11*, 184.

Mbaisi, A., Liyala, P., Eyase, F., Achilla, R., Akala, H., Wangui, J., et al. (2004). Drug susceptibility and genetic evaluation of Plasmodium falciparum isolates obtained in four distinct geographical regions of Kenya. *Antimicrob Agents Chemother, 48*(9), 3598-3601.

Mbaye, A., Dieye, B., Ndiaye, Y. D., Bei, A. K., Muna, A., Deme, A. B., et al. (2016). Selection of N86F184D1246 haplotype of Pfmrd1 gene by artemether-lumefantrine drug pressure on Plasmodium falciparum populations in Senegal. *Malar J, 15*(1), 433.

Menard, D., Khim, N., Beghain, J., Adegnika, A. A., Shafiul-Alam, M., Amodu, O., et al. (2016). A Worldwide Map of Plasmodium falciparum K13-Propeller Polymorphisms. *N Engl J Med, 374*(25), 2453-2464.

Milner, D. A., Jr., Montgomery, J., Seydel, K. B., & Rogerson, S. J. (2008). Severe malaria in children and pregnancy: an update and perspective. *Trends Parasitol, 24*(12), 590-595.

Mohon, A. N., Alam, M. S., Bayih, A. G., Folefoc, A., Shahinas, D., Haque, R., et al. (2014). Mutations in Plasmodium falciparum K13 propeller gene from Bangladesh (2009-2013). *Malar J, 13*, 431.

Muwanguzi, J., Henriques, G., Sawa, P., Bousema, T., Sutherland, C. J., & Beshir, K. B. (2016). Lack of K13 mutations in Plasmodium falciparum persisting after artemisinin combination therapy treatment of Kenyan children. *Malar J, 15*, 36.

Mwai, L., Kiara, S. M., Abdirahman, A., Pole, L., Rippert, A., Diriye, A., et al. (2009). In vitro activities of piperaquine, lumefantrine, and dihydroartemisinin in Kenyan Plasmodium falciparum isolates and polymorphisms in pfcrt and pfmdr1. *Antimicrob Agents Chemother, 53*(12), 5069-5073.

NMCP, K. a. I., international. (2016). National Malaria Control Programme (NMCP), Kenya National Bureau of Statistics (KNBS) and I, International. Kenya Malaria Indicator Survey 2015. Nairobi, Kenya, and Rockville, Maryland, USA: NMCP, KNBS, and ICF International: 2016.

Noedl, H., Se, Y., Schaecher, K., Smith, B. L., Socheat, D., & Fukuda, M. M. (2008). Evidence of artemisinin-resistant malaria in western Cambodia. *N Engl J Med, 359*(24), 2619-2620.

Nosten, F., & White, N. J. (2007). Artemisinin-based combination treatment of falciparum malaria. *Am J Trop Med Hyg, 77*(6 Suppl), 181-192.

Nsanzabana, C., Djalle, D., Guerin, P. J., Menard, D., & Gonzalez, I. J. (2018). Tools for surveillance of anti-malarial drug resistance: an assessment of the current landscape. *Malar J, 17*(1), 75.

O'Meara WP, B. P., Mwangi TW, et al. (2008). Effect of a fall in malaria transmission on morbidity and mortality in Kilifi, Kenya. *Lancet*, 372(9649): 1555-9662.

Odhiambo, F. O., Laserson, K. F., Sewe, M., Hamel, M. J., Feikin, D. R., Adazu, K., et al. (2012). Profile: the KEMRI/CDC Health and Demographic Surveillance System--Western Kenya. *Int J Epidemiol, 41*(4), 977-987.

Ogutu, B. R., Onyango, K. O., Koskei, N., Omondi, E. K., Ongecha, J. M., Otieno, G. A., et al. (2014). Efficacy and safety of artemether-lumefantrine and dihydroartemisinin-piperaquine in the treatment of uncomplicated Plasmodium falciparum malaria in Kenyan children aged less than five years: results of an open-label, randomized, single-centre study. *Malar J, 13*, 33.

Onyango, E. O., Ayodo, G., Watsierah, C. A., Were, T., Okumu, W., Anyona, S. B., et al. (2012). Factors associated with non-adherence to Artemisinin-based combination therapy (ACT) to malaria in a rural population from holoendemic region of western Kenya. *BMC Infect Dis, 12*, 143.

Otienoburu, S. D., Maiga-Ascofare, O., Schramm, B., Jullien, V., Jones, J. J., Zolia, Y. M., et al. (2016). Selection of Plasmodium falciparum pfcrt and pfmdr1 polymorphisms after treatment with artesunate-amodiaquine fixed dose combination or artemether-lumefantrine in Liberia. *Malar J, 15*, 452.

Phyo, A. P., Nkhoma, S., Stepniewska, K., Ashley, E. A., Nair, S., McGready, R., et al. (2012). Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. *Lancet, 379*(9830), 1960-1966.

Pickard, A. L., Wongsrichanalai, C., Purfield, A., Kamwendo, D., Emery, K., Zalewski, C., et al. (2003). Resistance to antimalarials in Southeast Asia and genetic polymorphisms in pfmdr1. *Antimicrob Agents Chemother, 47*(8), 2418-2423.

Picot, S., Olliaro, P., de Monbrison, F., Bienvenu, A. L., Price, R. N., & Ringwald, P. (2009). A systematic review and meta-analysis of evidence for correlation between molecular markers of parasite resistance and treatment outcome in falciparum malaria. *Malar J, 8*, 89.

Price, R. N., Uhlemann, A. C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., et al. (2004). Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. *Lancet, 364*(9432), 438-447.

Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K., & Cowman, A. F. (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. *Nature, 403*(6772), 906-909.

Rieckmann, K. H., Campbell, G. H., Sax, L. J., & Mrema, J. E. (1978). Drug sensitivity of plasmodium falciparum. An in-vitro microtechnique. *Lancet, 1*(8054), 22-23.

Roper, C., Alifrangis, M., Ariey, F., Talisuna, A., Menard, D., Mercereau-Puijalon, O., et al. (2014). Molecular surveillance for artemisinin resistance in Africa. *Lancet Infect Dis, 14*(8), 668-670.

Rosenberg, R., Andre, R. G., Ngampatom, S., Hatz, C., & Burge, R. (1990). A stable, oligosymptomatic malaria focus in Thailand. *Trans R Soc Trop Med Hyg, 84*(1), 14-21.

Sabbatani, S., Fiorino, S., & Manfredi, R. (2010). The emerging of the fifth malaria parasite (Plasmodium knowlesi): a public health concern? *Braz J Infect Dis, 14*(3), 299-309.

Sanchez, C. P., Dave, A., Stein, W. D., & Lanzer, M. (2010). Transporters as mediators of drug resistance in Plasmodium falciparum. *Int J Parasitol, 40*(10), 1109-1118.

Saunders, D. L., Vanachayangkul, P., & Lon, C. (2014). Dihydroartemisinin-piperaquine failure in Cambodia. *N Engl J Med, 371*(5), 484-485.

Shah, M., Omosun, Y., Lal, A., Odero, C., Gatei, W., Otieno, K., et al. (2015). Assessment of molecular markers for anti-malarial drug resistance after the introduction and scale-up of malaria control interventions in western Kenya. *Malar J, 14*, 75.

Sharom, F. J. (2011). The P-glycoprotein multidrug transporter. *Essays Biochem, 50*(1), 161-178.

Sidhu, A. B., Valderramos, S. G., & Fidock, D. A. (2005). pfmdr1 mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in Plasmodium falciparum. *Mol Microbiol, 57*(4), 913-926.

Sidhu, A. B., Uhlemann, A. C., Valderramos, S. G., Valderramos, J. C., Krishna, S., & Fidock, D. A. (2006). Decreasing pfmdr1 copy number in plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. *J Infect Dis, 194*(4), 528-535.

Sisowath, C., Stromberg, J., Martensson, A., Msellem, M., Obondo, C., Bjorkman, A., et al. (2005). In vivo selection of Plasmodium falciparum pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). *J Infect Dis, 191*(6), 1014-1017.

Sisowath, C., Ferreira, P. E., Bustamante, L. Y., Dahlstrom, S., Martensson, A., Bjorkman, A., et al. (2007). The role of pfmdr1 in Plasmodium falciparum tolerance to artemether-lumefantrine in Africa. *Trop Med Int Health, 12*(6), 736-742.

Snounou, G. (2002). Genotyping of Plasmodium spp. Nested PCR. *Methods Mol Med, 72*, 103-116.

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

Some, A. F., Zongo, I., Compaore, Y. D., Sakande, S., Nosten, F., Ouedraogo, J. B., et al. (2014). Selection of drug resistance-mediating Plasmodium falciparum genetic polymorphisms by seasonal malaria chemoprevention in Burkina Faso. *Antimicrob Agents Chemother, 58*(7), 3660-3665.

Straimer, J., Gnadig, N. F., Witkowski, B., Amaratunga, C., Duru, V., Ramadani, A. P., et al. (2015). Drug resistance. K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates. *Science, 347*(6220), 428-431.

Takala-Harrison, S., Clark, T. G., Jacob, C. G., Cummings, M. P., Miotto, O., Dondorp, A. M., et al. (2014). Genetic loci associated with delayed clearance of Plasmodium falciparum following artemisinin treatment in Southeast Asia. *Proc Natl Acad Sci U S A, 110*(1), 240-245.

Talundzic, E., Ravishankar, S., Kelley, J., Patel, D., Plucinski, M., Schmedes, S., et al. (2018). Next-Generation Sequencing and Bioinformatics Protocol for Malaria Drug Resistance Marker Surveillance. *Antimicrob Agents Chemother, 62*(4).

Taylor, A. R., Flegg, J. A., Holmes, C. C., Guerin, P. J., Sibley, C. H., Conrad, M. D., et al. (2017). Artemether-Lumefantrine and Dihydroartemisinin-Piperaquine Exert Inverse Selective Pressure on Plasmodium Falciparum Drug Sensitivity-Associated Haplotypes in Uganda. *Open Forum Infect Dis, 4*(1), ofw229.

Teklehaimanot, A., McCord, G. C., & Sachs, J. D. (2007). Scaling up malaria control in Africa: an economic and epidemiological assessment. *Am J Trop Med Hyg, 77*(6 Suppl), 138-144.

Torrentino-Madamet, M., Fall, B., Benoit, N., Camara, C., Amalvict, R., Fall, M., et al. (2014). Limited polymorphisms in k13 gene in Plasmodium falciparum isolates from Dakar, Senegal in 2012-2013. *Malar J, 13*, 472.

Trager, W., & Jensen, J. B. (1976). Human malaria parasites in continuous culture. *Science, 193*(4254), 673-675.

Tumwebaze, P., Conrad, M. D., Walakira, A., LeClair, N., Byaruhanga, O., Nakazibwe, C., et al. (2015). Impact of antimalarial treatment and chemoprevention on the drug sensitivity of malaria parasites isolated from ugandan children. *Antimicrob Agents Chemother, 59*(6), 3018-3030.

Tumwebaze, P., Tukwasibwe S, Taylor A, Conrad M, Ruhamyankaka E, Asua V, W. A., et al. (2016). Changing antimalarial drug resistance patterns identified by surveillance at three sites in Uganda. *J Infect Dis, 207(215):631-5*.

Tun, K. M., Imwong, M., Lwin, K. M., Win, A. A., Hlaing, T. M., Hlaing, T., et al. (2015). Spread of artemisinin-resistant Plasmodium falciparum in Myanmar: a cross-sectional survey of the K13 molecular marker. *Lancet Infect Dis, 15*(4), 415-421.

Valderramos, S. G., & Fidock, D. A. (2006). Transporters involved in resistance to antimalarial drugs. *Trends Pharmacol Sci, 27*(11), 594-601.

Veiga, M. I., Dhingra, S. K., Henrich, P. P., Straimer, J., Gnadig, N., Uhlemann, A. C., et al. (2016). Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies. *Nat Commun, 7*, 11553.

Venkatesan, M., Gadalla, N. B., Stepniewska, K., Dahal, P., Nsanzabana, C., Moriera, C., et al. (2014). Polymorphisms in Plasmodium falciparum chloroquine resistance transporter and multidrug resistance 1 genes: parasite risk factors that affect treatment outcomes for P. falciparum malaria after artemether-lumefantrine and artesunate-amodiaquine. *Am J Trop Med Hyg, 91*(4), 833-843.

White, N. J. (2008). Qinghaosu (artemisinin): the price of success. *Science, 320*(5874), 330-334.

White, N. J. (2016). Can new treatment developments combat resistance in malaria? *Expert Opin Pharmacother, 17*(10), 1303-1307.

WHO. (2002). The global malaria situation: current tools for prevention and control. 55th World Health Assembly. Global Fund to Fight AIDS, Tuberculosis and Malaria. WHO document no. A55/INF.DOC./6. Available at: http://www.who.int/gb/EB_WHA/PDF/WHA55/ea55id6.pdf.

WHO. (2008). WHO. Recommended Genotyping Procedures (RGPs) to identify parasite populations version 1, edition. Geneva: World Health Organization.

WHO. (2009a). Methods for surveillance of antimalarial drug efficacy.

WHO. (2009b). WHO. Methods for surveillance of antimalarial drug efficacy. Geneva: World Health Organization.

WHO. (2010a). WHO, Guidelines for the treatment of malaria (Second edition).

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

WHO. (2013). World Health Organization. World malaria report: France: World Health Organization.

WHO. (2016a). World Malaria Report 2016, Geneva. World Health Organisation.

WHO. (2016b). Artemisinin and artemisinin-based combination therapy resistance, World Health Organisation.

WHO. (2017a). World Malaria Report 2017, Geneva. World Health Organisation.

WHO. (2017b). Artemisinin and artemisinin-based combination therapy resistance, World Health Organisation,.

Williams, P. C., Martina, A., Cumming, R. G., & Hall, J. (2009). Malaria prevention in Sub-Saharan Africa: a field study in rural Uganda. *J Community Health, 34*(4), 288-294.

Witkowski, B., Amaratunga, C., Khim, N., Sreng, S., Chim, P., Kim, S., et al. (2013). Novel phenotypic assays for the detection of artemisinin-resistant Plasmodium falciparum malaria in Cambodia: in-vitro and ex-vivo drug-response studies. *Lancet Infect Dis, 13*(12), 1043-1049.

Wongsrichanalai, C., & Meshnick, S. R. (2008). Declining artesunate-mefloquine efficacy against falciparum malaria on the Cambodia-Thailand border. *Emerg Infect Dis, 14*(5), 716-719.

Wurtz, N., Fall, B., Pascual, A., Fall, M., Baret, E., Camara, C., et al. (2014). Role of Pfmdr1 in in vitro Plasmodium falciparum susceptibility to chloroquine, quinine, monodesethylamodiaquine, mefloquine, lumefantrine, and dihydroartemisinin. *Antimicrob Agents Chemother, 58*(12), 7032-7040.

WWARN. (2015). Artemether-lumefantrine treatment of uncomplicated Plasmodium falciparum malaria: a systematic review and meta-analysis of day 7 lumefantrine concentrations and therapeutic response using individual patient data. BMC Med. WWARN. Artemether-lumefantrine treatment of uncomplicated Plasmodium falciparum malaria: a systematic review and meta-analysis of day 7 lumefantrine concentrations and therapeutic response using individual patient data. BMC Med.;13:227-45;13:227-45.

Zongo, I., Dorsey, G., Rouamba, N., Tinto, H., Dokomajilar, C., Guiguemde, R. T., et al. (2007). Artemether-lumefantrine versus amodiaquine plus sulfadoxine-pyrimethamine for uncomplicated falciparum malaria in Burkina Faso: a randomised non-inferiority trial. *Lancet, 369*(9560), 491-498.

APPENDICES

Appendix 1: Primer Sequences used for Genotyping *P. falciparum*

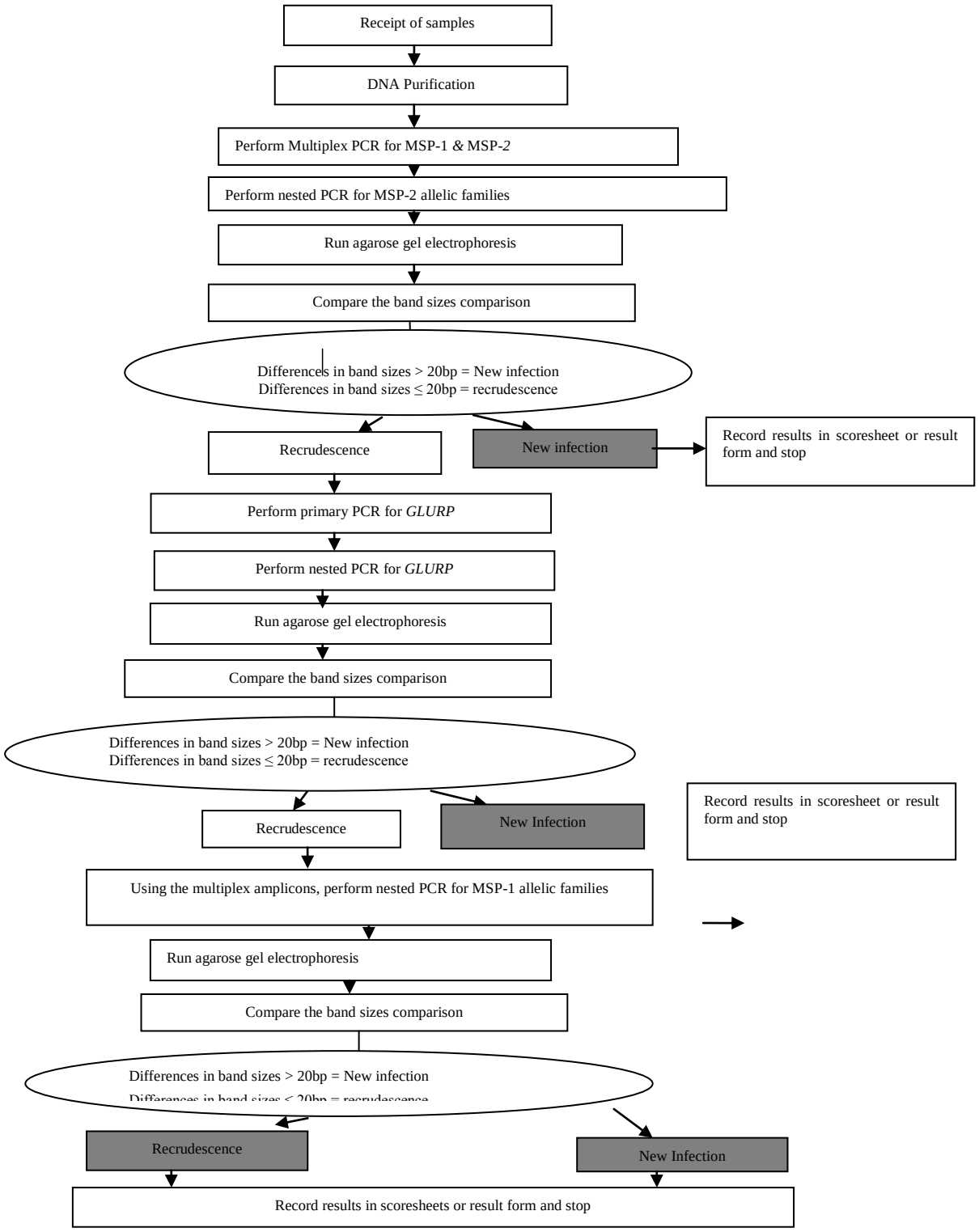
Primer name		Sequence	Amplicon size (bp)
MSP-l primary PCR			
M1-OF		5'-CTAGAAGCTTTAGAAGATGCAGTATTG-3'	
M1-OR		5'-CTTAAATAGTATTCTAATTCAAGTGGATCA-3'	
MSP-2 primary PCR			
M2-OF		5'-ATGAAGGTAATTAAAACATTGTCTATTATA-3'	
M2-OR		5'-CTTTGTTACCATCGGTACATTCTT-3'	
MSP-1 family specific nested PCR			
K1 allele	M1-KF	5'-AAA TGA AGA AGA AAT TAC TAC AAA AGG TGC-3'	125-250
	M1-KR	5'-GCT TGC ATC AGC TGG AGG GCT TGC ACC AGA-3'	
MAD 20 allele	M1-MF	5'-AAA TGA AGG AAC AAG TGG AAC AGC TGT TAC-3'	
	M1-MR	5'-ATC TGA AGG ATT TGT ACG TCT TGA ATT ACC-3'	
RO33 allele	M1-RF	5'-TAA AGG ATG GAG CAA ATA CTC AAG TTG TTG-3'	
	Ro33-R2	5'-CAA GTA ATT TTG AAC TCT ATG TTT TAA ATC AGC GTA-3'	
	M5 rev	5'- GCA TTG CCA GAA CTT GAA-3'	
MSP 2 family –specific nested PCR			
3D7 allele	S1-fw	5'- GCT TAT AAT ATG AGT ATA AGG AGA A-3'	250-450
	M5-rev	5'- GCA TTG CCA GAA CTT GAA-3'	
FC27 allele	S1-fw	5'- GCT TAT AAT ATG AGT ATA AGG AGA A-3'	
	N5-rev	5'-CTG AAG AGG TAC TGG TAG A-3'	
GLURP genotyping			
GLURP Primary PCR	N5 rev	5'-CTG AAG AGG TAC TGG TAG A-3'	
	G-F3	5'-ACATGCAAGTGTTGATCCTGAAG-3'	
	G-F4	5'-TGTAGGTACCACGGGTTCTTGTGG-3'	
GLURP Nested PCR	G-NF	5'-TGTTCACACTGAACAATTAGATTTAGATCA-3'	600-1200
	G-F4	5'-TGTAGGTACCACGGGTTCTTGTGG-3'	

Appendix 2: Thermoprofile for Genotyping *P. falciparum* (MSP1, MSP2 and GLURP)

Multiplex primary MSP1 AND MSP2 thermoprofile		**MSP2 alleles Nested PCR Thermoprofile**		**MSP1 alleles Nested PCR Thermoprofile**	
94^0C -2 min		94^0C -2 min		94^0C -2 min	
94^0C -30sec	30 Cycles	94^0C -30sec	30 Cycles	94^0C -30sec	30 Cycles
56^0C -1 min		56^0C -45 sec		59^0C -1 min	
72^0C -1 min		70^0C -1 min 30 secs		72^0C -1 min	
72^0C -5 min		70^0C -5 min		72^0C -5 min	
Go to 4^0 C-∞		Go to 4^0 C-∞		Go to 4^0 C-∞	

Primary PCR thermoprofile for GLURP		**Nested PCR thermoprofile for GLURP**	
94^0C -2 min		94^0C -2 min	
94^0C -30sec	30 Cycles	94^0C -30sec	30 Cycles
56^0C -1 min		58^0C -45secs	
72^0C -1 min		70^0C -1min 30 secs	
72^0C -5 min		70^0C -5 min	
Go to 4^0 C-∞		Go to 4^0 C-∞	

Appendix 3: Decision Tree for Genotyping *P. falciparum*



Source: (WHO, 2008)

Appendix 4: Classification of Treatment Outcomes in TES of Antimalarial Drugs

Classification of treatment outcomes **WHO, 2005**
Early Treatment Failure (ETF)- any of the following
• Development of danger signs or severe malaria on day 0, 1, 2 or 3 in the presence of parasitemia; • Parasitemia on day 2 higher than day 0 count irrespective of axillary temperature; • Parasitemia on day 3 ≥25% of count on day 0 without fever; or • Parasitemia on day 3 with axillary temperature ≥37.5 ºC
Late Treatment Failure (LTF)- defined as meeting criteria of either of the two below
Late Clinical Failure **(LCF)- any of the following** • Development of danger signs or severe malaria after day 3 in the presence of parasitemia, without previously meeting any of the criteria of Early Treatment Failure; • Presence of parasitemia and axillary temperature ≥37.5 ºC (or history of fever in low/moderate transmission areas) on any day from day 4 to day 42, without previously meeting any of the criteria of Early Treatment Failure. ***Late Parasitological Failure*** **(LPF)** • Presence of parasitemia on any day from day 7 to day 42 and axillary temperature <37.5 ºC, without previously meeting any of the criteria of Early Treatment Failure or Late Clinical Failure.
Adequate Clinical and Parasitological Response (ACPR)
• Absence of parasitemia on day 42 irrespective of axillary temperature without previously meeting any of the criteria of Early Treatment Failure or Late Clinical Failure or Late Parasitological Failure.

Source: (WHO, 2009b)

Appendix 5: Maseno University SGS Approval Letter

MASENO UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: MSC/00054/013

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 14th June, 2018

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR CHEBORE JEBIWOT WINNIE — MSC/00054/013

The above named is registered in the Master of Science in Medical Biotechnology in the School of Public Health and Community Development, Maseno University. This is to confirm that her research proposal titled "Molecular Markers of Plasmodium Falciparum Resistance to Artemisinin – Based Combination Therapies in Western Kenya" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

DEAN SCHOOL OF GRADUATE STUDIES MASENO UNIVERSITY 14 JUN 2018 P.O. BOX MASENO

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

Appendix 6: KEMRI/SERU Annual Approval Letter

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 **February 23, 2018**

TO: **DR. AARON SAMUELS,**
PRINCIPAL INVESTIGATOR.

THROUGH: **THE DIRECTOR, CGHR,**
KISUMU.

Dear Sir,

RE: **SSC PROTOCOL No: 2857 (*REQUEST FOR ANNUAL RENEWAL)* OPEN LABEL RANDOMIZED STUDY EVALUATING THE IN-VIVO EFFICACIES OF ARTEMETHER-LUMEFANTRINE AND DIHYDROARTEMISININ-PIPERAQUINE IN THE TREATMENT OF UNCOMPLICATED PLASMODIUM FALCIPARUM MALARIA IN CHILDREN UNDER FIVE YEARS OF AGE IN WESTERN KENYA.**

Thank you for the continuing review report for the period **7th March 2017** to **11th January 2018.**

This is to inform that during the 272nd Committee B meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **21st February 2018**, the Committee **conducted the annual review and approved** the above referenced application for another year.

This study is granted approval for continuation effective **March 7, 2018.** Please note that authorization to conduct this study will automatically expire on **March 6, 2019**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the SERU Secretariat by **January 23, 2019.**

You are required to submit any proposed changes to this study to SERU for Review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may continue with the study.

Yours faithfully,

THE HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

Appendix 7: Informed Consent Form

Annex IIIa-1

Parental Permission Form for Enrollment
(Will be used if funding is not sufficient to cover the sub-study)
English version: Flesh-Kincaid Level 7.7

Pls: Dr. Aaron Samuels and Dr. Simon Kariuki

Investigators and Institutions
Aaron Samuels (CDC), Simon Kariuki (KEMRI/CDC), Nelli Westercamp (CDC), Meghna Desai (KEMRI/CDC), Ann Buff (CDC), Tony Fiore (CDC), Kephas Otieno (KEMRI/CDC), Menno Smit (KEMRI/CDC, LSTM), Feiko Ter Kuile (MACEPA/LSTM), Andrew Nyandigisi (DOMC), George Wadegu (Siaya County Health Management)

CDC- Centers for Disease Control and Prevention, Atlanta, USA
KEMRI/CDC- Kenya Medical Research Institute and CDC, Kisumu, Kenya
DOMC- Kenya Division of Malaria Control, Ministry of Public Health and Sanitation, Nairobi, Kenya

Introduction

Malaria is a sickness caused by small parasites. These parasites can get into your body when you get bitten by a mosquito. It can cause fever, headache, body ache and lack of strength in the body. If not treated, it can make someone very sick, especially children. When malaria is treated with the right drugs, it can heal. Some malaria parasites can become resistant to these drugs.

The ministry of health. KEMRI research institute partnering with CDC in USA, are doing a research to see how drugs that are used to treat malaria work. This will help us treat children with malaria properly in future days. We ask that you put your children in this research because they have malaria. We will ask 350 children who have malaria to participate in this research. We will follow up on them for 42 days to see if the drugs they were given for this research work.

Procedure:

If you accept your child to be in this research, we will examine him or her. We will take a small amount of blood from the finger of your child to see if it has malaria and supposed to be in this research. We will preserve some blood. If the treatment does not work, we will have to give the child other drugs. We can examine that blood to know why the drugs did not work. Please understand that even if you do not want your child to be in this research, they will still be treated for malaria. If you accept your child to participate, we will treat the child with one of these two drugs usually used to treat malaria.

- Artemether-Lumefantrine (also known as AL or Coartem®)
- Dihydroartemisinin-Piperaquine (also known as Duocotecxin®)

You or a research worker cannot choose which drug to give, it will be decided by chance like tossing a coin. All these drugs have been used and they work well in these areas. These drugs have less effects that way less dangerous to your body. Your child will come to the clinic to receive the drugs and will be administered through the mouth everyday for the first 3 days. We will observe your child for 30 minutes after taking the drug to ensure there is no vomiting.
We will ask that you go back to the clinic 9 times in the following days. In the 1, 2, 3, 7, 14, 21, 28, 35 and 42 days to give him/her medicine or check your child to see if malaria has stopped.

We will give you a calendar to know which days to come. We will also call you to remind you to come. For all the days you will come to the clinic except tomorrow, we will remove blood from the finger of your child to test for malaria. Tests for shortage of blood will be done on the 0, 7, 14, 28 and 42 day. We will test for the level of drug in your child's body on day 7. For every visit to the clinic, we will test your child and ask medical questions. if you do not come to the clinic, someone from the clinic will come to visit you within one day. We expect these drugs will work to treat malaria to some children in this research. But if your child's malaria is not treated, we will use quinine to treat malaria. This is the international standard. This treatment is supposed to treat malaria. We will still examine your child to ensure they are fairing on well. If your child has a strong malaria, we will admit him/ her to hospital. We will also treat the child with drugs and offer medical assistance as is recommended by the health ministry. Every time we do a research on malaria, we will put a small amount of blood on paper. This blood will be used to learn how parasites cause infection. If the drugs do not work as required, some of the blood will be examined to know why it did not destroy all the parasites.

Danger:

There is little danger to your child for being in this research. Malaria drugs can have problems. All drugs can cause stomach problems, vomiting, diarrhea, headache, dizziness, itchiness and a little rash. These effects are mild and usually disappear without any medication. In very few times will these effects be harsh. If your child has harsh effects, like unending vomiting, bad rashes or heart problems, we will stop the drugs. We will also recommend that the child be treated with different medication in future days. You should bring your child to the clinic at once when the sickness increases or if he/ she gets effects from the drugs we treat them with. You can bring the child even if it is not the clinic day. There will be a doctor at the clinic everyday that can treat these problems. Bring the child to the child if he/ she has the following symptoms:

- Fever
- Convulsion
- Deep sleep
- Vomiting everything
- Lack of appetite
- Painful rashes or injuries on the mouth or red eyes
- Pain in the chest, difficulty in breathing or breathing quickly.

Danger from the removal of blood is minimal and can cause blood to flow, mark, fainting or infection, but this is very rare.

We will clean our hands or your child's finger before taking blood and we will use a new needle to remove blood.

Benefits:

During registration, your child will receive a treated net without any payment. All visits scheduled at the clinic and all treatments of malaria in the hospital during this research are free without payment.

Drugs for treatment are also free without payment. Your child will be followed up for 42 days to ensure the treatment is working.

There will be someone at the clinic. You can bring your child at any time at the clinic if you feel that he/ she is sick, even if it is at night, weekend or in the middle of the scheduled visits.

This research will also help the ministry of health to learn which drugs work best in this area. This can help you or anyone else you know in future days.

Compensation:

You will receive two hundred shillings for every visit to the clinic to cater for your travelling

Privacy:

Information that we will receive about your child will not be seen by anyone else except the researchers in this research and will be written on the clinic card of your child. The results of this research will be reported briefly. The name of your child will not appear in the report of this research.

Contacts

If you have questions or concerns about your child's participation in this study, you may speak with one of our staff.

If you have any questions about the study, or you feel your child has been harmed, please contact Dr. Meghna Desai at KEMRI/CDC, P.O. Box 1578-40100, Kisumu, or at 057 20 22902. If you have any questions about your child's rights as a study patient, or if you want to talk about the study with someone who is not part of this research project, please contact Secretary of KEMRI NERC on (020) 2722541 or 0722205901, fax (254) (020) 2720030, P.O. Box 54840-00200 Nairobi-Kenya.

Voluntary participation, refusal, and withdrawal

It is important that you have been told about several things about taking part in this study:

- You are free to choose to have your child in this study or not. If you choose for your child not to participate, your child will still be treated for malaria.
  - Your child may not benefit from taking part in the study. But knowledge may be gained that will benefit others.
  - You may remove your child out of the research at any time. This will not affect your child's medical benefits and your child will still receive malaria treatment for free. If you take your child out of the research before he or she has received all the medication from this research, then your child will receive the usual medicine of AL.
  - If the doctors need to take your child out of the research for any reason, then we will not continue to follow up on your child. If your child is removed from the research before all the medicine has been given, or if the research medicine did not make work as required, then your child will be referred to the clinic and treated with the most appropriate medicine.

Your Choice:

You can allow your child to be in this research and receive one of the two medicines that we told you about, OR

Your child does not have to be in this research. He/she will receive normal treatment (AL) in this area.

This project has been reviewed and approved by CDC Institutional Review Board and the Ministry of Health who work to ensure that research participants are protected from harm.

Consent to take photographs

We would like to take a photograph of you with your child so that when you return with your child we will know that your child is part of the study. This photograph will not be shared with anyone outside of the research team.

Please mark one of the boxes below to indicate whether you do or do not allow us to take a picture of you and your child.

□ YES, I agree to allow you to take a photo of me and my child

□ NO, I do NOT agree to allow you to take a photo of me and my child

Statement of permission

By signing or placing my finger below, I am saying that:
I have read this form, or it has been read to me; I have been able to ask questions about it, and my questions have been answered;
I understand that some of the blood samples may be sent to the United States or to Europe for testing not available in Kenya.
I voluntarily agree for my child to be in this research and
I have been told that I have the right to take my child out of the study at any time without in any way affecting his/her medical care.

If you agree to have your child be in this research, please put your fingerprint at the bottom of this page or sign your name.

_____ _____ _____
fingerprint - or - Guardian Signature Date

_____
Name of person who read the consent Date

_____
Name of person who witnessed* Date

A copy of this Parental Permission Form has been provided to parent _____
(researcher initials)

** A witness is a person independent from the study. A witness is only needed if the parent/guardian cannot read. In that case, the parent/guardian can provide a thumbprint (in the box below) or verbally state his/her consent in the presence of a witness who will then sign that the form was accurately read out.*

Consent for long-term storage of blood samples for future research

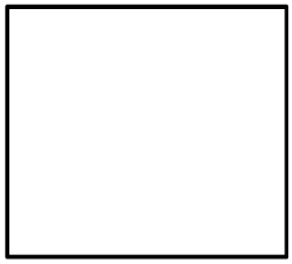
We are asking people who join this study if they will let the researchers' use their blood sample for future research. These future research will help find new ways to prevent malaria. If you say yes, we will store your child's sample with a unique number not with your child's name in a safe place in the laboratories in KEMRI/ CDC. We may send blood to CDC Atlanta or LSTM in Liverpool, but we will not give them your name, address, or any information that could identify your child. After the study period has ended, we will remove any means to associate the sample to your child, and we will not be able to identify the sample. If you do not wish to have your child's blood stored, your child may still

participate in our study. Your child will still be examined for malaria and shortage of blood. If your child has malaria or low blood, we will still treat him/her.

I have been told about the storage of my child's blood and i have agreed KEMRI/ CDC to preserve my child's blood for a period not less than 15 years for future research. I also understand and agree that my blood samples may be sent to laboratories at CDC Atlanta or LSTM Liverpool for tests that cannot be done in Kenya. I understand that I can change my mind to not have my child's blood sample sent, stored and used for future research. To do this, I may tell Dr. Meghna Desai of my request and he will tell the other study staff at KEMRI/CDC.	If you agree, circle "YES". If you do not agree, circle "NO"	
	YES	NO

Parent/Guardian providing consent for a child	Name:	Signature	Today's date (DD/MM/YY) _____
Witness*	Name:	Signature	Today's date (DD/MM/YY) _____
research staff consenting participant	Name:	Signature	Today's date (DD/MM/YY) _____

**A witness is a person independent from the study. A witness is only needed if the parent/guardian cannot read. In that case, the parent/guardian canprovide a thumbprint (in the box below) or verbally state his/her consent in the presence of a witness who will then sign.*



Wabiro kwayi mondo i dwogi e klinik diochiko e ndalo 1, 2, 3, 7, 14, 21, 28, 35, kod 42 mondo wa mi nyathini yath kod ng'iye mondo wabed gi adieri ni malaria orumo/ochango. Wabiro miyi kalenda mondo ing'e ndalo ma onego ibi. Wabiro gochoni e simu mondo wapar ni limbe gi.

E limbe ka limbe mak mana kiny, wabiro golo remo e lith lwedo mondo wa pim malaria. Wabiro pimo nok mar remo e ndalo gi 0, 7, 14, 28, kod 42. Wabiro pimo kiwango mar thieth ma nitie kuom nyathini odichieng mar abiriyo. E limbe gi wabiro pimo nyathini kod penjo penjo mag ngima. Ng'ato ma aye klinik biro manyi e dala ni ka oki biro e klinik.

Wageno ni yedhe gi biro tiyo e thietho malaria kuom ng'eny nyithindo ei nonro ni.Wabiro tiyo gi quinine thietho nyathini kendo, ka malaria mar nyathini ok orumo. Mae en national standard. Thieth ni onego o chang/tiek malaria. Pod wabiro ng'iyo nyathini mondo wabed gi adieri ni othi maber.

Ka nyathini ni gi malaria ma teko ne ngeny, wabiro kete e kar thieth. Wabiro thiedhe gi yedhe kendo miye rit kaka Migawo mar Thieth opiedho. Seche duto ma wa timo pim mar malaria ka watiyo gi remo, wabiro keto remo matin e otas. Remo ni ibiro tigo somo kute ma malaria ma kelo tuo. Ka yath ok otiyo maber kaka onego oti, remo moko ibiro pim mondo wang'e gima omone nego kute mar malaria.

Inyruok/Andaki
Nitie inyruok/andaki matin ne nyathini ka odonje nonro ni.

Yedhe mag malaria nyalo kelo chandruok. Yedhe duto nyalo kelo chandruok mar ich, ng'ok, wich bar, nur, ilo, kod del mo ruotho matin.Side effects/gik mabathe gi tin kendo gi rumo ma onge thieth. Ok yot mondo yedhe gi okel side effects mokadho.

Ka nyathini ni gi side effects mokadho, kaka ng'ok ma ok rum, del mo ruotho mokadho kata chandruok mar chuny, wabiro chungo yath. Kendo wabiro piedho ni nyathini othieth gi yedhe mar malaria mopogore e ndalo ma biro.

Onego ikel nyathini e klinik ka tuo omedore kata ka obedo gi side effects kuom yedhe mag thieth. Onego ikele kata ka og nogego ikele chieng no. Laktar biro bedo e klinik pile ka pile/ndalo duto ma nyalo thietho chandruok gi.

Kel nyathini e klinik ka:

- Ndende ochwakore
- Oriere
- Ka en gin nindo mang'eny kendo ok owinji gimoro amora
- Ong'ogo chiemo duto
- Ok onyal metho kata chiemo
- Pien dende oruotho kendo lit kata ka dhoke odholore kata ka wang'e olokore makwar
- En gi kor maremo kata en gi pek kuom yueyo kata oyueyo piyo ahinya

Inyruok manyalo wuok kuom golo remo tin kendo gin kaka chwer remo, rithruok, wang' lil kata yamo.Magi tek mondo otimre. Wabiro yweyo lwet nyathini ka pok wa golo remo kendo wabiro tiyo gi sindan manyien golo remo.

Ber

Saa donjo e nonro, nyathini ibiro net mar otanda mo okete yath ma nego suna kuom kinde mageny maonge chudo moro amora. Ting tudo mar malaria e klinik kod hosiptal e kinde mar nonro ni ibiro chul ni.Yedhe mi ibiro thieth go malaria mar nyathini ibiro yudo nono. Wabiro limo nyathini kuom ndalo 42 mondo wabed gi adiera ni thieth tiyo.

Ng'ato biro bedo e klinik kae pile pile. Inyalo kelo nyathini e limbe saa moro amora ka ineno ni otuo, gotieno kata giko juma kata kind limbe.

Nonro ni biro konyo Migawo mar Thieth ng'eyo yedhe matiyo maber kae. Ma nyalo konyi kata ng'ama ing'eyo e ndalo ma biro.

Chudo

Ibiro yudo siling mia ariyo mar chulo wuoth klinik.

Weche nonro mopondo

Weche duto ma iwachonwa kuom nyathini ok bi tang'ne ng'ato makmana jo taa nonro kendo weche gi ibiro ndik e kadi mar nyathini. Duoko mar nonro ni ibiro gol e summary form. Nying nyathini okbiwuok e ripot moro amora mar nonro ni.

Yor tudruok

Ka bang'e in gi penjo kuom bedo nyathini e nonro ni , inyalo wuoyo gi achiel kuom jotich nonro.

Ka in gi penjo kuom nonro kata iparo ni nyathini oinyore tudri kod Dr. Meghna Desai KEMRI/CDC, P.O. Box 1578-40100, Kisumu, kata e 057 20 22902. Ka in gi penjo moro amora ewi ratiro mar nyathini kaka ng'ama ochiwore ndonjo e nonro kata ka idwa loso gi ng'at ma ok ja nonro ni to inyalo tudri kod Jagoro mar KEMRI NERC e (020) 2722541 kata 0722205901, fax (254) (020) 2720030, P.O. Box 54840-00200 Nairobi-Kenya.

Chiewruok, Tamruok kod Wuok

En gima maber ni ose ng'isi gik mageny kuom donjo e nonro ni: In thuolo yiero mondo nyathini odonj e nonro ni kata kik odonj e nonro ni. Ka ok idwar mondo nyathini odonj e nonro to pod ibiro miyie thieth mar malaria. Nyathini ok bi yudo ber kuom donjo e nonro makmana rieko ma idhi yudi ma biro konyo jok moko.

- Inyalo golo nyathini e nonro saa moro amora.Ma ok bi ketho ber ma dine nyathini oyudo kendo pod ibiro yudo thieth mar malaria nono. Ka igolo nyathini e nonro ka pok oyudo yedhe duto mag nonro, ibiro miye artemether-lumefantrine.
    - Ka laktar dwaro golo nyathini e nonro ne wach moro amora, ok wabi thi nyime gi limo nyathini. Ka ogol nyathini e nonro to pok no miye yedhe duto kata ka

yedhe mag nonro ok no miyo nyathini owinjo maber, nyathini ibiro or e klinik kendo ibiro thiethe gi yedhe mowinjore.

Yiero ni:
Inyalo yie ne nyathini mondo odonje nonro ni to obiro yudo achiel kuom yedhe ariyo ma ne wang'isi kata nyathini ok nyal donjo e nonro ni. Inyalo mie thieth mar malaria mo piedhi mar,artemether-lumefantrine.

Weche nonro ni ose ne ma opiedhi gi CDC Institutional Review Board kod Migawo mar Thieth ma en kamiti ma ng'iyo ni joma ochiwore e nonro ok yudi inyruok moro amora.

Ayie mar picha
Wabiro dwaro kawo picha ni gi nyathini mondo ka iduogo wabiro ng'eyo ni nyathini nitie e nonro. Picha ni ok bi tang'e ne ng'ato ma onge e nonro ni.

Wakwayi mondo iket alama ei achiel kuom sanduge gi mang'iso ni iyie kata idagi mondo wakaw picha ni gi nyathini.

☐ AYIE, Ayie ni ikaw picha na gi nyathina

☐ ADAGI, Ok ayie mondo ikaw picha na gi nyathina

Statement of permission
Keto lweta kata go sei piny ka, ng'iso ni:
Asesomo kata osesomna weche manitie e oboke ni; oseduok penjo ga e yo makare;
Ang'eyo ni inyalo or remo moko United States kata Europe ne pim ma ok nyal tim Kenya;
Ang'eyo ni nyathina ka nigi kute mageny e rembe to onyalo nindo kar thieth mondo otimne pim moko;

Ka iyie donjo e nonroni, ket lweti e giko mar otas ni (Kaka itimo seche ma ikao kipande) kata igo sei

_____ _____ _____
Lwedo - kata - Sei mar Jarit Tarik

_____
Nying ng'ama ne osomo ayie Tarik

_____
Nying Janeno mar ayie* Tarik

Otas ma chal kama osemi janyuol _____

(Ja taa nonro initials)

** Janeno en ng'ama okotudore eyo moro amora gi nonro. Janeno dwarore mana ka Janyuol/Jarit nyathi ok nyal somo. Ka otimore kamano to janyuol/jarit nyalo keto lwete(e sanduku manitie piny ka) kata wacho ayanga ni oyie e mbele janeno ma biro goyo sei.*

Ayie mar kano remo kuom ndalo mang'eny ne nonro ma ndalo mabiro

Wakwayo jo mo donjo e nonro ni kagibiro yie ne jo taa nonro mondo oti gi remb gi ne nonro ma ndalo ma biro. Nonro gi nyalo konyo yudo yore manyieny mag geno malaria. Ka iyie wabiro kano remb nyathini unique code ok gi nying nyathini e kama opondo e lab mar KEMRI/CDC. Remb nyathini inyalo ne gi jo taa nonro ma nitie CDC Atlanta, LSTM Liverpool makmana ok wabi miyo gi nyingi, adres ni kata wach manyalo identify nyathini. Ka nonro orumo, wabiro golo nyinge duto ma onge wach moro amora mabiro miyo ngato ng'eyo weche kata remo mogol kuom nyathini. Ka ok idwar mondo okan remb nyathini, nyathini pod nyalo bedo e nonro ni. Nyathini pod ibiro timne pim mar malaria gi nok mar remo. Ka nyathini nitie gi malaria kata nok mar remo, pod wabiro thiedhe

Keno mar remb nyathina ne olerna kendo ayie ne KEMRI/CDC mondo okan remb nyathina kuom higni 15 ne nonro ma ndalo ma biro. Ang'eyo kendo ayie ni inyalo or remba e lab ma CDC Atlanta kata LSTM Liverpool ne pim ma ok nyal tim Kenya. Ang'eyo ni anyalo loko pacha mondo kik or, kan remb nyathina ne nonro ma ndalo ma mabiro. Mondo atim ma, anyalo ng'iso laktar Dr. Meghna Desai kwayo na to obiro ng'iso jotich nonro moko mag KEMRI/CDC.	Ki iyie luor "AYIE", ka ok iyie to luor "ADAGI	
	AYIE	ADAGI

Janyuol/Jarit machiwo ayie ne nyathi	Nyingi:	Sei	Tarik ma kawuono (DD/MM/YY) _____
Janeno*	Nyingi:	Sei	Tarik ma kawuono (DD/MM/YY) _____
Jatich nonro machiwo ayie ne ng'ama ochiwore donjo e nonro	Nyingi:	Sei	Tarik ma kawuono (DD/MM/YY) _____

* *Janeno en ng'ama okotudore eyo moro amora gi nonro. Janeno dwarore mana ka Janyuol/Jarit nyathi ok nyal somo. Ka otimore kamano to janyuol/jarit nyalo keto lwete(e sanduku manitie piny ka) kata wacho ayanga ni oyie e mbele janeno ma biro goyo sei.*