Temporal frequency of *Plasmodium falciparum* Kelch-13 N-terminal region and its background mutations; *arps10, crt, fd* and *mdr2*, over a 20-year period of declining malaria transmission in Kilifi, Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree or any other award in any University.

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I dedicate this work to my parents, Charles Okanda and Anna Chaka and my beloved siblings. You are my most prized possessions.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABC	ATP-binding cassette
ACT	Artemisinin based combination therapy
AL	Artemether Lumefantrine
AMA1	Apical membrane antigen 1
arps10	apicoplast ribosomal protein s10
Bp	base pair
BTB/POZ	Broad-complex, tramtrack, bric-a-brac/poxvirus and zinc finger
CDC	Centre for Disease Control
CQ	Chloroquine
Crt	chloroquine resistance transporter
Dhfr	dihydofolate reductase
Dhps	dihydropteroate synthase
DMAPP	Dimethylallyl pyrophosphate
DNA	Deoxyribonucleic acid
dNTPS	deoxynucleoside triphosphates
DP	Dihydroartemisinin Piperaquine
EDTA	Ethylenediamine tetraacetic acid
Fd	ferredoxin
FNR	Ferredoxin NADP+ reductase
FPIX	Ferriprotoporphorin IX
IARC	International Agency for Research on Cancer
IC_{50}	half maximum inhibitory concentration
ILRI	International Livestock Research Institute
INDELS	Insertions and deletions
IPP	Isoptenyl pyrophosphate
K13	kelch 13
Kb-	Kilo- base pair

KEMRI	Kenya Medical Research Institute
KHDSS	Kilifi Health and Demographic Surveillance System
LLINs	Long lasting insecticide nets
mdr2	multidrug resistance gene 2
Mdrp1	multidrug resistance protein 1
MEP	Mevalonate Independent Pathway
$Mgcl_2MgCl_2$	Magnesium chloride
MSP	Merozoite surface protein
NADH	Nicotinamide adenine dinucleotide
P. falciparum	*Plasmodium falciparum*
PCR	Polymerase Chain Reaction
Pfcrt	*Plasmodium falciparum* chloroquin resistance transporter
Pfdhfr	*Plasmodium falciparum* dihydrofolate reductase
Pfmdr1	*Plasmodium falciparum* multidrug resistance gene1
Pfmdr2	*Plasmodium falciparum* multidrug resistance gene 2
Pfmpr2	*Plasmodium falciparum* multidrug resistance protein 2
RSA	Ring stage survival assay
SEA	South East Asia
SP	Sulphadoxine pyrimethamine
Spp	Species
TBE	Tris/borate/EDTA
TBVs	Transmission Blocking Vaccines
tRNA	transfer ribonucleic acid
UK	United Kingdom
WHO	World Health Organization

ABSTRACT

Malaria remains a major challenge in many countries with the World Health Organization (WHO) documenting about 435,000 deaths from the disease in 2017. Efforts to control malaria using artemisinin-based combination therapy (ACT) have had a drawback due to emerging resistance by *Plasmodium falciparum* in South-East Asia (SEA). This was attributed to polymorphisms in the propeller domain of the kelch 13 (*k13*) gene which were linked to slow parasite clearance in infected individuals. It was also reported that mutations in four background genes i.e. apicoplast ribosomal protein s10 (*arps10*), chloroquine resistance transporter (*crt*), ferredoxin (*fd*) and the multidrug resistance 2 (*mdr2*), were precursors to the emergence of *k13* resistance mediating SNPs in *P. falciparum* isolates from SEA. The mutations in *k13* associated with artemisinin resistance have not yet been identified in Kenya and the rest of sub-Saharan Africa. Therefore, the objective of this study was to identify the presence and frequency of mutations in the N-terminal region of *k13* as well as its precursor genes in, *P. falciparum* isolates from Kilifi County over a twenty-year period. Parasite DNA was extracted from blood samples obtained from patients presenting with malaria to the Kilifi County Hospital. Polymerase chain reaction (PCR) amplification of the DNA was carried out using primers targeting the N-terminal region of *k13* and the four background genes. The amplified products were sequenced using the Sanger-capillary method and the CLC workbench software used to analyze the sequences and identify single nucleotide polymorphisms (SNPs). In the N-terminal region of *k13*, mutations were identified in 11 loci. The predominant mutation was the K189T substitution, which persisted over the 20 year-period at a relatively high frequency (>5%). The remaining SNPs were mainly singletons and inconsistent across the different years (1995, 1999, 2005, 2013 and 2015). No SNPs were identified in three of the background genes namely *arps10*, *crt* and *fd*. However, a SNP (I492V), with a frequency of more than 10%, was identified in *mdr2* across the five time points of the twenty-year period. It is unlikely that the mutations observed were as a result of ACT pressure as they were identified before and after the introduction of ACT treatment in Kenya. The results suggest that there is currently no threat of artemisinin resistance in Kilifi parasites. However, more research is needed to ascertain the role of *mdr2* I492V SNP in ACT treatment. This study provides the baseline frequency of mutations in *P. falciparum k13* N-terminal region and background genes that are precursors to *k13* resistance in SEA.

CHAPTER ONE : INTRODUCTION

1.1. Background of the study

Malaria is a global health challenge and among the main causes of morbidity and mortality annually. The disease is caused by *Plasmodium* parasites which are transmitted to humans by the bites of female *Anopheles* mosquitoes. Five species of *Plasmodium* cause malaria in humans but *Plasmodium falciparum* is associated with the most severe form of the disease. The burden of malaria is evidenced by the 219 million cases reported in 2017, an increase from the 216 million cases in 2016 (WHO, 2018). Sub-Saharan Africa carries the better share of the malaria burden as 92% of the total cases reported occurred in the region. In 2017 alone, approximately 435,000 deaths from malaria were recorded and sub-Saharan Africa accounted for 93% of these mortalities. Additionally, children below five years of age contributed to the highest proportion (61%) of deaths reported in 2017. The disease presents a huge burden to the global economy considering a total funding of 3 billion US dollars was spent on control and elimination efforts in 2017 alone (WHO, 2018).

Malaria control may involve vector management to prevent transmission of the disease. Use of insecticides in the form of Long-lasting Insecticide Nets (LLIN) and indoor residual spraying are among the most common vector control strategies (Mutuku *et al*., 2013; WHO, 2018). However, in the case of infection, antimalarial drugs may be used to eliminate the parasite. Antimalarial drugs can be used by travelers to endemic regions for prevention purposes. Pregnant women are also put on intermittent preventive therapy in regions of medium or high malaria transmission using drugs such as Sulphadoxine

pyrimethamine (SP). Combination of both vector control and use of chemotherapy has seen tremendous progress in malaria control since 2000. However, insecticide resistance as well as resistance to nearly all existing antimalarial drugs presents a major setback to the gains realized in the past years (WHO, 2018). For instance, Chloroquine (CQ) was initially used as the preferred drug for resolving uncomplicated malaria. Resistance to the drug emerged and rapidly spread across the globe resulting to its replacement with Sulphadoxine pyrimethamine (Fidock *et al*., 2000; Cui *et al*., 2015). Similarly, resistance to SP emerged and was subsequently replaced by the Artemisinin based combination Therapies (ACT), which is currently the regimen for uncomplicated malaria, due to their high therapeutic index (Cui *et al*., 2015).

The crucial role played by ACTs in malaria control is however threatened by emerging resistance to this class of drugs. Resistance has already been documented in more than five countries in South East Asia (SEA) including Cambodia where it was originally reported (Noedl *et al*., 2008; Ariey *et al*., 2014; Miotto *et al*., 2015). Resistance to antimalarial drugs has previously been shown to arise due to mutations is certain parasite genes that enable *P. falciparum* to survive in the presence of the drugs. Molecular studies are therefore important as they enabled identification of the origin and spread of mutations responsible for CQ, SP and currently artemisinin resistance (Cui *et al*., 2015).

Artemisinin resistance in South East Asia was reported to arise due to mutations in *P. falciparum* Kelch gene on Chromosome 13 (*k13*). Specific mutations namely F446I, N458Y, M476I, Y493H, R539T, I543T, P553P, R561H and C580Y in the propeller

region of *k13* were linked to delayed clearance after artemisinin therapy (Ariey *et al*., 2014). These mutations are now presently employed in surveillance studies to track artemisinin resistance and are yet to be identified in Africa (Ashley *et al*., 2014; Kamau *et al*., 2015; Muwanguzi *et al*., 2016; De Laurent *et al*., 2018). Further studies also revealed that mutations in other loci predisposed *P. falciparum* to acquiring *k13* resistance mediating mutations. These mutations were identified in apicoplast ribosomal protein s10 *(arps10)* (V127M), chloroquine resistance transporter(*crt)* (I356T), ferredoxin (*fd)* (D193Y) and multidrug resistance protein 2 (mdr2) (T484I). Although the precursor mutations have been identified in Africa, they occurred at relatively low frequencies (MalariaGEN *Plasmodium falciparum* Community Project, 2016). Africa carries the greater share of malaria burden and ACTs are generally still effective in the continent (WHO, 2018). With no ready replacement to the therapy, resistance would present a major stumbling block to the elimination milestones already realized in the region. Considering that resistance to SP and CQ both originated and spread to Africa from South East Asia, it is crucial for surveillance studies to be carried out in the region (Cui *et al*., 2015). This would be important in tracking any emerging resistance and maintaining the gains realized in malaria control over the past years.

In this study, molecular surveillance was carried out on *P. falciparum* isolates from patients presenting with malaria to the Kilifi County Hospital between 1995 and 2015. The study was conducted to assay the presence of mutations in the N-terminal region of k13 which is rarely the focus of most studies compared to the C-terminal region of the

gene (Ménard *et al*., 2016; Muwanguzi *et al*., 2016; Madamet *et al*., 2017; De Laurent *et al*., 2018). In addition, the presence and frequency of mutations in the precursor genes namely; *arps10, crt, fd* and *mdr2* was assessed to ascertain that there was no possible emerging resistance to artemisinin in Kilifi County, Kenya. This study provides information on the baseline frequency of mutations in *P. falciparum k13* N-terminal region and four other genes that are precursors to *k13* resistance mediating SNPs in Kilifi. This information will be useful in subsequent surveillance studies for ACT treatment failure in Kilifi County, Kenya.

1.2. Problem statement

Artemisinin-based combination therapies are at present, the most effective malaria control drugs, hence their widespread use. In Kenya, the use of ACTs was adopted in 2004 after two changes in drug policy, CQ to SP (1998) and SP to ACTs (2004) (Ministry of Health, 2016). Resistance to artemisinin has already been reported in South East Asia (Noedel *et al*., 2008; Ariey *et al*., 2015; Miotto *et al*., 2015) and this is due to mutations in the propeller domain of *P. falciparum K13* gene. Since resistance to CQ and SP originated and spread to Africa from south East Asia, it is feared that this might be the case for ACTs. This would present a major setback to the gains realized in malaria control in Kenya and the rest of Africa. Currently, Artemether + Lumefantrine (AL) is used as the first choice remedy for uncomplicated malaria and Dihydroartemisinin + Piperaquine (DP), the second choice (Ministry of Health, 2016). Previous surveillance studies on possible resistance to ACTs in Kenya have focused on variations in the propeller domain of *k13* (Kamau *et al*., 2015; Ashley *et al*., 2015; De Laurent *et al*., 2018), but very little is known about the presence of high frequency SNPs

in the N-terminal region of the gene. In this study, the N-terminal region of the gene was sequenced to confirm the presence of SNPs that may help understand the genetic background of *k13* and the possible role it plays in response to antimalarial drug treatment.

1.3. Justification

So far, no resistance mediating mutations in *k13* have been identified in Africa (Kamau *et al*., 2015) but temporal monitoring of loci commonly influenced by drug pressure is important in tracking and curbing emerging resistance. Additionally, temporal analysis provides insightful information on the adaptive changes acquired by the parasite under drug pressure over time (Okombo *et al*., 2014). Mutations in four other *Plasmodium falciparum* genes have also been identified as precursors to the emergence of resistance mediating mutations in *k13* (Miotto *et al*., 2015). Assessing the presence of any precursor mutations will also be useful in determining whether there is a threat of artemisinin resistance. This is important in Kilifi where there have been two policy changes in antimalarial drug use and ACTs are still generally effective. Timely detection of emerging resistance is important to public health authorities as they will be able to effect control strategies before resistance becomes established.

1.4. Objectives

1.4.1. General objective

To investigate the temporal prevalence of background mutations associated with polymorphisms in *k13* and screen the N-terminal region of *k13* upstream from the propeller domain for samples obtained over a 20-year period of declining malaria transmission in Kilifi County.

1.4.2. Specific objectives

The specific objectives of this study were to:

1. To assess the temporal frequency of polymorphisms at the N-terminal region of *k13* in *P. falciparum* isolates across five time-points between 1995 and 2015.
2. To analyze polymorphisms in *arps10*, *crt*, *fd* and *mdr2* genes of *P. falciparum* isolates between 1995 and 2015.

CHAPTER TWO : LITERATURE REVIEW

2.1. Malaria

Malaria is a life-threatening illness with about 219 million cases and 435,000 deaths in 2017 of which 93% occurred in Africa. This was a marked increase from the 216 million cases reported in 2016 (WHO, 2018). The disease is caused by parasites from the genus *Plasmodium*, which was discovered in 1880 by the French army officer Alphonse Laveran (Lalchhandama, 2014). In Kenya, 70% of the population is at risk of malaria infection, majority of whom are at the coast along the Indian Ocean and the Lake Victoria region (Ministry of Health, 2016; World Health Organization, 2018). Five species of *Plasmodium* cause malaria and have relatively similar life cycles. These include *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*. Of the five, *Plasmodium vivax* and *Plasmodium falciparum* have been documented by WHO to be the most virulent. *Plasmodium vivax* is common in countries outside sub-Saharan Africa, while *Plasmodium falciparum* which is liable for most malaria related fatalities globally is dominant in sub-Saharan Africa (Cox, 2010) and was the focus of this study.

2.2. *Plasmodium falciparum*

Plasmodium falciparum is a single celled, intracellular parasite, belonging to the kingdom protozoa. The *P. falciparum* genome was sequenced by the Malaria Genome Project and first reported in 2002. It is approximately 23 megabases large with about 80% composed of Adenine + Thymine (AT) pairs. As such it was described as one of the most AT rich genomes. The genome is composed of 14 chromosomes that encode about 5,300 genes. It was further detailed that a large majority of the described genes are

involved in evading immune responses from the host and aid host-parasite interactions (Gardner *et al*., 2002).

Plasmodium falciparum cause falciparum malaria in humans (Rich *et al*., 2009; Perkins *et al*., 2011)**.** It is known to contribute to about 50% of all cases of the disease and results to the highest number of fatalities (Roberts and Janovy, 2005). In 2017 for instance, malaria caused an approximated 435,000 deaths worldwide. An estimated 219 million people globally, were infected with malaria in 2017, majority of who came from sub-Saharan Africa (WHO, 2018). The parasite is present in all the continents except Europe which was declared free from malaria in 1974 (Piperaki and Daikos, 2016). This was achieved through rigorous control and eradication strategies including use of insecticides and antimalarial drugs. However, *P. falciparum* infection is most common in Africa as it accounted for 92% of the total number of cases reported in 2017 alone. Children under five years suffer the most from malaria since 61% of all the fatalities in 2017 occurred within this age group. India accounts for about 4.5% of the total incidence of the disease globally and has the highest incidence rates outside Africa (WHO, 2018).

Plasmodium falciparum was originally identified as the causative agent of malaria in humans by Alphonse Laveran in 1880 (Lalchhandama, 2014). It is believed to have originated from the malarial parasite of gorillas belonging to the genus *Laverania* (Liu *et al*., 2010; Holmes, 2010). Alphonse originally named it *Oscillaria malariae* before it

was renamed to *Plasmodium falciparum* in 1897 by William Welch (Loy *et al*., 2017). It is transmitted to humans by female anopheles mosquitoes, a discovery that was made by Ronald Ross and Giovanni Grassi (Loy *et al*., 2017). Being an apicomplexan parasite, *P. falciparum* possesses an apical complex which is an assortment of organelles. The apical complex notably contains rhoptries and micronemes, essential for secretion, mobility, adhesion and invasion of the host. It also harbors an apicoplast that helps in synthesis of compounds for the parasite including lipids. Most drugs aimed at eliminating the parasite are usually targeted to the apicoplast. In addition, the apicoplast aids in the Mevalonate Independent Pathway (MEP) pathway during the asexual stage of the life cycle, by synthesizing dimethylallyl pyrophosphate (DMAPP) and isoptenyl pyrophosphate (IPP) (Yeh and DeRisi, 2011). In addition to malaria, *Plasmodium falciparum* is believed to cause cancer in humans and has therefore been cartegorized as a group 2 cancer causing agent by the International Agency for Research on Cancer (IARC). It has previously been associated with transformation of lymphocytes in patients diagnosed with Burkitt's lymphoma further augmenting the progression of disease to severity (Thorley-Lawson *et al*., 2016). Interestingly, certain genetic traits in humans confer resistance to *P. falciparum* infection. These include conditions such as thalassemia, glucose-6-phosphate dehydrogenase deficiency and sickle cell (Kwiatkowski, 2005; Hedrick, 2011). Compared to other *Plasmodium* parasites, it has the highest morbidity and mortality rates. It therefore presents a considerable setback to public health and economic development in several regions of the world. Consequently, it was the focus of this study.

2.2.1. Life cycle of *Plasmodium falciparum* and malaria pathogenesis

The parasite has a complex life cycle that presents as different stages with distinct characteristics (Figure 2.1). It is transmitted to humans by infected female *Anopheles* mosquitoes. Infection begins when sporozoites are introduced into the host by an infected mosquito during a blood meal. The sporozoites are spindle shaped with a length of between 10 and 15 µm. They are known as the infective stage of the parasite to humans. About 45 minutes after infection, sporozoites migrate into the liver and develop into ovoid schizonts. A schizont has an approximate diameter of 30 – 70 µm. Individual schizonts then rapture to produce multiple merozoites measuring about 1.5 µm long and 1 µm in diameter. The process is termed as exoerythrocytic schizogony and is usually asymptomatic. From the liver, merozoites migrate to capillaries in the lungs. Merozoites then develop into trophozoites, which have a ring-like morphology. These vesicles eventually rapture releasing multiple trophozoites that in turn invade the red blood cells. In the erythrocytes, trophozoites feed on the hosts hemoglobin resulting into a pigment referred to as haemozoin. This process within the red blood cells is referred to as erythrocytic schizogony (Cox, 2010; Bartoloni and Zammarchi, 2012; Mawson, 2013; WHO, 2017).

The repeated multiplication in the red blood cells is responsible for the symptoms of malaria that manifest about 10 - 15 days after the bite of an infected mosquito. Symptoms occur due to the release of parasite remains such as hemozoin from raptured, infected erythrocytes. The disease might manifest in the form of mild fever, chills and headache in the case of uncomplicated malaria. However, severe malaria might occur

affecting several tissues in the body. Severe cases may be characterized by metabolic acidosis, anemia, vomiting, renal failure, anorexia, respiratory distress and cerebral malaria. Without immediate medical attention, these complications may rapidly become fatal. Many of the complications appear concurrently or may closely follow each other within hours. Immunocompromised individuals including the elderly, pregnant women and those on immunosuppressive therapy are at a higher risk of severe malaria (Trampuz *et al*., 2003).

As infection progresses in the erythrocytes, trophozoites mature into schizonts and gametocytes. Gametocytes present in the shape of a crescent and are usually elongated. They circulate in the blood stream and are taken up by mosquitoes during a blood meal. In the mosquito mid-gut, gametocytes mature into male and female gametes. Mature gametocytes measure about 8-12 µm long and 3-6 µm wide. Within the mosquito, gametocytes reproduce sexually, giving rise to a zygote which develops into an ookinete. Similar to the gametocyte, ookinetes have an elongated morphology and measure between 18-24 µm.The ookinetes penetrate the mid-gut wall and develop into oocysts, that are round in shape and can reach a diameter of up to 80um. Oocysts then multiply to produce several sporozoites, which begin the life cycle again (Figure 2.1) (Lanzer *et al*., 2006; Cox, 2010; Mawson, 2013; WHO, 2017; Lucius *et al*., 2017).

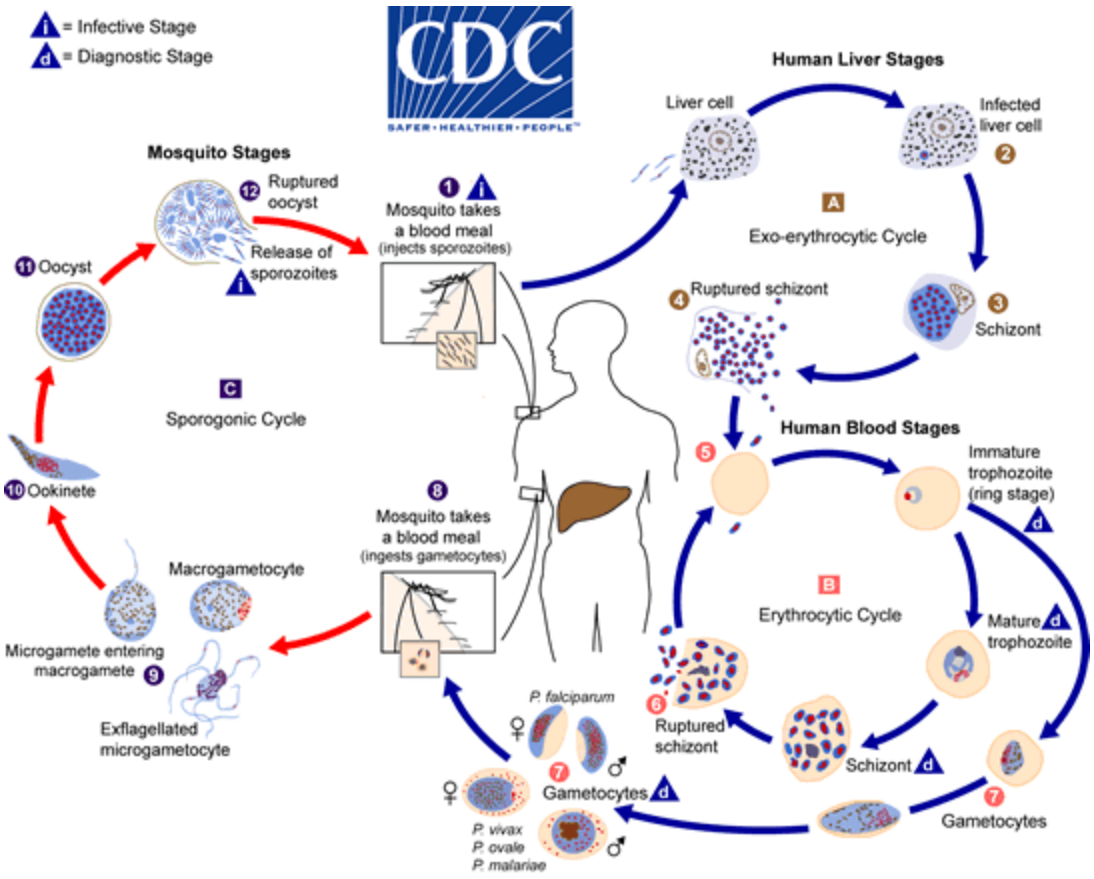

Figure 2.1: The life cycle of *Plasmodium falciparum* (CDC, 2016)

2.3. Interventions to control malaria

Multiple efforts have been put in place to control and possibly do away with malaria. Vector control strategies, targeting the *Anopheles* mosquitoes using either insecticides or biological agents may be used. Alternatively, measures directed towards *P. falciparum* using antimalarial drugs or vaccines may be employed.

2.3.1. Vector control

Vector control is mainly directed towards *Anopheles* mosquitoes, the vectors of *Plasmodium spp*. In regions where malaria eradication has been achieved, vector control has been cited as one of the key reasons for the success (Karunamoorthi, 2011).

Common practice involves the use of insecticides such as the pyrethroids in the form of indoor residual sprays or long lasting insecticide nets (LLINs). They are able to prevent malaria transmission by interfering with the feeding patterns and the life span of mosquitoes. The strategies are also considered effective with regard to cost and are therefore favorable for most low income regions (Shiff, 2002; Mutuku *et al*., 2013; Okumu and Moore, 2011; Childs *et al.,* 2016). Use of repellants may also help limit contact between mosquitoes and humans (Benelli and Beier, 2017). However, resistance to insecticides by mosquitoes limits the effectiveness of this intervention (Riveron *et al*., 2015).

Integrated vector management which involves a combination of insecticide use and various other control strategies like use of nanotechnology and genetic engineering are recommended for better control of mosquitoes. Biologically synthesized silver nanoparticles have been demonstrated to contain potential larvicidal properties (Arokiyaraj *et al*., 2015). Concerns over the safety of nanoparticles may nevertheless limit the benefits of nanotechnology as a vector control strategy. Genetic engineering approaches such as the CRISPR-Cas9 gene drive technology may be used to produce transgenic mosquitoes that block transmission of *Plasmodium* (Gantz *et al*., 2015). However, questions over the ecological consequences of the CRISPR-Cas9 system may delay its widespread use. For instance, it is feared that possible uncontrollable traits may be introduced. These traits might create disequilibrium in the ecosystem by either wiping out mosquitoes or introducing more of the insects resulting in potential plagues (Neves and Druml, 2017). The gene drive technology therefore needs to be better understood

before being rolled out as a vector control strategy. Due to these shortcomings, other malaria control methods such as use of vaccines might be used to help in the fight against malaria.

2.3.2. Antimalarial vaccines

Vaccines are a proven fundamental tool in public health. They have previously been used in eradication campaigns against measles, polio and small pox (Henderson, 1987; Moss and Griffin, 2006; John, 2009; Breman *et al*., 2011). Moreover, they were successfully utilized in the control of diseases such as neonatal tetanus (Roper *et al*., 2008). The history of antimalarial vaccines dates as far back as 1967 when it was shown by Nussenzweig *et al*. (1967) that some level of protection could be achieved in mice after exposing them to radiation attenuated sporozoites of *Plasmodium berghei*. This animal model study pioneered the use of sporozoites weakened by radiation to challenge human hosts in a bid to develop immunity against malaria. These studies demonstrated that patients could be protected from *Plasmodium falciparum* and *Plasmodium vivax* infection. This was after being exposed to bites from a considerable number of irradiated mosquitoes harboring sporozoites in their salivary glands (Clyde *et al*., 1973; Rieckmann *et al*., 1974; Clyde *et al*., 1975). However, immunization with irradiated sporozoites was considered impractical on a large scale. This is because the sporozoites needed to be delivered while still alive, through the bites of infected mosquitoes or through injections as was done in mice models. Due to this challenge, research has since focused on understanding the mechanisms required for protective immunity, the target antigens for the vaccines and effective delivery avenues that could offer sufficient protection against malaria (Hoffman *et al*., 2002). A licensed vaccine which offers about

80% protection by 2020 is now the target of control programs including the WHO (The malERA Consultative Group on Vaccines, 2011).

Antimalarial vaccines can be categorized into different groups based on the target stage in the *P. falciparum* life cycle (Greenwood and Targett, 2011). The first group of vaccines targets the asexual blood stages of *P. falciparum*. In a study by Osier *et al*. (2008), it was shown that chances of developing malaria in Kenyan children were significantly lower in the existence of antibodies targeting the *P. falciparum* Apical membrane antigen 1 (AMA1), and merozoite surface protein antigens 1, 2 and 3 (MSP1, MSP2 and MSP3). Additionally, they reported that the risk of contracting malaria reduced with a higher antibody titer against the assayed antigens AMA1, MSP1, MSP2 and MSP3. This study therefore implied that the antibody combinations significantly reduced the parasite load in infected children and prevented progression to severe disease (Osier *et al*., 2008). Vaccines that target the blood stage of *P. falciparum* are mostly based on the merozoite surface antigens AMA1 and MSP1 (Osier *et al*., 2008; Longley *et al*., 2015). They largely rely on antibodies against the surface antigens to block invasion and subsequent infection of the red blood cells (Bull *et al*., 1998).

The second category of vaccines interferes with transmission of malaria. They are referred to as the Transmission Blocking vaccines (TBVs). The vaccines target the mosquito stages of the parasite as well as the asexual stages (The malERA Consultative Group on Vaccines, 2011). Within the mosquito midgut, antigens presented by the

sexual stages of the parasite trigger immune mechanisms that block fertilization. Candidate antigens for developing TBVs include the P25 and P28 present on ookinates (Sauerwein, 2007; Saul, 2007). Antibodies generated against the candidate antigens are able to block the maturation of ookinates into oocysts (Tomas, 2001). The TBVs might alternatively target gametocytes within human hosts, making them non-infectious to mosquitoes (The malERA Consultative Group on Vaccines, 2011). Another group of vaccines target the sporozoites. A bottleneck is presented in the life cycle of *P. falciparum* by the number of sporozoites a mosquito is able to inoculate during an infective bite. This idea of a bottleneck was used as the logic behind the formulation of pre-erythrocytic vaccines (Sinden, 2010). The RTS, S/AS01 is an example of a vaccine in this category. Combining all the vaccine categories into a multistage vaccine would however serve an essential role in preventing malaria infection and transmission (Sauerwein, 2007).

Vaccines can be used as complementary options to other interventions in malaria control. An example is the RTS, S/AS01 vaccine candidate that was shown previously to confer protective immunity. The vaccine is a recombinant protein composed of the *P. falciparum* circumsporozoite protein, the Hepatitis B surface antigen and AS01 as an adjuvant. Use of RTS, S/AS01 was first licensed by the European Medicines Agency in 2015 (Gosling and Seidlein, 2016). During phase three of the clinical trials, it was shown to substantially prevent cases of clinical malaria in African infants. Nonetheless, a rapid decline in protection after vaccination was reported (RTS,S Clinical Trials Partnership, 2015) . Therefore, there is still a need for dedicated efforts towards designing a safe and

effective vaccine for use in future. In addition to insecticides and vaccines, antimalarial drugs have also been widely utilized in control of malaria.

2.3.3. Antimalarial drugs

Antimalarial drugs are mostly employed to manage the blood stage infection of *Plasmodium*, which is responsible for the clinical manifestations of malaria. Additionally, they may be used to prevent malaria in endemic areas through approaches such as prophylaxis or intermittent preventive therapy. They can be categorized as 4-aminoquinolines, antifolates, quinine and the sesquiterpene lactone endoperoxides.

2.3.3.1. 4-aminoquinolines

Among the antimalarial drugs, the 4-aminoquinolines were considered the most vital in malaria prevention and treatment. Chloroquine (CQ), the most widely known in this class was first synthesized in 1934 as Resochin before it was later renamed. It was however found to be too toxic and was abandoned for over 10 years (Pussard and Verdier, 1994; Kumar *et al*., 2003; WHO, 2008). The high efficacy of CQ was confirmed at the beginning of the 2nd world war and later recommended for both prophylaxis and treatment of uncomplicated and severe malaria. Further research into the 4-aminoquinolines then led to the identification of other drugs in this group. They included amodiaquine and amopyroquine (Pussard and Verdier, 1994).

The mechanism of action of this class of drugs remains largely debatable. Numerous hypotheses have however been put forward to explain their mode of action. Initial studies showed that CQ was able to exert its therapeutic effect by inhibiting parasite DNA and RNA synthesis. However, antimalarial activity of the drug and its selective

toxicity could not be explained by the interaction between CQ and DNA (Cohen and Yielding, 1965; Hahn *et al*., 1966; O'Brien *et al*., 1966). Other mechanisms were suggested including hindering of digestive vacuole lipase, protein synthesis as well as aspartic protease. These would nevertheless require high drug concentrations that were difficult to achieve *in vivo*. These suggestions were thus disregarded (Ridley, 2002, Bray *et al*., 2006, Schlitzer, 2007, Kaur *et al*., 2010).

An important hint to understanding the mode of action of CQ was the observation that it was only potent against the erythrocytic stages of *P. falciparum.* Thus research focused on the interaction between CQ and ability of the parasite to degrade hemoglobin (Aikawa, 1972; Deshpande and Kuppast, 2016). It was shown that *Plasmodium* degrades hemoglobin to a toxic form known as heme. Heme in its free state is damaging to membranes and vacuolar proteases of *Plasmodium*. The parasite therefore detoxifies heme through an enzyme independent fashion, into a polymer referred to as hemozoin. Hemozoin is visible microscopically as the malaria pigment (Gligorijevic *et al*., 2006). The 4-aminoquinolines are known to cause parasite death by blocking the formation of hemozoin. They achieve this by combining with ferriprotoporphorin IX (FPIX) preventing polymerization to hemozoin (Egan, 2003; Egan, 2006). Different modes can be used to administer the drugs. However, the oral route is the preferred routine of administration of these drugs although the parenteral way may also be used.

The 4-aminoquinolines are mainly administered through the oral route, particularly chloroquine and amodiaquine. The two drugs were shown to be well absorbed and had a high bioavailability following oral administration and nasogastric application in comatose patients (Salako and Adelusi**,** 1983**;** Gustafsson *et al*., 1983**;** White *et al*., 1988**)**. In rabbit models, oral amopyroquine was also shown to be well absorbed and had a bioavailability of 69% (Pussard *et al*., 1988). However, intramuscular administration of CQ resulted in fatalities because of such side effects as short term hypertension (Scott, 1950; Pussard and Verdier, 1994). Due to these potential toxic effects, it was recommended by the WHO in 1984 that CQ no longer be administered through the parenteral route (WHO, 1894). It was later shown that parenteral CQ could be administered in smaller, more frequent doses or by continuous infusions when being injected through the intravenous route (Looareesuwan *et al*., 1986). Despite the therapeutic importance of these drugs, resistance form *P. falciparum* presented a clear drawback in utilizing them for malaria elimination.

Chloroquine was originally introduced for therapeutic and preventive use against malaria in 1934 and widely used in the subsequent years. Resistance to the drug was initially reported between 1960-1961 in both South America and South East Asia. In Africa, resistance to CQ was first reported in 1977 and proliferated gradually from one country to another until it was established in the continent (Pussard and Verdier, 1994). It was originally demonstrated that erythrocytes infected with CQ resistant *P. falciparum* isolates accumulated less of the drug in their digestive vacuoles compared to those harboring CQ sensitive strains (Fitch, 1970; Verdier *et al*., 1985). Moreover, evidence

showed that efflux of CQ in resistant parasites was about 50-fold faster than that in sensitive isolates (Krogstad *et al*., 1987). This rapid discharge phenotype was found to be similar in all resistant clones across the regions where resistance was documented. The phenotype was also reported to be reversed by certain drugs such as the ion channel blockers, tricyclic antihistamines and antidepressants hence exhibiting similarities with multi- drug resistant mammalian neoplastic cells (Pastan and Gottesman, 1987)**.**

Mutations in certain *P. falciparum* genes have been implicated in CQ resistance. Nucleotide changes in *P. falciparum* chloroquine resistance transporter (*Pfcrt*; PF3D7_0709000) gene were linked with treatment failure. The resistant phenotype was characterized by reduced accumulation of the drug in the cell and transport out of the food vacuole. Chloroquine resistant parasites have a lysine substituted with threonine at position 76 (K76T) of the *crt* protein. The K76T is also thought to be accompanied by several other compensatory mutations that help restore the physiological function of *crt* following the mutation (Fidock *et al*., 2000; Wellems, 2004; Bray *et al*., 2005; Lakshmanan *et al*., 2005; Winstanley and Ward, 2006). In Kenya, use of CQ as the first line drug for uncomplicated malaria was ruled out in 1998 and was replaced by sulfadoxine/pyrimethamine (SP) (Ministry of Health, 2016).

2.3.3.2. Antifolates

The antifolates were first discovered by researchers seeking to find a cure for leukemia. In the 1940s, it was hypothesized that the disease occurred due to lack of folate in the serum of patients. Consequently, folate was administered to patients suffering from leukemia in an attempt to resolve the condition. However, instead of curing leukemia,

folate accelerated progression of the disease and proved to be ineffective against it. It was consequently withdrawn as a potential regimen for leukemia (Farber, 1974). Since it was shown to speed up proliferation of leukemia, methods of inhibiting folate were sought in order to reduce the amount of the compound in cancerous patients. The first folate analogue, aminopterin was developed and evidenced to temporarily reduce acute leukemia in some patients. This observation then pioneered the use of antimetabolites as agents of inhibiting the growth and spread of malignant cells (Farber, 1974). Although aminopterin was a potent antineoplastic agent, it was highly toxic. It was later replaced with methotrexate which is currently used as the regimen for leukemia (Greenwood, 1995). Due to the success of these antifolate agents in the treatment of cancer, researchers assayed their ability to resolve infections caused by bacteria in addition to other parasites including *Plasmodium falciparum*, the causal agent of malaria (Nzila, 2006).

The antifolates used in antimalarial chemotherapy may be grouped into 2 classes. Class I antifolates inhibit dihydropteroate synthase (*dhps*) while those in class II inhibit the dihydrofolate reductase (*dhfr*) enzyme. However, both *dhps* and *dhfr* inhibitors work in synergy and are sometimes used as combinations for treating malaria (Nzila, 2006).

2.3.3.3. Antifolates that inhibit *dhfr*

Antifolates that bind to *P. falciparum dhfr* are known for their high therapeutic index because they have a higher binding affinity to the parasite enzyme than the human *dhfr*. The first antimalarial that inhibits *dhfr* was discovered in 1945. It is now known as proguanil. Proguanil was identified to have a higher efficacy than quinine in treating avian malaria. It was also shown to possess a better therapeutic index than quinine in

animal models and was therefore used as a treatment for human malaria during the Second World War (Curd *et al*., 1945). Proguanil metabolizes to chlorcycloguanil, the active component that inhibits *P. falciparum dhfr* (Carrington *et al*., 1951). Due to its efficacy, it was used for malaria prophylaxis in combination with chloroquine (Peters, 1971; Peterson *et al*., 1988; Onori and Majori, 1989**;** Kain *et al*., 2001). It has also been used together with atovaquone to form the formulation popularly known as Malarone® for prevention of malaria in endemic regions (Kain, 2003). Chlorination of the phenyl ring in proguanil results in the antifolate chlorproguanil (Peters 1971; Onori and Majori, 1989; Wernsdorfer 1990). Chlorproguanil works in a similar way to proguanil by metabolizing to chlorcycloguanil which inhibits parasite *dhfr*. It was recommended for prophylaxis at lower doses due to the high potency (Watkins *et al*., 1987). Another antifolate, Clociguanil was an analogue of Chlorproguanil but was phased out due to its instability and short activity (Knight and Peters, 1980).

Pyrimethamine is another *dhfr* binding antifolate. It belongs to the 2, 4-diaminopyrimidine family which were initially valued for their anti-tumor properties (Hitchings *et al*., 1950). Pryimethamine is mostly used in combination with Sulphadoxine in malaria chemotherapy or less often as a monetherapy under the product name Daraprim® (Nzila, 2006). In addition to Pyrimethamine, proguanil, chlorcycloguanil and clociguanil, WR99210 was also used as a *dhfr* binding compound. However, developing it into an antimalarial failed because very little amounts of the drug were available in circulation in turn limiting its therapeutic efficacy (Rieckmann *et al*., 1996).

2.3.3.4. Antifolates that block *dhps*

They are mostly sulphur drugs and are valued for their ability to block biosynthesis of folate. These drugs can be subdivided further into the sulphomides and the sulphonamides. However, attempts to use them as monotherapies against malaria failed due to their high toxicity levels and low efficacy (Michel, 1968). Presently, they are used in combination with the *dhfr* inhibitors because their modes of action are in synergy. The anti-*dhps* Dapsone is recorded as the most potent antimalarial in this group. The compound was first synthesized in 1908 and originally tested for its antimicrobial activity in late 1930's (Buttle *et al*., 1937). It was shown to prevent growth of certain bacteria as well as the malaria parasite. Development of the compound against malaria was nonetheless abandoned because it contained unacceptable levels of toxicity and also had limited efficacy (Rieckmann *et al*., 1968; Sheehy, 1967). Other than malaria, it is used in treating other infectious diseases such as leprosy and Pneumonia (Britton and Lockwood, 2004). Currently, it appears to be the most largely utilized *dhps*-binding chemotherapeutic agent.

More often, antifolates are combined to give potent and longer acting formulations. For instance, pyrimethamine is mixed with sulphadoxine to give suphladoxine-pyrimethamine (SP), commonly referred to as Fansidar®. Fansidar was used for uncomplicated malaria in many parts of Africa but was phased out in Kenya as the first line antimalarial drug in 2004 due to resistance (Ministry of Health, 2016). However, it is up to date used for intermittent preventive therapy in expectant women (Cui *et al*., 2015). Pyrimethamine was also combined with sulfalene to give Metakelfin® which is no longer in use due to reduced efficacy. Most antifolates are now no longer extensively

used for managing malaria because of resistance from the parasite. Mutations in the *dhfr* (PF3D7_0417200) and *dhps* (PF3D7_0810800) genes of *P. falciparum* were shown to play a role in reduced sensitivity to antifolates (Vinayak *et al*., 2010). They were replaced by the more effective artemisinin based combination therapies (ACT).

2.3.3.5. Quinine

The discovery of quinine as an agent for treating malaria dates as far back as early 1600's. The drug was initially obtained from the back of the Cinchona (Quina quina) tree. At the time, it was referred to as the 'Jesuits' bark, a term that originated from its use by Jesuit missionaries from South America (Achan *et al*., 2011). Legend however suggests that quinine was used earlier by Indians to manage fever. According to the legend, an Indian lost in the jungle, with fever, accidentally consumed stagnant water contaminated by neighboring quina quina trees. He initially thought that he had consumed poison but to his surprise, his high fever was resolved. He shared this intriguing experience with his fellow villagers who begun using the quina quina extracts in treating fever (Achan *et al*., 2011). In Europe however, the accepted legend is that of a Spanish Countess from Chinchon. The Countess contracted a fever while in Peru and used the bark of a tree to cure it. In 1638, the countess carried the therapeutic bark back (now quinine) to Spain, hence introducing the regimen in Europe. In honor of The Countess, Carl Linnaeus named the bark Cinchona in 1738 (Achan *et al*., 2011).

Until 1820, the medicine was formulated by grinding the back of the cinchona tree into a powder, mixing it with a drink, mostly wine and consuming it as a concoction. In 1820, Pierre Joseph Pelletier and Joseph Caventou extracted and named quinine from the bark, and replaced it with purified quinine as the routine treatment for malaria (Achan *et al*.,

2011). Between 1866 and 1868, quinine and other cinchona alkaloids were found to be effective against malaria in clinical trials involving around 3600 participants. The alkaloids included cinchonidine, quinidine and cinchonine but in 1890, quinine was preferred as the standard cure for malaria up until the 1920s when more potent synthetic antimalarial drugs were adopted (Abdi *et al*., 1995; Achan *et al*., 2011). It still plays a fundamental role in the management of malaria especially severe forms of the disease.

Quinine can be administered both orally and through the parenteral route. It reaches maximum concentration in the body after about 1 - 3 hours (Salako and Sowunmi, 1992), but is also rapidly excreted from the system. The extent of absorption into the body is relatively high and can be found both in the cerebrospinal fluid and across the placental barrier (White, 1996; Esamai *et al*., 2009; Achan *et al*., 2011). The drug acts against the erythrocytic stages of *P. falciparum* and can also be used as a pain reliever (Achan *et al*., 2011). Despite the therapeutic benefits of quinine, a number of side effects are associated with it. Mild side effects include headache, hearing disability and nausea. However, patients might experience adverse side effects ranging from vomiting, diarrhea, marked loss of hearing, loss of vision and dizziness. When administered rapidly, the drug might cause low blood pressure and intravenous administration might lead to thrombosis in the veins (Karlsson, *et al*., 1990; Achan *et al*., 2011). Severe pain is associated with intramuscular administration which may lead to sterile abscesses. In pregnant women, hypoglycemia may result from quinine therapy (Okitolonda*et al*., 1987; Achan *et al*., 2011). Less frequently, serious side effects such as psychosis, liver injury, skin eruptions and asthma might occur (Achan *et al*., 2011).

Quinine works in a similar way to chloroquine by binding to haem and blocking its crystallization to haemozoin. Resistance to antimalarial drugs by *P. falciparum* is an important issue affecting the efficacy of nearly all the drugs ever introduced. Resistance to the drug was first reported in Brazil in 1908 (Da Silva and Benchimol, 2014). Quinine resistance was widely reported in South America and South East Asia before eventually spreading to Africa. Resistance was associated with mutations in the *P. falciparum* multidrug resistance 1 (*pfmdr1*), *pfcrt* and the multidrug resistance protein 1 (*mdrp1*) genes. Resistance was however shown to be mild since the drug maintained some potency but with delayed activity (Mayxay *et al*., 2007; Legrand *et al*., 2007; Achan *et al*., 2011). In fact, the drug is mostly used in managing severe malaria in most regions (Hüttinger *et al*., 2010).

2.3.3.6. Sesquiterpene lactone endoperoxides

Artemisinin and its derivatives artemether, dihydroartemisinin and artesunate are collectively known as the sesquiterpene lactone endoperoxides. Artemisinin was discovered by the Chinese scientist Tu Youyou in 1972. She was awarded the Nobel Prize in Medicine for this discovery (Voorhi *et al*., 2015)**.** Artemisinin is an extract of a Chinese herbal plant scientifically known as *Artemisia annua*. However, yeast can also be used more efficiently to produce an artemisinin precursor through genetic engineering (Arsenault *et al*., 2008). They are highly potent and are used for the management of uncomplicated malaria and helminth infections (Whirl-Carrillo *et al*., 2012). They are gametocidal and may block transmission of the parasites to mosquitoes (Winstanley and Ward, 2006). They are also active against the ring stages of *P. falciparum* hence help in reducing parasite burden during infection. Nevertheless, their potency is short lived and

are usually combined with other longer acting drugs to give the artemisinin-based combination therapies (ACT). Moreover, use of the drugs as single therapies is discouraged by the WHO due to chances of *P. falciparum* developing resistance against the potent treatment (WHO, 2018).

2.3.3.7. Artemisinin based combination therapies (ACT)

Resistance to previously effective drugs like SP and CQ prompted the WHO to recommend use of ACT for the treatment of uncomplicated malaria (WHO, 2017). These therapies combine artemisinin derivatives with drugs from other classes. Artemisinin derivatives are highly potent and rapidly eliminate most parasites within a short period. However, they are equally cleared fast from the body. The partner drugs being longer acting, therefore eliminate any remaining parasites (Cui *et al*., 2015). By persisting in the body longer, partner drugs as well reduce the chances of recrudescence and the gradual development of resistance (Nosten and White, 2007). Common ACTs recommended for use by WHO include artemether+lumefantrine (AL), artesunate+amodiaquine, artesunate+mefloquine, dihydroartemisinin+piperaquine (DP) and artesunate+sulfadoxine-pyrimethamine (WHO, 2017). In Kenya, AL is used as the first line regimen for uncomplicated malaria and DP is the second line drug (Ministry of Health, 2016).

Despite the integral role of ACTs in malaria control, emergence of resistance by *P. falciparum* presents a major setback to their therapeutic efficacy. Resistance was originally reported in Cambodia in 2008 and was characterized as delayed parasite

clearance from infected individuals (Noedl *et al*., 2008). Genome wide studies of *P. falciparum* identified regions on chromosome 13 that mediated resistance to artemisinin (Cheeseman *et al*., 2012). In addition, mutations in the propeller domain of *P. falciparum* kelch (*k13*) gene were linked to delayed clearance of parasites after treatment with artemisinin in South East Asia (Ariey *et al*., 2014). The mutations associated with resistance have not yet been found in the African *P. falciparum* parasites (Ashley *et al*, 2014; Kamau *et al*., 2015; Muwanguzi *et al*., 2016; De Laurent *et al*., 2018;Amambua-Ngwa *et al*., 2019). Nonetheless, De Laurent *et al*. (2018) identified a mutation in Kenya, at codon 539, which was previously reported in South East Asia to habour changes that result in artemisinin resistance. However, the SNP coded for an amino acid substitution (R539K) that was different from that reported in SEA (R539T).

Resistance to most antimalarial drugs has been shown to arise due to mutations in specific parasite genes. This highlights the importance of molecular genotyping in testing the therapeutic efficacy of antimalarial drugs. The technique involves sequencing genes that have been proven as markers of resistance and identifying nucleotides changes that drive treatment failure. This approach has high sensitivity, specificity and reproducibility. Additionally, protocols can be scaled up to handle multiple samples at a particular time (Cui *et al*., 2015).

2.4. Markers of antimalarial drug resistance

A number of genes have previously been identified as markers of resistance to current and previously used antimalarial drugs. They include the *Pfdhfr*, *Pfdhps, Pfcrt, Pfmdr1,* multidrug resistance gene 2 (*Pfmdr2), Pfmrp1,* multi drug resistance protein 2 (*Pfmpr2)*

and *Pfk13*. *Pfdhfr* (located on chromosome 4) codes for dihydrofolate reductase enzyme involved in the synthesis of thymidine nucleotides by reducing dihydrofolate to tetrahydrofolate. Due to this, it is usually the target for most antifolates including sulfadoxine and pyrimethamine (Peterson *et al*., 1988). Mutations in the gene were shown to confer resistance to antifolates. They include the S108N SNP identified by Peterson *et al*. (1988) to drive pyrimethamine resistance. Additionally, triple mutations in the gene i.e. N51I, C59R and S108N were associated with sulfadoxine-pyrimethamine treatment failure (Kublin *et al*., 2002). Mutations in the gene might therefore be used for surveillance of potential resistance to SP in areas where the drug is still being used.

Pfdhps codes for dihydropteroate synthase, an enzyme involved in folate biosynthesis. Drugs such as SP exhibit their therapeutic activity by inhibiting the enzyme (Brooks *et al*., 1994). The following mutations in the gene were identified to be responsible for resistance to sulfadoxine including S436A, A437G, L540E, A581G, S436F, A613T/S (Brooks *et al*., 1994; Wang *et al*., 1997). These mutations have since been extensively identified in Africa including Kenya, and they correlate with resistance to SP (Plowe *et al*., 1993; Kublin *et al*., 2018). The *Pfcrt* gene codes for a protein located on the food vacuole of the parasite and is believed to be a member of the drug/metabolite superfamily of proteins (Fidock *et al*., 2000; Tan *et al*., 2004). Several resistance related polymorphisms have been previously identified in the gene. However, the K76T SNP appears to be the principle mediator of resistance to CQ and was identified in a number of studies (Plowe *et al*., 1993; Fidock *et al*., 2000) .

Pfmdr1, *Pfmdr2*, *Pfmrp1*, and *Pfmrp2* code for proteins that belong to the ATP-binding cassette (ABC) superfamily. They are known to play a role in the outflow of drugs through active transport and ATP hydrolysis. They are therefore well known for actively contributing to resistance to multiple drugs (Veiga*et al*., 2014). Mutations and changes in copy numbers of the genes have been previously linked to resistance to antimalarial drugs. Reducing the copy number of *Pfmdr1* for instance, was shown to increase sensitivity to artemisinin, quinine, lumefantrine, mefloquine and chloroquine. Additionally, the N86Y and D1246Y SNPs were linked to reduced sensitivity to quinine, amodiaquine and CQ particularly in Africa. However, it was noted that the mutations elevated the sensitivity of *P. falciparum* to other drugs such as lumefantrine, and artemisinin (Mu *et al*., 2003; Cui *et al*., 2015). The T484I SNP in *mdr2* was recently reported as a background (precursor to *k13* resistance-mediating mutations) marker of artemisinin resistant parasites in South East Asia (Miotto *et al*., 2015). Similar to the other transporters, the *mrp1* gene has previously been implicated in the efflux of chloroquine and mefloquine leading to reduced efficacy (Wu *et al*., 2006). A synonymous SNP at codon 1138 (A1138A) of *mpr2*, was found be associated with increased IC_{50} values on treatment with chloroquine. Insertions and deletions in the gene were also demonstrated to mediate sensitivity to piperaquine and mefloquine (Veiga *et al*., 2014). Additionally, insertions and deletions (indels) in the gene were found to correlate with responses to lumefantrine treatment *In Vitro* (Okombo *et al*., 2013). These genes are therefore useful in tracking emerging or existing resistance in different populations hence guiding decisions on antimalarial drug use policies.

The Kelch gene (PF3D7_1343700) is located in chromosome 13 of *P. falciparum* and has only one exon with 2181 nucleotide base pairs. It codes for the kelch protein, composed of three domains namely the N-terminal, the broad-complex, tramtrack, bric-a-brac/poxvirus and zinc finger (BTB/POZ) and the propeller domain with 6 blades at the carboxyl terminal region (Figure 2.2). The function of k13 in *P. falciparum* however remains largely unclear. The N-terminal region of k13 is very well conserved among the *Plasmodium* species and is composed of about 225 amino acid residues (Ariey *et al*., 2014). Mutations in the propeller region of *k13* were shown by Ariey *et al*. (2014) to be drivers of resistance to artemisinin in South East Asia. Four mutations in the propeller region namely Y493H, R539T, I543T and C580Y (Figure 2.2) were verified as markers of artemisinin resistant parasites. Further studies also identified these mutations in resistant parasites but have been reeported to be absent in artemisinin sensitive clones (Miotto *et al*., 2015; Thuy-nhien *et al*., 2017). In Africa, ACTs are still effective and mutations that mediate resistance are yet to be identified in African *P. falciparum* isolates. Nonetheless, *k13* propeller mutations have still been identified in *P. falciparum* isolates from Africa, the predominant one being the A578S polymorphism. None of them has been associated with response to ACT treatment and are simply thought to be geographical signatures (Ashley *et al*., 2014; Kamau *et al*., 2015; Muwanguzi *et al*., 2016; De Laurent *et al*., 2018). At the N-terminal region of *k13*, mutations tend to be less frequent compared to the C-terminal region where the propeller domain is located. Despite there having been non-synonymous mutations at the N-terminal region of the gene, none has been linked to sensitivity or resistance after treatment with artemisinin

(Conrad *et al*., 2014; Ashley *et al*.,2014; Takala-harrison *et al*., 2015; Agathe *et al*., 2016).

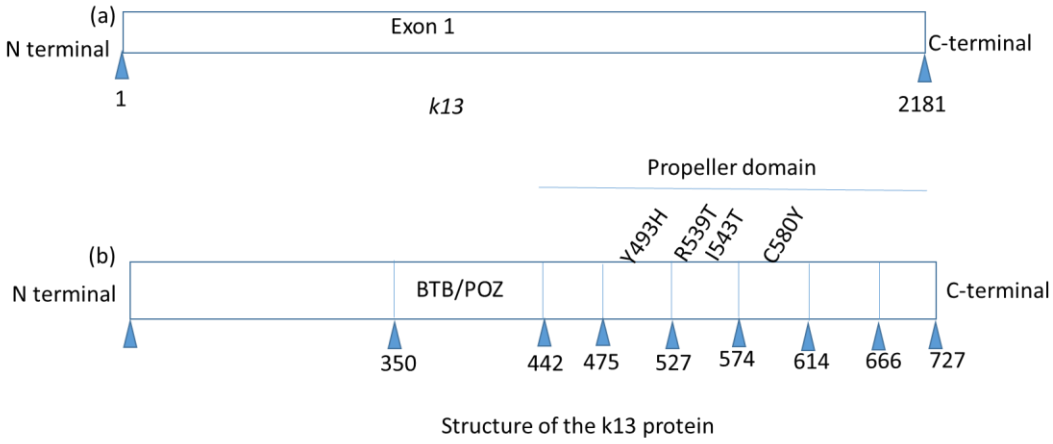

Figure 2.2: (a) The *k13* gene. (b) Structure of the k13 protein and the genetic alterations linked to artemisinin resistance.

2.5. Background mutations leading to polymorphisms in k13

A study by Miotto *et al*. (2015) revealed that mutations in *k13* have a genetic background upon which they arise and mediate resistance. In this study conducted across 6 countries of South East Asia, nonsynonymous nucleotide changes were identified in apicoplast ribosomal protein s10, *arps10* (PF3D7_1460900) on chromosome 10, chloroquine resistance transporter, *crt* (PF3D7_0709000) on chromosome 7, ferredoxin, *fd* (PF3D7_1318100) on chromosome 13 and multidrug resistance protein 2 (*mdr2*; PF3D7_1447900) gene on chromosome 14 (Figure 2.3). The polymorphisms were as follows, valine was substituted with methionine at codon 127 of *arps10* (V127M), isoleucine with threonine at codon 356 of *crt* (I356T), asparagine with tyrosine at codon 193 of *fd* (A193T) and threonine with isoleucine at codon 484 of *mdr2* (T484I). These mutations displayed

significant association with the observed polymorphisms in *k13* that played a role in slow parasite clearance after treatment with ACTs in the region. They were also found to exhibit higher levels of differentiation in regions of high resistance. However, their frequency was considerably low or entirely absent in areas where resistance was yet to be documented. Interestingly, the mutations were not implicated in driving resistance in isolation as they had negligible effects on response to ACTs in parasites without the resistance polymorphisms in their *k13* propeller domain. Miotto *et al*. (2015) therefore suggested that the mutations may be precursors of *k13* resistance mutations in parasites initially sensitive to artemisinin. The background mutations have not been identified in African isolates (Balikagala *et al*., 2017; Ikeda *et al*., 2018).

Given that the Kelch protein is highly conserved among the *Plasmodium* species, a loss of fitness is suggested to be the consequence of mutations in *k13*. The background mutations are therefore thought to be crucial in easing the fitness burden by providing compensatory mutations in other regions of the genome. Two of the four identified background mutation genes i.e. *arps10* and *fd* are associated with the apicoplast as they affect a number of proteins in the organelle. Several antimalarial drugs such as clindamycin and tetracycline target the apicoplast ribosomal protein complex which includes *arps10* (Miotto *et al*., 2015).

Ferredoxin is involved in the electron transport chain of the parasite. It is reduced by ferredoxin NADP+ reductase (FNR), which transfers electrons from NADH to fd. Reduced ferredoxin then facilitates other reactions such as reduction of sulphite, nitrites

and in fatty acid biosynthesis (Kimata-Ariga *et al*., 2007). Ferredoxin in its reduced state is also thought to help *P. falciparum* withstand the oxidative stress associated with artemisinin treatment. The *mdr2* belongs to the ABC superfamily of genes which are known to be involved in the efflux of drugs by active transport. The gene has also previously been linked to resistance to some antifolates (Martinelli *et al*., 2011). Mutations in *crt* were also found to confer resistance to chloroquine (Fidock *et al*., 2000). Both genes encode transport proteins located in the digestive vacuole of the parasite. The background mutations are therefore generally thought to confer a survival advantage to the parasite when subjected to artemisinin pressure. Consequently, this study set out to identify the presence and frequency of mutations in the background genes as well as the N-terminal region of *k13* in Kilifi isolates over a twenty-year period. This is because there has been no reported study on the genes, yet they might be crucial in detecting early signs of ACT treatment failure in Kenya. Additionally, the N-terminal region of *k13* has been scarcely studied over time and therefore this study provided insights on the mutations that occurred in the region.

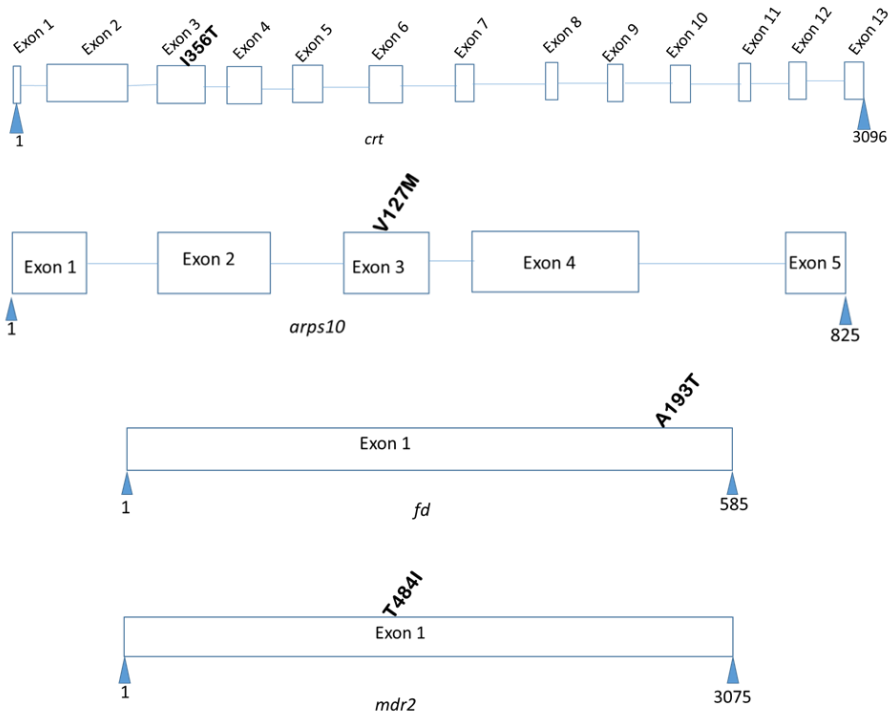

Figure 2.3: The background mutation genes with the specific changes (in bold) and their locations, associated with resistance to artemisinin

CHAPTER THREE : MATERIALS AND METHODS

3.1. Description of study area and samples

This study was conducted at the KEMRI-Wellcome Trust Research Program laboratories in Kilifi County. Kilifi lies at the Coast of Kenya and spans about 65 km along the coast line. Malaria is endemic in the region with peak transmission between April and June after the long rains or October to November following the short rains (O'Meara *et al*., 2008). Samples collected over a twenty-year period (1995 - 2015) of declining malaria transmission in Kilifi County were used. The samples were obtained from patients presenting with malaria to the Kilifi County Hospital. The hospital is located 3° south of the equator in Kilifi town and inside the Kilifi Health and Demographic Surveillance System (KHDSS) (Figure 3.1), which covers about 260,000 people majority of whom are subsistence farmers. The KHDSS occupies an area of about 891 km^2 and spans approximately 31 km along the coastal strip from Kilifi (Scott *et al*., 2012). The samples were clustered into two time points (1995 and 2015) and 150 samples were used at each time point. However, where high frequency (>10%) mutations were observed in any of the genes, three additional time points (1999, 2005 and 2012) were assessed to obtain a broader picture of variation. Five points in time (1995, 1999, 2005, 2012 and 2015) were analyzed for *k13*, since previous work on the propeller domain covered all the five time points (Wamae *et al*., 2019).

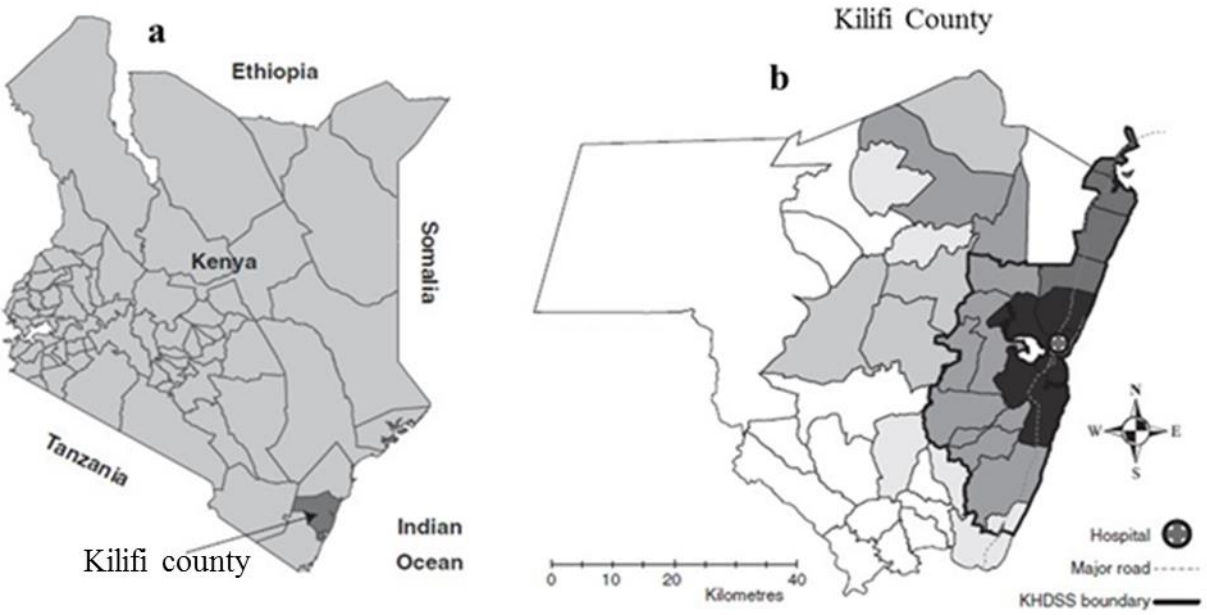

Figure 3.1: (a) The map of Kenya indicating the location of Kilifi County. (b) A map of Kilifi showing the location of Kilifi County Hospital within the KHDSS (Scott *et al*., 2012)

3.2. Extraction of DNA

For the 1995 - 2012 samples, parasite genomic DNA extracted from frozen erythrocytes by Okombo *et al*. (2014) was used. Genomic DNA of the parasites from the 2015 whole blood samples was extracted by Wamae *et al*. (2019), using the QIAamp® DNA Blood Mini kit (QIAGEN) following the manufacturer's instructions. Two hundred microliters of whole blood was mixed with 40 µl of protease and 200 µl of lysis buffer (AL) before incubating at 56 °C for 10 minutes. After incubation, 400 µl of absolute ethanol was added to the mixture. Six hundred and fifty microliters of the mixture was then loaded into QIAamp® spin columns and allowed to pass through the membrane, before adding 750 µl of wash buffer AW1. Following this step, 750 µl of wash buffer AW2 was added

to the columns and allowed to soak for 2 minutes. In the final step, DNA was eluted using 50 µl of 10 mM Tris solution, centrifuged at 13,000 ×g for 3 minutes.

The concentration and quality (A_{260}/A_{280} ratios) of the extracted genomic DNA was determined using the Nanodrop, ND 1000 Spectrophotometer (inqaba biotec). The isolated DNA samples were diluted in sterile double-distilled water to produce working concentrations of 5 ng/µl which were stored at -20 °C for use in further steps.

3.3. Generation and purification of amplicons

3.3.1. Polymerase chain reaction (PCR)

The five genes namely *arps10, crt, fd, k13* and *mdr2* were amplified using primers in Table 3.1. Specific target regions and their respective sizes have also been indicated in Table 3.1. In addition to the genes, the study included the serine-tRNA ligase (PF3D7_0717700) gene as a control. The gene is considered neutral, as it is not under selective pressure from antimalarial drugs (Anderson *et al*., 2005).

Table 3.1: Target genes, their primers, annealing temperatures and the specific regions sequenced

Gene name	Gene ID	Size of gene (bp)	Size of target region (bp)	Target exon	Primers	Annealing temperature (°C)	Reference
ARPS10	PF3D7_1460900	1477	165	3	F- CACAATATTATGTTTCATTTTAG R- GTATAATTTATTCTGCTTACATTC	55	This study
CRT	PF3D7_0709000	3096	109	3	F- GATTATCGACAAATTTTCTAC R- CTTTTTAATTCTTACGGCTAAG	55	This study
FD	PF3D7_1318100	585	246	1	F- GATGCTAGTGAAAGACAGAATG R- CACATATTTTTGATTGAGGAC	55	This study
MDR2	PF3D7_1447900	3075	1349	1	F- GAGGTTTGTGGTGTATTATTTTC R- GTTAAACCTATAAATAATACAC	55	This study
K13	PF3D7_1343700	2181	618	1	F- ATGGAAGGAGAAAAAGTAAAAAC R- CTCTTTTTTGTTGGTATTCATAATTG	56	This study
Serine-tRNA ligase	PF3D7_0717700	1620	1620	1	F- ATGGTTTTAGATATAAATTTATTTCG R- TCTTGTTCACTCCATAAAGGGA	51	This study

F-forward R-reverse

The Expand ™ High fidelity PCR system (Roche) was used for this study. Annealing temperature for each primer pair was determined using gradient PCR and utilized *P. falciparum* 3D7 DNA as the template in a 10 µl reaction volume. A range of 47 °C to 58 °C was used to obtain the optimal annealing temperature for each of the primer pairs in Table 3.1. The cycling conditions for PCR were 94 °C for 2 minutes, 10 cycles of 94 °C for 15 seconds, annealing temperature- 30 seconds and 72 °C for 2 minutes, 25 cycles of 94 °C for 15 seconds, annealing temperature- 30 seconds and 72 °C for 2 minutes then a final extension at 72 °C for 2 minutes.

A single total reaction volume of 10 µl constituted of 0.5 µl of template DNA (5 ng/µl), 6.56 µl of ultrapure DNase/RNase free distilled water, 0.2 µl of 10 mM deoxynucleotides triphosphate (dNTPs), 1 µl of 25 mM $MgCl_2$, 0.3 µl of 10 pmol/µl forward primer, 0.3 µl of 10 pmol/µl reverse primer, 1µl of 10 × PCR buffer with 15 mM $MgCl_2$ and 0.14 µl of 3.5 U/µl Expand ™ High fidelity PCR Taq polymerase (Roche). The thermal conditions for PCR were as follows, 94 °C for 2 minutes then 15 cycles of 94 °C for 15 seconds, annealing temperature for 30 seconds and 72 °C for 2 minutes, 25 cycles of 94 °C for 15 seconds, annealing temperature for 30 seconds and 72 °C for 2 minutes then a final extension at 72 °C for 2 minutes. The positive control was DNA from *P. falciparum* 3D7 clone (PF3D7_1343700) retrieved from Plasmo DB as the reference (www.plasmodb.org).

3.3.2. Agarose gel electrophoresis

Amplified products were visualized on 1% (w/v) agarose gels. One gram of agarose powder (SIGMA) was dissolved in 100 ml of 0.5× Tris Borate EDTA (TBE) buffer. The mixture was boiled in a microwave for 2 minutes and cooled to about 40 – 45 °C after which 5 µl of 20,000 × RedSafe DNA staining solution was added before pouring it into a casting tray, fitted with gel electrophoresis combs. The gel was allowed to solidify for 30 minutes and the tray was immersed in the electrophoresis tank containing TBE buffer. The combs were removed then 5 µl of loading dye and 2 µl of amplified products were used for loading into the gel wells. Electrophoresis was run at 100 volts for 40 minutes after which the DNA bands were visualized under ultraviolet illumination in the Bio-Rad ChemiDoc XRS+^{TM} imaging system.

3.3.3. Purification of amplicons

Following PCR, the amplified products as observed from agarose gel electrophoresis results were purified using the ExoSap-IT^{TM} (Affymetrix^{TM}) reagent. The reagent comprises of two enzymes; Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a particularly formulated buffer by Affymetrix^{TM}. Purification was done to remove excess primers and unincorporated nucleotides during the amplification process. One microliter of the ExoSap-IT® (Affymetrix) reagent was mixed with 5 µl each of the PCR products to make a reaction volume of 6 µl. These were then incubated at 37 °C for 20 minutes for the enzymes to hydrolyze extra nucleotides and primers. Thereafter, a second incubation at 80 °C for 20 minutes was done to inactivate the enzymes.

3.4. Sequencing

After purification, the products were sequenced directly with the primers utilized for PCR in Table 3.1 and the BigDye® Terminator v3.1 cycle sequencing kit from Applied Biosystems, UK. An additional primer pair (F- AGAAAAATAGGTGGAGCTA and R-GTAATAAGAAACCTGCACCTG) was used for sequencing the serine-tRNA ligase, the control gene. A single reaction volume was 10 µl consisting of 2 µl of purified template DNA, 0.5 µl BigDye® Terminator enzyme mix, 1.75 µl 5× BigDye® sequencing buffer, 4.75 µl ultrapure DNase/RNase free distilled water and 1 µl of 5 pmol/µl sequencing primer. Thermal conditions for cycle sequencing included an initial denaturation at 95 °C for 30 seconds, 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes followed by a final hold at 15 °C for 10 minutes.

3.4.1. Ethanol precipitation

To purify and concentrate the sequencing products, ethanol precipitation was done. Briefly, using a micropipette, 1 µl of 125 mM Ethylenediaminetetraacetic acid (EDTA), 1 µl of 3 M sodium acetate and 25 µl of absolute ethanol was mixed with the cycle sequencing products and incubated at room temperature for 15 minutes. They were then centrifuged at 3200 ×g for 20 minutes at 20 °C. After centrifuging, the supernatant was drained off and the pellet re-suspended in 100 µl of 70% ethanol. These were centrifuged again at 320 ×g for 5 minutes at 20 °C. The supernatant was drained off again and the pellet air dried for 30 minutes. Ten microliters of Hi-Di™ Formamide (Thermo Fisher Scientific) was then added and samples stored at 4 °C before capillary electrophoresis.

3.4.2. Capillary electrophoresis and sequence assembly

Sequence chromatograms were obtained by capillary electrophoresis which was outsourced from the International Livestock Research Institute (ILRI), Nairobi, Kenya, where the ABI 3730xl DNA analyzer (Applied Biosystems), was used. Sequence assembly, editing and alignment was done using CLC workbench version 7.7.1 (QIAGEN) after which SNPs and their relevant amino acids were noted. Briefly, raw sequence data (with the AB1 file format), from ILRI was imported into the CLC workbench software. Under the 'sequence analysis tools' option, the forward and reverse sequences for each sample were grouped using the 'sort sequences by name' command. After sorting, the sequences were trimmed using the 'trim' command to remove low coverage regions with poorly defined chromatograms. The sequences were then assembled using the 'assemble to reference' option, which generated a subfolder for each sample after assembly. The Pf3D7 sequence for each respective gene, imported from Plasmo DB (www.plasmodb.org), was used as the reference. In each assembly step, a consensus sequence was extracted using the 'extract consensus sequence' command. Each consensus sequence was named according to the unique sample ID, originally sequenced. The consensus sequences were then used for alignment using the 'create alignment' option. Sequence positions that differed from the rest were marked as SNPs and these were ascertained using sequence assemblies of the respective samples. Synonymous and non-synonymous amino acid substitutions were also marked by translating the nucleotide sequences and comparing the translation with the 3D7 sequence to confirm the positions.

3.5. Statistical analysis

The frequency of each SNP was obtained by dividing the number of samples bearing the particular mutation by the total number of samples analyzed for each gene. The quotient was then multiplied by 100 to get the prevalence of each mutation.

3.6. Ethical clearance

Approval to use the samples was granted by the Ethics Review Committee of the Kenya Medical Research Institute (KEMRI); protocol number SERU 3149.

CHAPTER FOUR : RESULTS

4.1. PCR amplification and gel electrophoresis

4.1.1. k13 N-terminal region

A total of 150 samples at each time point were amplified except for 2012, where only 145 samples were available. Out of the total samples used for PCR, amplicons were successfully generated in 144, 131, 131, 120 and 144 samples from 1995, 1999, 2005, 2012 and 2015 time points, respectively. All the amplified products gave the expected band size of 618 bp (Figure 4.1).

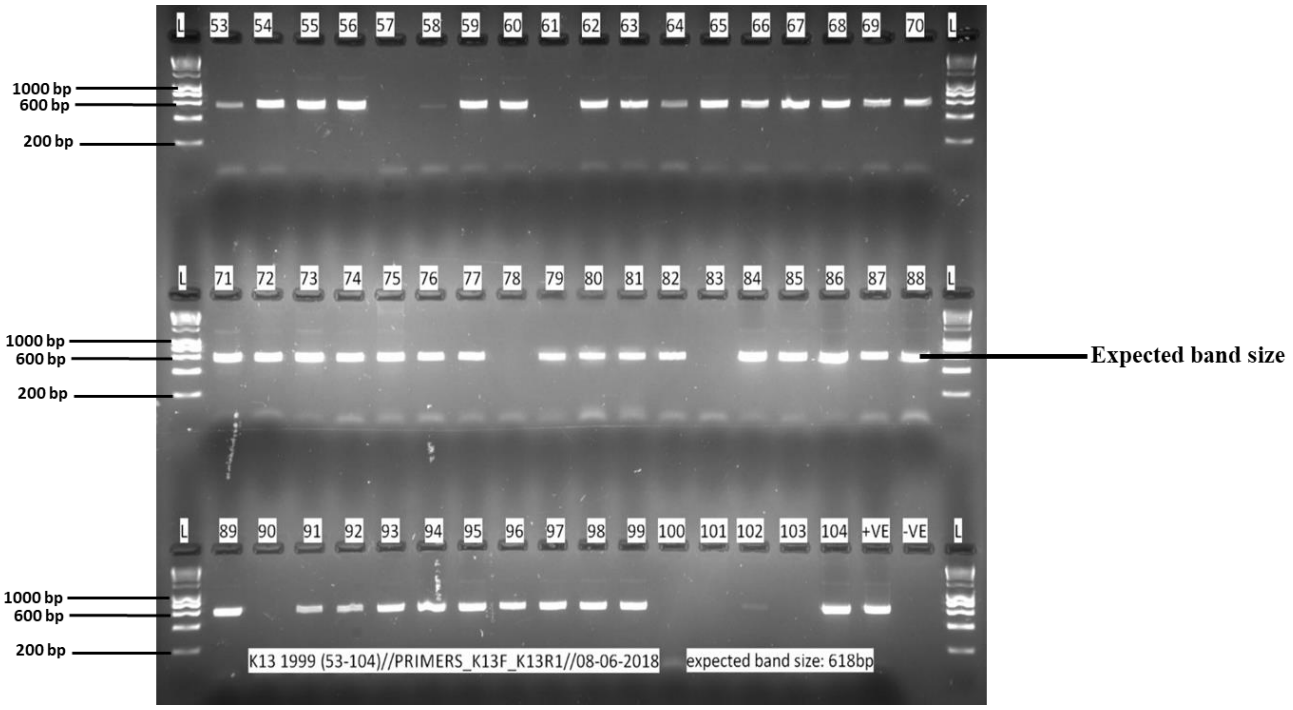

Figure 4.1: A gel image representative of the *k13* amplicons obtained and their expected band size (618 bp).

53-104 L L represents 1 kb molecular weight marker (New England Biolabs). The numbers 53-104 represent the individual samples, loaded into the gel. The samples whose gel image is represented here were obtained from the 1999-time point. +ve and –ve represent positive and negative controls, respectively. Bp represents base pairs.

4.1.2. Background mutation genes

All the four genes shown to contain background mutations were successfully amplified and the expected amplicon band sizes of 165, 246, 109 and 1349 bp for *arps10, fd, crt* and *mdr2* genes, respectively, were obtained. For *arps10* gene, 144 and 137 amplicons were generated in 1995 and 2015 time points, respectively. The amplification of *fd* and *crt* genes generated 125 and 146 amplicons for 1995 and 98 and 139 amplicons for 2015, respectively. Five time points were analysed for *mdr2* with 131, 117, 134, 145 and 130 samples being PCR positive in 1995, 1999, 2005, 2012 and 2015, respectively. The representative gel images for each of the target genes and the expected band sizes are shown in Figure 4.2.

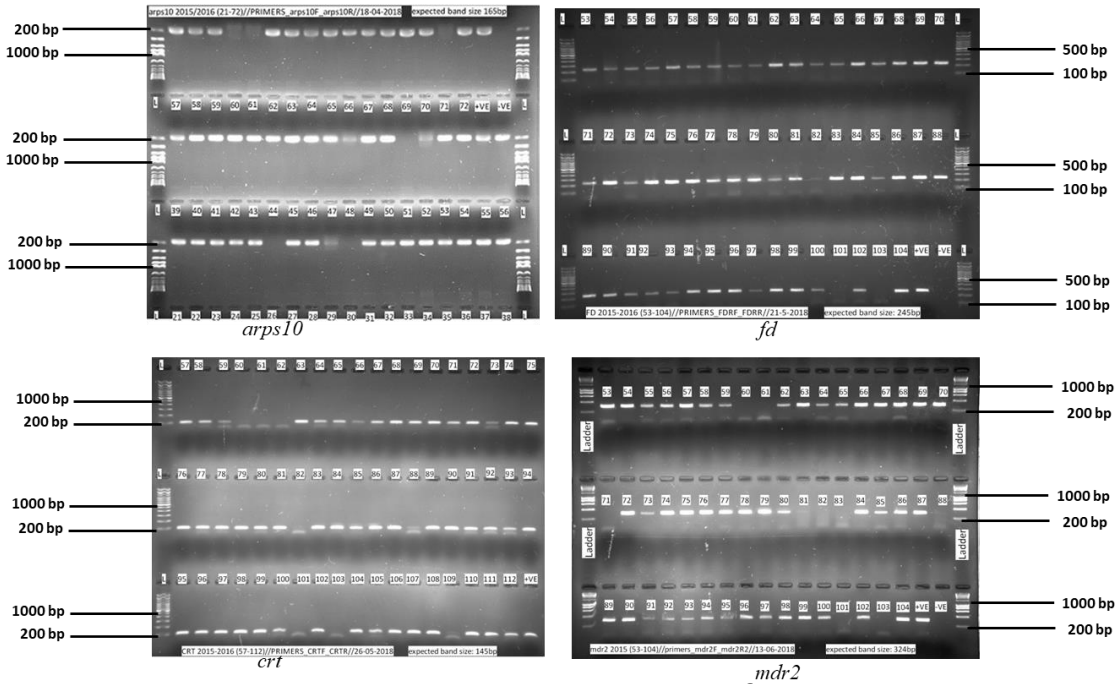

Figure 4.2: Representative gel images showing the amplification products obtained from *arps10, fd, crt* and *mdr2*.

L, represents 1 Kb molecular weight marker for *arps10*, crt and *mdr2* and 100 bp molecular weight marker for *fd*. The numbers adjacent to the wells indicate the individual samples loaded into the gel. 2006, 2015 and 2016 represent the years from where the samples were obtained. The positive and negative controls are denoted by +ve and –ve, respectively. Bp stands for base pairs.

4.2. Sequencing, sequence assembly and identification of SNPs

4.2.1. Prevalence of polymorphisms at the N-terminal region of Pfk13

Positive PCR products were sequenced and good quality sequences, with well-defined chromatogram peaks were obtained from 144 (100 %), 128 (97.7 %), 131 (100 %), 118 (98.3 %) and 144 (100 %) samples in 1995, 1999, 2005, 2012 and 2015 time points, respectively. The *P. falciparum* 3D7 clone (PF3D7_1343700) sequence was used as a reference for sequence assemblies and to ensure correct orientation of the sequences. An alignment of the sequences was used to identify single nucleotide polymorphisms (SNP), which were defined as positions in the sequences that differed with the reference. The alignment yielded single nucleotide polymorphisms (SNP) at 11 different loci. Out of the 11, only one locus was synonymous while the rest were non-synonymous substitutions (Table 4.1). However, a large majority of the SNPs occurred at very low frequencies (<1%) and were not consistent across all the time points. They were mainly singletons, occurring in a single sample in either one or two time points. Nonetheless, a mutation at codon 189 substituting lysine with threonine (K189T) persisted from 1995 to 2015 and at a relatively high frequency (>5%) in all the years apart from 1995, in which the frequency was 4.17% (Table 4.1). In addition to the SNPs, an asparagine (N) repeat (Figure 4. 3) was identified from codon 136 in 1999, 2005 and 2013. The most predominant repeat was a 7N i.e. NNNNNNN (3D7 reference 6N), which was observed in all the three years at frequencies of 0.78%, 3.05 % and 3.39 % in 1999, 2005 and 2012, respectively as shown in Table 4.1.

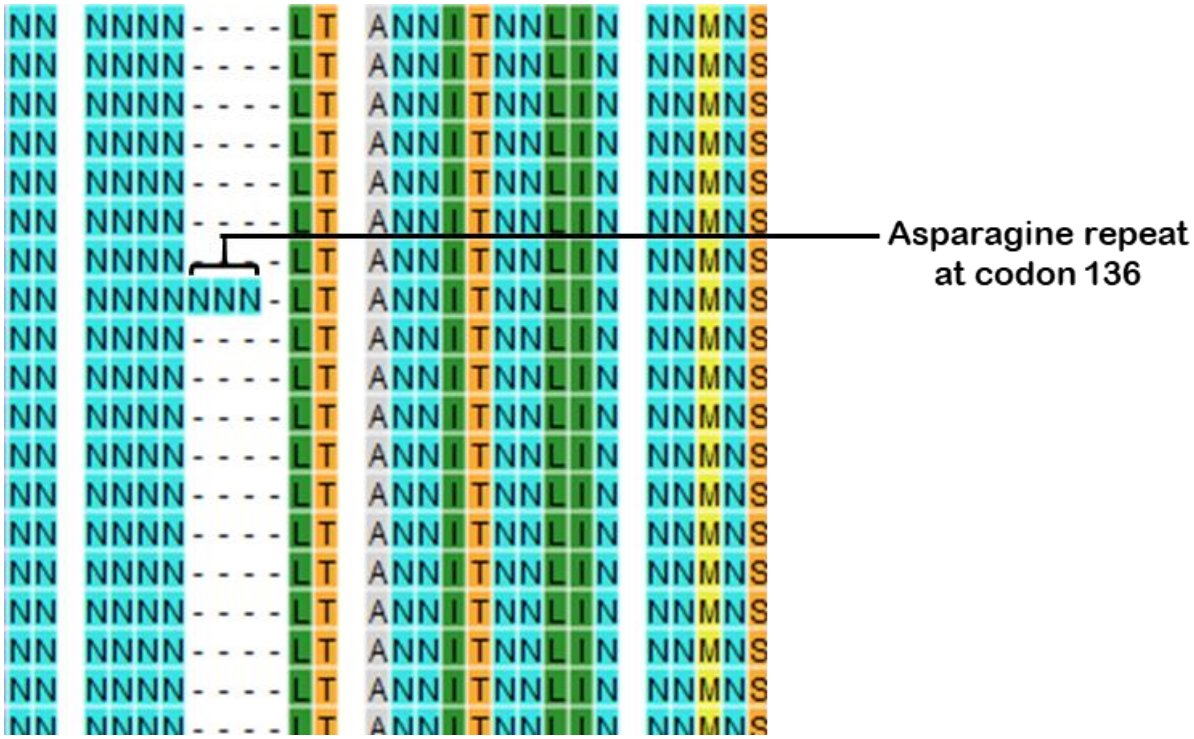

Figure 4.3: A section of an alignment file indicating a sequence with an asparagine (N) insertion.

The letters represent different amino acids as follows: L - leucine, I - isoleucine, S - serine, T - threonine, A - alanine. Each horizontal line is representative of the individual sequence for each sample analyzed.

Table 4.1: Temporal frequency of polymorphisms identified in N-terminal region of *Pfk13* in Kilifi County between 1995 and 2015

				1995	1999	2005	2012	2015
Codon position	SNP position	3D7 reference aa (Nucleotide)	Non-reference aa (Nucleotide)	Frequency % (n)	Frequency % (n)	Frequency % (n)	Frequency % (n)	Frequency % (n)
96	287	P (C)	Q (A)	0	0.8 (1)	0	0	0
108	322	K (A)	E (G)	0	0.8 (1)	0.8 (1)	0	0
119	355	L (T)	L (C)	0.8 (1)	0	0.8	0	0
126	377	T (C)	N (A)	0.8 (1)	0.8 (1)	0	0	0
134	401	I (T)	S (G)	0	0.8 (1)	0	0	0
136	406	H (C)	N (A)	0		0.8 (1)	0.9 (1)	0
136	409	6×N (6×AAT)	7× N (7× AAT)	0	0.8 (1)	3.8 (4)	3.4 (4)	0
			9× N (9×AAT)	0	0	0	1.7 (2)	0
			10× N (10× AAT)	0	0	0	0.9 (1)	0
149	445	T (A)	S (T)	0	0.8 (1)	0	0	0.7 (1)
157	469	M (A)	V (G)	0	0.8 (1)	0	0	0
174	520	A (G)	S (T)	0	0	0	0.9 (1)	0
182	544	S (T)	T (A)	0	3.1 (4)	1.5 (2)	0	0
189	566	K (A)	T (C)	4.2 (6)	14.8 (19)	6.9 (9)	8.5 (10)	6.3 (9)

The total number of samples were 144, 128, 131, 118 and 144 for 1995, 1999, 2005, 2012 and 2015, respectively; aa and n represents amino acid and number of samples, respectively.

4.2.2. Prevalence of polymorphisms on background mutation genes

Good quality sequence reads were obtained from 131 (90.97 %) and 135 (98.54 %) in 1995 and 2015, respectively for *arps10*. However, neither SNPs nor insertions/deletions (INDELS) were observed in all the sequences obtained. For the *fd* gene, 104 (83.2 %) and 144 (99.3 %) sequences in 1995 and 2015 time points, respectively, were analyzed and no mutations were observed at both time points. Similar to *arps10* and *fd*, no mutations were observed in the *crt* gene for all the 88 (89.9%) and 131 (94.2 %) sequences analyzed from 1995 and 2015 time points, respectively.

For the *mdr2* gene, 131 (100%), 111 (94.9 %), 140 (94.6 %), 129 (96.3%) and 130 (100%) sequences analyzed for 1995, 1999, 2005, 2012 and 2015 time points, respectively. A high frequency (>10%) SNP (I492V) was identified in both 1999 and 2015 time points. Polymorphisms were identified in three different loci but only one (I492V) (Figure 4.4), persisted across all the years and at high frequencies (Table 4.2). The highest frequencies occurred in 1995 (30.53%) and 2015 (23.08%). Polymorphisms in two other loci namely, I495V and V506I occurred in 1999 and 2005 years, respectively.

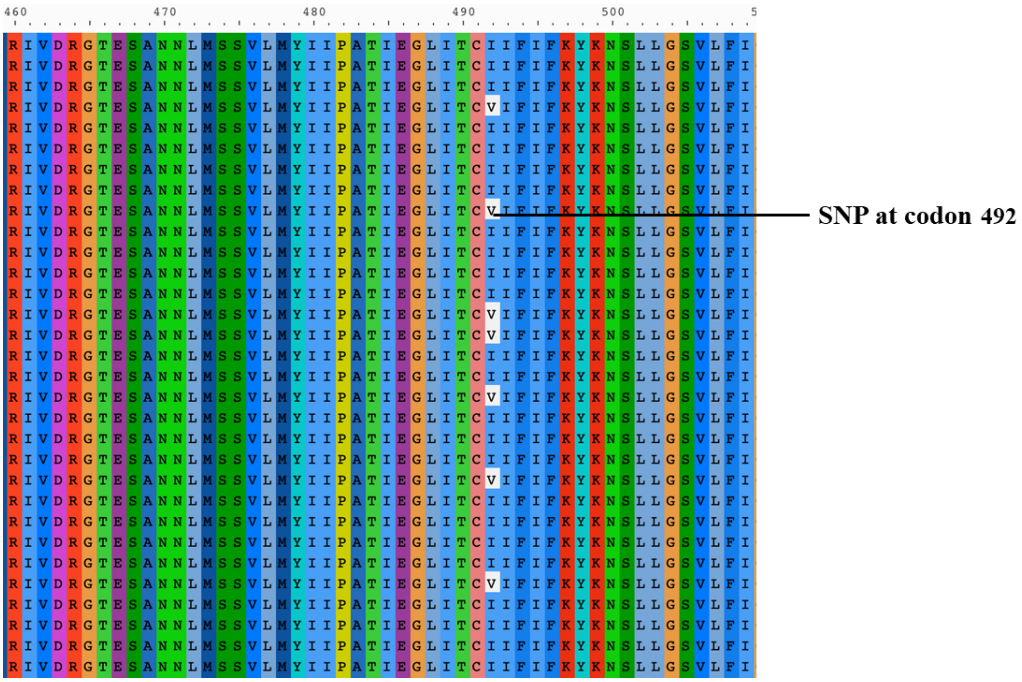

Figure 4.4 A section of an alignment file indicating the I492V mutation in *mdr2*

The letters represent different amino acids as follows: L - leucine, I - isoleucine, S - serine, T - threonine, A – alanine. Each horizontal line is representative of the individual sequence for each sample analyzed.

Table 4.2: Temporal prevalence of polymorphisms identified in *Pfmdr2* in Kilifi County between 1995 and 2015

				1995	1999	2005	2012	2015
Codon position	SNP position	3D7 reference aa (Nucleotide)	Non-reference aa (Nucleotide)	Frequency % (n)	Frequency % (n)	Frequency % (n)	Frequency % (n)	Frequency % (n)
492	1474	I (A)	V (G)	30.5 (40)	21.6 (24)	13.6 (19)	14.0 (18)	23.1 (30)
495	1483	I (A)	V (G)	0	0.9 (1)	0	0	0
506	1516	V (G)	I (A)	0	0	0.71 (1)	0	0

The total number of samples was 131 in 1995, 111 in 1999, 140 in 2005, 110 in 2012 and 130 in 2015; aa and n represents amino acid and number of samples, respectively.

4.2.3. Control gene (serine-tRNA ligase)

The *P falciparum* serine--tRNA ligase gene which is not under selective pressure from antimalarial drugs was incorporated into the study as a control. Mutations were observed in 19 loci but at relatively low frequencies (<5%). The predominant SNP occurred at codon 84 (L84V) which was present across all the time points. The frequency of mutations observed in the gene over the 20-year period is represented in Table 4.3.

Table 4.3: Temporal prevalence of polymorphisms identified in Pfserine-tRNA ligase in Kilifi County between 1995 and 2015

				1995	1999	2005	2012	2015
Codon position	SNP position	3D7 reference aa (Nucleotide)	Non-reference aa (Nucleotide)	Frequency % (n)	Frequency % (n)	Frequency % (n)	Frequency % (n)	Frequency % (n)
31	92	N (A)	S (G)	0	0	0	0.8 (1)	2.8 (4)
71	211	D (A)	N (G)	2.5 (3)	0	0	0	0
84	250	L(T)	V (G)	3.4 (4)	2.9 (4)	5.3 (7)	4.9 (6)	2.1 (3)
86	258	I (T)	M (G)	0	0	0	0	1.4 (2)
88	264	E (A)	D (T)	0	5.9 (8)	0.8 (1)	1.6 (2)	2.8 (4)
94	280	Q (C)	E (G)	0	0	0	0.8 (1)	2.1 (3)
133	399	T (A)	T (G)	0	2.2 (3)	0.8 (1)	0	0.7 (1)
140	418	L (C)	F (T)	0	0.7 (1)	0	0	0
155	463	V (G)	L (T)	0	0	0	1.6 (2)	0
175	524	I (T)	K (A)	0	0	0.8 (1)	0	0
199	596	A (C)	V (T)	0	0	0	0	0.7 (1)
221	662	A (C)	G (G)	0	0.7 (1)	0.8 (1)	1.6 (2)	0.7 (1)
267	799	T (A)	P (C)	0	0	0	4.9 (6)	1.4 (2)
284	851	A (C)	V (T)	0	0.7 (1)	0	0	0
318	952	E (G)	K (A)	0	0.7 (1)	0	0	0
372	1116	F (C)	F (T)	0.8 (1)	0	0	0	0
417	1249	S (T)	A (G)	0	1.5 (2)	0	0	0.7 (1)
430	1290	N (T)	N (C)	0	1.5 (2)	0	0	0
528	1584	Y(C)	Y(T)	0	0	0	1.6 (2)	0

The total number of samples was 118 in 1995, 136 in 1999, 130 in 2005, 123 in 2012 and 143 in 2015; aa and n represents amino acid and number of samples, respectively.

CHAPTER FIVE :DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1. Discussion

The study presents findings on the frequency of mutations in the *Pfk13* N-terminal region and four background genes that are precursors to *k13* resistance mediating SNPs, over a 20-year period in Kilifi County. Assessing the frequency of antimalarial drug resistance-associated mutations over time has previously been shown to help in detecting changes acquired by parasites when subjected to drug pressure (Okombo *et al*., 2014; Achieng *et al*., 2015). Additionally, mutations in *k13* were identified by Ariey *et al*. (2014) as markers of artemisinin resistance in South East Asia. None of the resistance driving mutations have been identified in African *P. falciparum* isolates to date (Ashley *et al*., 2014; Kamau *et al*., 2015). Nonetheless, De Laurent *et al*. (2018) identified a mutation at codon 539, which was previously reported in South East Asia to harbour changes that result in artemisinin resistance. However, the SNP coded for an amino acid substitution (R539T) that was different from that reported in SEA (R539K).

In this study, the N-terminal region of *k13* which has hardly been the focus of most studies in Africa, was examined (Ménard *et al*., 2016; Muwanguzi *et al*., 2016; Madamet *et al*., 2017; De Laurent *et al*., 2018). Out of the 11 loci where SNPs were identified, the K189T mutation conspicuously persisted across the five time points between 1995 and 2015. This mutation has been identified in previous cross-sectional studies from Bangladesh, Nigeria, Senegal and Uganda (Conrad *et al*., 2014; Takala-harrison *et al*., 2015; Ashley *et al*., 2015; Agathe *et al*., 2016; Safeukui *et al*., 2017). A correlation between the mutation and response to artemisinin was not established (Ashley *et al*., 2015). The data presented in this study similarly indicates no association between the

mutation and ACT response since its pattern of occurrence is similar to that of the L84V mutation in *P falciparum* serine-tRNA ligase, a neutral gene with regard to antimalarial drug pressure. Additionally, the SNP was present at a relatively high frequency before and after the introduction of ACT in Kilifi (Ministry of Health, 2016). This is unlike SNPs in *crt* which were shown to increase in frequency between 2005 and 2012 with increasing AL pressure in Kilifi (Okombo *et al*., 2014).

A mutation at codon 149 (T149S) was observed in the *Pfk13* N-terminal region at relatively low frequencies and at only two time points (1999 and 2015). This mutation was also identified in previous studies in South East Asian isolates (Miotto *et al*., 2015), Kilifi (Ashley *et al*., 2015) and in Dakar, Senegal (Torrentino-Madamet *et al*., 2014; Agathe *et al*., 2016). The highest frequency was reported in Senegal between 2012 and 2013 at 4.7%, contrary to the findings in this study of 0.78%. However, no evidence of its role in artemisinin resistance was shown (Miotto *et al*., 2015, Ashley *et al*., 2014). There is also no indication of its role in potential ACT resistance in Kilifi County considering it was present at the same frequency before (1999) and after (2015) the therapy was introduced. The 8 remaining mutations in the N-terminal region occurred at relatively low frequencies and were not consistent as they appeared and disappeared from one time point to another. Others were also present in only one time point. These might be considered as random mutations with no effects on the response of *P. falciparum* to artemisinin treatment.

In addition to the SNPs, asparagine repeats were noted at codon 136. These are yet to be documented in other studies (Conrad *et al*., 2014; Safeukui *et al*., 2017; Takala-harrison *et al*., 2015) although Agathe *et al*. (2016) reported similar insertions within the same

repeat locus, at codon 142 of *k13*. Notably, the insertions occurred in the mid time points (1999, 2005 and 2012) but were absent in both 1995 and 2015. They might hence be considered as random changes in the genome of the parasites which have no influence from drug pressure.

Unlike the propeller domain where several polymorphisms have been identified in previous studies (Ménard *et al*, 2016; Madamet *et al*., 2017; De Laurent *et al*., 2018), mutations in the N-terminal region of *k13* tend to be rare. This may be due to the fact that *k13* is highly conserved in this region (Ariey *et al*., 2014). In humans, it has been shown that mutations in conserved regions of genes may confer deleterious consequences with regard to fitness (Kryukov *et al*., 2005) and this might also be the case for *P. falciparum*. An alternative reason might be that the N-terminal region is less well studied compared to the C-terminal propeller domain that contributes to a large number of the published reports.

Nucleotide changes that confer resistance to artemisinin were shown to arise on a background of four other SNPs in *P. falciparum arps10, fd, mdr2* and *crt* (Miotto *et al*., 2015). The study sequenced the four background genes to assess the presence and frequency of these mutations. No polymorphisms were identified in three of the genes namely; *arps10, fd* and *crt*, consistent with preceding data by Balikagala *et al*. (2017) and Ikeda *et al*. (2018) in Ugandan isolates. This is because there may be no threat of resistance to artemisinin in Kilifi parasites. Contrary to their findings, three SNPs (I492V, I495V and V506I) were identified in *mdr2* with the I492V persisting at high frequency (>10%) across all the years. This mutation (I492V) was identified at a

similarly high frequency (100%) in Suriname between 2013 and 2014 (Chenet *et al*., 2017). However, none of the mutations found in this study matched the T484I resistance conferring mutation identified by Miotto *et al*. (2015) which was a characteristic of artemisinin resistant founder populations in South East Asia. It is unlikely that the mutations in *mdr2* arose due to artemisinin drug pressure since the I492V SNP for instance was present before 2004, the time when ACT use was effected in Kenya (Ministry of Health, 2016). The remaining two mutations; I495V and V509I were novel, occurring in one sample each and are therefore rare variants.

The *mdr2* gene belongs to the ABC superfamily of transporters which were shown to be involved in the efflux of drugs or heavy metals out of the cell, initiating resistance (Koenderink *et al*., 2010). A mutation (F423Y) in *mdr2* was also shown by Briolant *et al*. (2012) to be associated with pyrimethamine resistance *In Vitro*. It is therefore possible that continuous antimalarial drug pressure might prompt the emergence of resistance associated mutations in *mdr2*.

In Kenya and the rest of Africa, the ACTs in current use are still effective (Kamau *et al*., 2015; Muwanguzi *et al*., 2016). The absence of the *k13* background mutations implies that there is presently no threat of resistance to the ACTs in use. Alternatively, it might be possible that resistance in African isolates might arise on a completely different background as suggested by Cerqueira *et al*. (2017). This is because to date, none of the markers have been identified in most *P. falciparum* clones from Africa, yet there have been reports of delayed clearance and high ring stage survival assay (RSA) rates after treatment (Lu *et al*., 2017; Ikeda *et al*., 2018). Cases of treatment failure after ACT

treatment in Africa have been reported but mutations validated to confer resistance have not been found to be responsible (Sutherland *et al*., 2017). The *k13* A578S mutation that is frequent in African isolates has also been shown, through site-directed mutagenesis to have no effect on clearance of parasites following treatment (Ménard *et al*., 2016). It is therefore possible that *k13* might eventually not be the marker of artemisinin resistance for African *P. falciparum* isolates (Madamet *et al*., 2017). If that is the case, it might explain why no *k13* background mutations were identified in the Kilifi parasites. It might also be possible that the lack of the background mutations is due to lack of as much drug pressure as there was in South East Asia where artemisinin was used as a monotherapy for a very long time before the introduction of ACT. Additionally, the high diversity previously seen among African (Kamau *et al*., 2015; Amambua-Ngwa *et al*., 2019) *P. falciparum* isolates compared to the South East Asian parasites (Ariey *et al*., 2014; Miotto *et al*., 2015; Shetty *et al*., 2019) implies that resistance to artemisinin in Africa might arise due to a different mechanism other than mutations in *k13*.

5.2. Conclusions

The study findings reveal that there are currently no background markers of artemisinin resistance in *P. falciparum* isolates in Kilifi, therefore no threat of emerging resistance in the region. Mutations identified at the N-terminal region of k13 have not been shown to mediate resistance to artemisinin. This further indicates that there is no impeding resistance to artemisinin in Kilifi *P. falciparum* isolates. The SNPs observed in the studied genes might therefore be considered as random changes in the genome of the parasites with no impact on response to ACT treatment. This study gives the baseline

frequency of mutations in *P. falciparum k13* N-terminal region and four other genes that are precursors to *k13* resistance mediating SNPs in Kilifi. This information will be useful in subsequent surveillance studies for ACT treatment failure in Kilifi County.

5.3. Recommendations

While ACTs are still effective in Kenya, there is no ready alternative in the event of treatment failure. This calls for continuous surveillance of loci commonly known to mediate resistance to enable early identification and containment of emerging drug resistance. Given that this study was only conducted for *P. falciparum* isolates circulating in Kilifi County, similar studies in other areas within Kenya where malaria is endemic need to be carried out, to get a clear picture of the status of artemisinin resistance in the country. Furthermore, additional research is needed to test the significance of the single high frequency I492V SNP in *mdr2* to ascertain the role it plays in response to antimalarial treatment. Further studies are also needed to identify potential markers of resistance in African *P. falciparum* isolates, since none of the validated markers have been implicated despite a few cases of ACT treatment failure.

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