TEMPORAL TRENDS IN THE PREVALENCE OF *PLASMODIUM FALCIPARUM* DRUG RESISTANCE MARKERS OVER FIVE YEARS OF CHANGING ANTIMALARIAL DRUG POLICY IN WESTERN KENYA

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL BIOTECHNOLOGY

DEPARTMENT OF BIOMEDICAL SCIENCE AND TECHNOLOGY

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ABSTRACT

Malaria remains one of the most deadly infectious disease in sub-Saharan Africa accounting for high rates of mortality and morbidity especially in children less than 5 years. *Plasmodium falciparum* (*Pf*) parasite causes the most virulent form of malaria partially due to development of high levels of resistance against most anti-malaria drugs used. In malaria holoendemic areas of western Kenya, *P. falciparum* drug resistance has been noted since 1980s. Various genetic mutations have been identified and associated with *P. f* resistance to drugs, such as chloroquine (CQ), sulphadoxine-pyrimethamine (SP) and recently, Artemisinin-based combination therapy (ACT). Even though previous studies in Kanyawegi in western Kenya have addressed the presence of the genes associated with antimalarial resistance at one time point, the prevalence and temporal stability of the genes associated with ACT resistance in this malaria holoendemic population have not been reported. As such the temporal stability in the prevalence of *pfcrt* (K76T), *pfmdr1* (N86Y), *pfdhfr* (C59R) and *pfdhps* (K540E), and the association within and between the mutations that confer resistance against anti-malarials and those considered predictive of ACT treatment failure was investigated at the height of SP resistance just before administration of the ACT drug (July 2004), four weeks after administration of ACT (August 2004), and 5 years (July 2009) after SP withdrawal as front line treatment for uncomplicated malaria infections. A total of 95 paired retrospective blood samples from children under 5 years, confirmed positive for *P. f* were used. Using Polymerase Chain Reaction (PCR) and Nested PCR, *pfcrt* K76T, *pfmdr1* N86Y, *pfdhfr* (C59R) and *pfdhps* (K540E) genes were amplified and presence of mutations determined by gel electrophoresis after Restriction Fragment Length Polymorphism. Using chi-square analysis to determine the prevalence and Pearson's Correlation Co-efficient to determine the association between the genotypes and drug failures, the prevalence of *Pfdhps* wild type 540K increased from 14.7% (n =14/95) in July 2004 to 53.7% (n=51/95) in August 2004 (p=0.0004) and subsequently to 94.1% (16/17) in July 2009 (p=0.015). For *Pfdhfr* wild type C59, the prevalence in July 2004 was 0.0% (n =0/95) and 1.1% (n=1/95) (p=0.144) in August 2004 and 0.0% (n = 0/17) in July 2009. The prevalence of *Pfmdr1* wild type 86N was insignificant (p=0.223) from 0.0% (n=0/95) in July 2004 to 2.1% (n =2/95) in August 2004 and reduced to 0.0% (n = 0/17) in July 2009 (p = 0.759). Prevalence of *Pfcrt* wild type 76T remained constant in July and August 2004 at 2.1% (n=2/95) and rose to 5.9% (n=1/17) in 2009 (p=0.138). The prevalence of the K76T mutation was persistent in isolates from this highly holoendemic area indicating that selection for the mutant codon is in progress while results showed the absence of the *pfmdr1* N86Y isolates from both the baseline and the follow up isolates suggesting that the parasites harbouring this mutation are not widespread in this area and there was continued rise in the prevalence of the mutations associated with SP resistance while there was no association between the *pfcrt* K76T and *pfmdr1* N86Y pre- and post-adoption of ACT. Findings presented here suggest that resistant markers against CQ and SP have not faded and as such not recommended antimalarials in this *P.f* holoendemic region. This study will complement existing data on anti-malarial drug resistance monitoring and enhance future prediction of resistance levels that would be critical in informing anti-malarial drug policy aimed at reducing malaria-related morbidity and mortality.

CHAPTER ONE: INTRODUCTION

1.1 Background Information

Worldwide, there are 300 to 450 million reported cases of malaria each year, with the most severe form of human malaria caused by the apicomplexan parasite *Plasmodium falciparum* (WHO, 2012). Moreover, the prevalence of malaria infection fails to abate worldwide due to drug resistance, leading to high malaria-related morbidity and mortality (Snow *et al.*, 2005). Studies have reported that about 90% of all malaria deaths in the world today occur in sub-Saharan Africa, with children below 5 years and pregnant women bearing the greatest disease burden (WHO, 2012). Although progress has been made in the last 10 years towards developing malaria vaccines, there is currently no effective malaria vaccine on the market, leaving chemotherapy as the most effective strategy to combat malaria (Schwartz *et al.*, 2012). Due to evolutionary changes, malaria parasites, especially *P. falciparum,* are capable of surviving and even multiplying despite administration of anti-malarial drugs to a malaria patient, normally given in equal or higher doses than those usually prescribed (Cheng *et al.*, 2012). In recent years, resistance to anti-malarial drugs has seriously compromised the efforts to eliminate malaria in sub-Saharan Africa and has inflicted a huge economic burden in regions where the scourge is endemic (Amin *et al.*, 2007).

Anti-malarial drug policy in Kenya has gone through a lot of policy changes since the 1960s due to drug resistance when chloroquine (CQ) was the only drug recommended as a first line anti-malarial therapy in the country, replacing quinine (Shretta *et al.*, 2000; Mwai *et al.*, 2009). *P. falciparum* CQ resistant genes were originally detected in South America and South East Asia in the 1950s and by 1970 CQ was proven ineffective in these parts of the world (Sidhu *et al.*, 2002). In Kenya, CQ resistant isolates first emerged in 1977 and resistance steadily rose to about 70% a

decade later. The high rate of resistance forced the country to replace CQ with sulphadoxine/sulphalene pyrimethamine (SP) as the first line therapy for uncomplicated malaria in 1998 (Terlouw *et al.*, 2002). The main genetic locus of SP resistance has been traced to *pfcrt* K76T in chromosome seven, position 540 (Cooper *et al.*, 2005). Studies carried out with *pfcrt* K76T in western Kenya region have focused mainly on its association with susceptibility to different drugs other than ACT at one time point (Zhong *et al.*, 2008; Cheruiyot *et al.*, 2014), however no study has been done to assess its temporal prevalence and its association to ACT.

During the mid-2003, SP resistant parasites rapidly arose rendering SP ineffective leading to its replacement with artemether-lumefantrine (AL) in 2004 (Amin *et al.*, 2007; Figueiredo *et al.*, 2008). Studies carried on the SP drug resistant markers in western Kenya have focused on investigating the prevalence of this point mutations among field isolates of *P. falciparum* (Khan *et al.*, 1997; Figueiredo *et al.*, 2008) at one point and therefore there was limited knowledge on the trends in occurrence of the antifolate drug resistance genes.

Studies since 2006 show that the presence of artemisinin-resistant malaria in South East Asia (Cambodia) has been confirmed (Phyo *et al.*, 2012). The detection of ACT resistant parasites in SEA and the possible threat of antimalarial drug resistance spreading to other regions has put an increasing pressure to sustain the efficacy of existing treatments. Part of this is through monitoring and surveillance of the malaria drug resistant markers in areas of high malaria transmission globally. In Kenya, Nyanza region is one of the regions with high malaria transmission hence a potential area of early detection of antimalarial drug resistance in the country. Therefore detecting and tracking the spread of resistance markers in regions within Nyanza, including Kanyawegi, especially for the recommended ACT drugs is important for the overall surveillance and rational change of drug policy by the Kenyan government. Genetic point

mutations in the homologue of the multi-drug transporter, *P. falciparum* multi-drug resistance gene 1 (*pfmdr1*) in codon 86, also known as P-glycoprotein homologue 1, (*Pgh-1*) has been shown to modulate levels of resistance to CQ and artemisinin (Farooq and Mahajan, 2004). Various studies have shown vital although inconclusive associations between resistance to the anti-malarials CQ and mutations in the *pfmdr1* gene in both field and laboratory isolates (Mackinnon *et al.*, 2009). Several studies have also shown the mutation in the gene is also associated with quinine resistance and enhance mefloquine and artemisinin sensitivity in *P. falciparum* (Sidhu *et al.*, 2005; Lim *et al.*, 2009). The *Pfmdr1* N86Y mutation is a vital regulator of resistance to other hydrophobic anti-malarials that have replaced CQ as standard treatment for uncomplicated malaria (Duraisingh and Cowman, 2005). *Pfmdr1* alleles are involved in the development of tolerance/resistance to ACT (Chavchich *et al.*, 2010) and because of this it is important to investigate the prevalence and temporal change of *pfmdr1* in considering schemes aimed at the reversal of resistance to ACT.

The *P. falciparum* chloroquine resistance transporter gene (*pfcrt)* is a key determinant in *P. falciparum* resistance to CQ (Figueiredo *et al.*, 2008). The discovery of the genetic basis of CQ resistance in *P. falciparum* through the identification of *pfcrt* mutation, where there is change of lysine to threonine at position 76 (K76T) in the gene, has shed light on its molecular function of encoding a putative transporter or channel protein (Figueiredo *et al.*, 2008). *Pfcrt* CQ sensitive strains consistently demonstrate a wild type allele. The K76T mutation seems necessary for the resistance phenotype and is deemed the most authentic molecular marker of resistance to CQ among the many *pfcrt* polymorphisms (Ochong *et al.*, 2003).

Resistance to SP was a common problem in Kenya when previously used in combinations with SP in the chemotherapy of CQ resistant malaria (Gregson and Plowe, 2005). *P. falciparum*

resistance to SP is initiated by variations in the dihydropteroate synthase and dihydrofolate reductase genes (Fortes *et al.*, 2011). The mutation at 108N in the *pfdhfr* gene has been shown to bestow resistance to pyrimethamine but the presence of variations at locations 59 (C59R), 51 (N51I) in concert with S108N result in a massive increase in the level of resistance to pyrimethamine than when polymorphisms in the 108 act alone (Gregson and Plowe, 2005). In regard to sulphadoxine, variations in codons 540 (K540E) and 437 (A437G) in the *P. falciparum dhps (Pfdhps)* have been linked to resistance to this drug (Gregson and Plowe, 2005). Epidemiological studies also show that occurrence of mutation 59R in *dhfr* and 540E in *dhps* are the most strongly associated with resistance to anti folate anti malarials (Wang *et al.*, 1997; Fortes *et al.*, 2011).

South-East Asia and western Cambodia has been the key area for the evolution of drug resistance which then spreads afar. Former mainstay anti-malarials such as CQ and SP also experienced their first resistance challenges in West Cambodia before spreading to other parts of Asia and to Africa. Thus monitoring Kanyawegi sub-location in Kisumu County, a malaria holoendemic area, was needed to monitor changes in common drug resistant allele frequencies during the different stages of policy change in anti-malarial drug administration in Kenya.

1.2 Problem Statement for Study

Research has shown that *P. falciparum* is currently becoming more resistant to arylaminoalcohols (mefloquine and lumefantrine) and endoperoxides (artesunate and artemether) in parts of western Cambodia (Phyo *et al.*, 2012). CQ resistance has also been seen in the area since 1977 (Sidhu *et al.*, 2002) . The drug-resistant parasites within the South East Asia will inevitably spread to East Africa. *Pfmdr1, pfcrt, pfdhfr* and *pfdhps* have been identified as important genetic determinants of anti-malarial resistance and should be used to confirm potential cases of drug resistance. However, the prevalence and trajectory of *Plasmodium falciparum* drug resistance markers *pfcrt* (K76T), *pfmdr1* (N86Y), *pfdhfr* (C59R) and *pfdhps* (K540E) in malaria holoendemic area in western Kenya has not been evaluated. Therefore, the current study evaluated the prevalence and frequency of mutation of the 4 genes in 3 time points, that is, at the height of SP resistance just before administration of the ACT drug (July 2004), four weeks after administration of ACT (August 2004), and 5 years (July 2009) after SP withdrawal as front line treatment for uncomplicated malaria infections in Kanyawegi sub-location in western Kenya.

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1.3. Objectives

1.3.1 General Objective

To investigate the prevalence and trajectory of *Plasmodium falciparum* drug resistance markers *pfcrt* (K76T), *pfmdr1* (N86Y), *pfdhfr* (C59R) and *pfdhps* (K540E) in malaria holoendemic area in western Kenya.

1.3.2 Specific Objectives

i. To determine the prevalence and change of the chloroquine resistance *pfcrt* (K76T) polymorphism in children presenting with *P. falciparum* malaria in a malaria holoendemic area of western Kenya pre and post adoption of ACT.

ii. To determine the prevalence and change of the polymorphism in *P. falciparum* multi-drug resistant allele 1 (*pfmdr1*) (N86Y) in children presenting with *P. falciparum* in a malaria holoendemic area of western Kenya pre and post adoption of ACT.

iii. To determine the prevalence and change in occurrence of the antifolate drugs resistance alleles *pfdhfr* (C59R) and *pfdhps* (K540E) in children presenting with *P. falciparum* malaria in a malaria holoendemic area of western Kenya pre and post adoption of ACT.

iv. To compare the prevalence of alleles within *pfcrt* K76T and *pfmdr1* N86Y pre and post adoption of artemether lumefantrine as first line of treatment against malaria in western Kenya.

1.4 Research Questions

i. What is the frequency and change of the chloroquine resistance *pfcrt* (K76T) polymorphism in children presenting with *P. falciparum* malaria in a malaria holoendemic area of western Kenya?

ii. What is prevalence and change of occurrence of the polymorphism in *P. falciparum* multi drug resistant allele 1 (*pfmdr1*) (N86Y) in children presenting with *P. falciparum* malaria in a malaria holoendemic area of western Kenya?

iii. What is the prevalence and change of the antifolate drugs resistance alleles' *pfdhfr* (C59R) and *pfdhps* (K540E) in children presenting with *P. falciparum* malaria in a malaria holoendemic area of western Kenya?

iv. What are the prevalence of alleles within *pfcrt* K76T and *pfmdr1* N86Y pre- and post-adoption of artemether lumefantrine as first line of treatment against malaria in western Kenya?

1.5 Significance of the Study

In this malaria holoendemic region in western Kenya, transmission is intense and is an everyday occurrence with an average parasite prevalence of up to 50% between the wet and dry season (Dent *et al.*, 2009). This causes considerable mortality and morbidity especially in children below 5 years (Moormann *et al.*, 2012). Malaria has also been proven to be a significant burden on health system, accounting for about 70% of out-patient attendance and 40% of in hospital admissions in Chulaimbo Health Centre, which is the local hospital in the area (Gerald, 2007). The majority of mortality malaria cases in Western Kenya are as a result of a combination of high transmission, drug resistance to therapy and limited health services in the this area (Gerald, 2007). Malaria morbidity presents a substantial economic burden on households having to pay for treatment and prevention which is also hampered by drug resistance. Malaria control is also a problem in this area since most public services break down and this leads to significant disease resurgence (Imbahale *et al.*, 2010).

Monitoring the prevalence and incidence of drug resistance is critical in guiding drug policy to reduce malaria-related morbidity and mortality in Kenya and specifically in malaria holoendemic areas of western Kenya.

CHAPTER TWO: LITERATURE REVIEW

2.1 Malaria Transmission, Morbidity and Mortality

Malaria is a vector borne disease that is caused by protozoan parasites of the genus *Plasmodium* and transmitted from one person to another through bites of an infected female *Anopheles* mosquito (Hisaeda *et al.*, 2005). There are five species of *Plasmodium* that cause malaria in humans; *P. falciparum, P. vivax, P. malariae, P. ovale* and recently identified *P. knowlesi* (Cox-Singh, 2012). Most cases of malaria in sub-Saharan Africa, are caused by the *P. falciparum* species (Cox, 2010). Malaria affects over 3 billion people worldwide, with about 207 million people reporting clinical cases of malaria each year, with an estimated 627,000 deaths, mostly children in Africa (WHO, 2012). Approximately 90% of malaria deaths take place in Africa, where malaria accounts for about one in six of all childhood deaths. The disease also contributes greatly to cases of anaemia among children, a major cause of poor growth and development (Murray *et al.*, 2012).

In Kenya, regions of stable high malaria transmission have altitudes of between 0 to 1300 meters above sea level, and this falls mainly in specific regions of the coast and around Lake Victoria in western Kenya (Figure 1) (Noor *et al.*, 2009). The country with a population of around 40 million has about 25 million of its population at risk of Malaria (KEMRI, 2012). Malaria transmission in Kanyawegi is intense throughout the year with an annual entomological range of between 30 and 300 (Wanjala *et al.*, 2011). Malaria epidemics in Nyanza region have been noted since the 1980s (Baliraine *et al.*, 2010) and in addition to morbidity and mortality other consequential effects have been related to each step of infection and disease process for instance cerebral malaria. Chronic subclinical infections cause anaemia or may encourage malnutrition, which in turn increases susceptibility to severe clinical outcomes of subsequent malarial or other

pathogenic infections (Baliraine *et al.*, 2010).

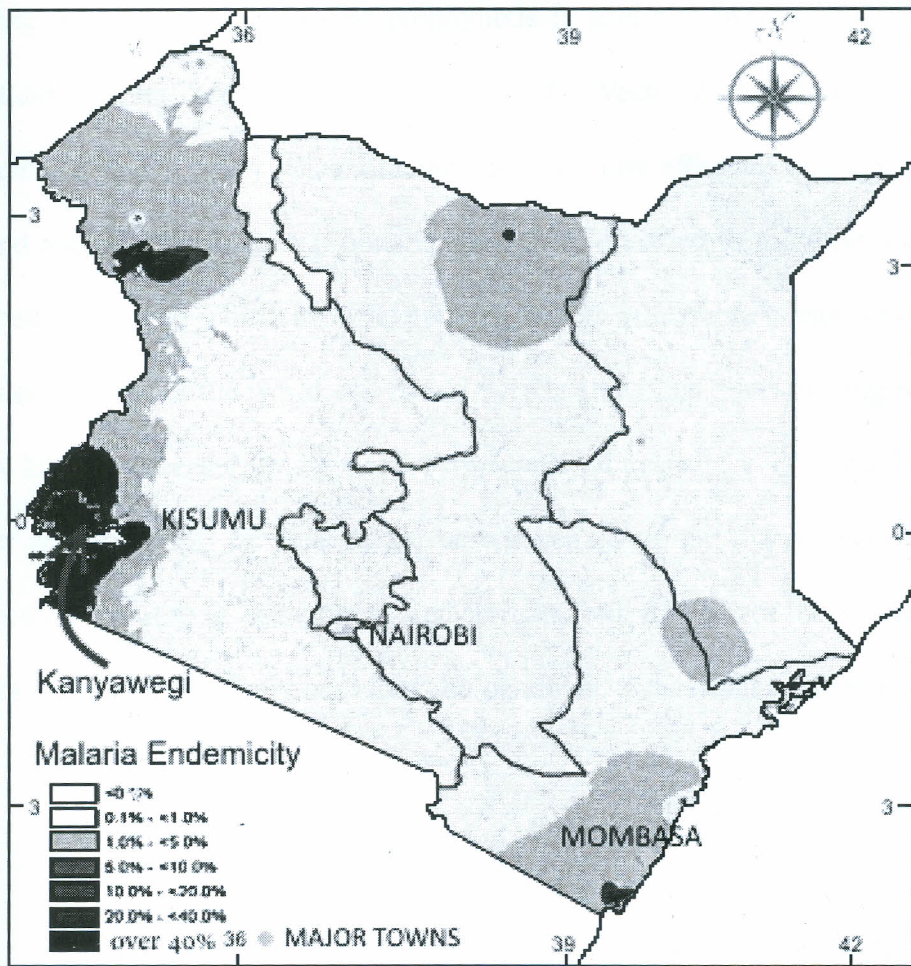

Figure 2.1: Map showing malaria endemicity in Kenya (Noor *et al.*, 2009). Location of Kanyawegi is indicated by the red arrow.

Measures that are currently in place to control malaria are focused on malaria patients, exposed individuals and the mosquito vectors. Methods for control are through early diagnosis and treatment using anti-malarials. Exposure prophylaxis is administered through the use of long lasting insecticide-treated mosquito nets (WHO, 2010). Vector control is achieved by indoor residual spraying (WHO, 2001). Chemotherapy as the most effective strategy to control the emergence and spread of resistance (Ponnampalam, 1981) has led to the promotion of ACT as the first line drug of choice. This leaves the population very vulnerable in case drug resistance to ACT spreads to this part of the world, as reports already show that signs of drug resistance have been seen in hotspots for the emergence of anti-malarial resistance like the Thai-Cambodia border (Alker *et al.*, 2007). Lessons of the past in regard to anti-malarials resistance should provoke effective monitoring systems to detect increased prevalence of molecular markers related to drug resistance especially in malaria holoendemic areas, such as Kanyawegi in western Kenya.

2.1.1 *Plasmodium falciparum* Chloroquine Resistant Transporter (*pfcrt*)

Figure 2.2: Drug resistance to *P. falciparum* from studies in sentinel sites, up to 2004. (WHO 2005)

In many parts of the continent, *P. falciparum* has proven resistant to most of the drug therapies introduced since chloroquine was first used as the first line drug of treatment for uncomplicated malaria in the late 1950's (Lopes *et al.*, 2002)(Figure 2.2). Chloroquine resistant strains first appeared 50 years ago simultaneously in south America and South East Asia (Hand and Meshnick, 2011). Significant progress has been made to understand how CQ resistance occurred but many aspects of this problem still remain unknown, largely due to the fact that the genetic mechanisms responsible for this phenomenon still remain unclear. Evidence shows two main genes, the *P. falciparum* chloroquine resistance transporter (*pfcrt*) and the *P. falciparum* multi-drug resistance *1 (Pfmdr-1)* are major players in causing resistance to CQ (Figueiredo *et al.*, 2008).

The change of lysine to threonine at position 76 (K76T) in the *pfcrt* has been proven to mediate drug resistance in CQ (Sidhu *et al.*, 2002). *Pfcrt* confers resistance to CQ by separating the drug from the target (heme) and thus reducing the capability for specific CQ-heme adherence which would prove toxic to the parasite (Foote *et al.*, 1989).

Different studies have shown various polymorphic amino acid positions in *pfcrt* and all these have been shown to be associated with CQ drug resistance (Dokomajilar *et al.*, 2006b). These polymorphisms have been shown to vary depending on selection history and geographical location but CQ resistant strains have a constant wild type genotype (Dokomajilar *et al.*, 2006a). The K76T mutation appears necessary for the resistance allele to have an effect on the drug and thus is the most authentic marker of drug resistance associated with *pfcrt* polymorphisms (Figueiredo *et al.*, 2008).

Scientists have surmised that regulation of the endogenic transporter activity related to the *pfcrt* alters the CQ movement and the digestive vacuole pH in the malaria parasite and the changes in

the final transport properties of the digestive vacuole can explain the reason why drugs such as artemisinin, halofantrine and mefloquine are also sensitive to *pfcrt* polymorphisms (Cooper *et al.*, 2005). Studies carried out with *pfcrt* K76T in Kanyawegi sub location have focused mainly on its association with susceptibility to different drugs other than ACT at one time point (Zhong *et al.*, 2008; Cheruiyot *et al.*, 2014) but no study has been done to assess its temporal prevalence over a certain period and its relation to current drug of choice ACT. Other studies (Terlouw *et al.*, 2003) done in the area have compared the *pfcrt* mutations between the highlands and lowlands but not the prevalence over time in western Kenya. An understanding of the presence of the *pfcrt* marker between varying time points since CQ withdrawal should give an idea of its prevalence and association to drug resistance and whether CQ resistance has faded a decade after its withdrawal from use since resistance monitoring in several African countries such as Zambia and Mali show that malaria parasites are succumbing to the formerly used drug chloroquine.

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2.1.2 *Plasmodium falciparum* Multi Drug Resistance-1 (*Pfmdr-1*)

P. falciparum multi drug resistance 1*(Pfmdr1)* also known as P-glycoprotein homologue 1 (*Pgh-1*) is a molecular marker associated with anti-malarial resistance (Duraisingh and Cowman, 2005). *Pfmdr1* is an ATP-Binding Cassette (ABC) that confers drug resistance by pumping the drug meant to kill the parasite out of the erythrocyte using an energy dependant mechanism through specific efflux pumps (Sanchez *et al.*, 2005). The efflux system in *Pfmdr1* is unfortunately not drug-specific but accommodates multiple drugs and thus its significance to multi- drug resistance (Ferreira *et al.*, 2011).

P. falciparum multi drug resistance 1 polymorphisms have been associated with resistance to CQ, mefluoquine and artemisinin (Farooq and Mahajan, 2004). *Pfmdr1* is not only associated with artesunate-mefloquine but it is also strongly linked to failure in the Coartem (artemether-lumefantrine) combined treatment (Chavchich *et al.*, 2010). Point mutations present in *pfmdr1,* especially in codon 86 have been associated with increased sensitivity to various anti-malarials (Andrea Ecker, 2012). The polymorphism is as a result of a transposition of an asparagine with tyrosine in amino acid 86 (Figueiredo *et al.*, 2008). The plasmodial homologue of P-glycoprotein a transporter that regulates *in vitro* and *in vivo* drug susceptibility (Ferreira *et al.*, 2011) but likely role of the *pfmdr1* in transport and resistance mechanisms still remains unclear (Purfield *et al.*, 2004).

In this regard, understanding and estimating the potential artesunate-mefloquine medical failure in Kenya and the molecular basis, polymorphisms and gene amplifications in this gene would be very crucial in implementing integrated measures to overcome the drug resistance problem.

2.1.3 Dihydrofolate reductase (*pfdhfr*) and Dihydropteroate synthase (*pfdhps*)

In Kenya, the use of sulphadoxine/pyrimethamine (also known as Fansidar or PSD) as the first line drug for use in the case of uncomplicated malaria was introduced in 1998 after CQ was deemed ineffective to combat malaria (Terlouw *et al.*, 2003). This drug policy was short-lived after drug resistant strains of SP were discovered in various parts of the world 5 years later (Terlouw *et al.*, 2003) (Figure 2.2). Mutations in two genes, dihydrofolate reductase *(pfdhfr)* and dihydropteroate synthase *(pfdhps)* have been strongly associated with *P. falciparum* resistance to sulphadoxine and pyrimethamine respectively (Terlouw *et al.*, 2002). Point mutations in *pfdhfr* at position 51, 59, 108 and 164 have been linked to resistance to pyrimethamine while mutations in codon 540 (540E) and 437 (437G) are linked to resistance to sulphadoxine (Hastings, 2004).

Antifolate anti-malarials drugs interpose with folate synthesis, a pathway very essential for the *P. falciparum* survival (Fortes *et al.*, 2011). Interference with folate metabolism by *dhfr* and *dhps* inhibitors leads to reduced levels of fully reduced tetrahydrofolate, which is a necessary co-factor in vital one carbon transfer reactions in the purine, pyrimidine and amino acid biosynthetic pathways (Fortes *et al.*, 2011). The reduced levels of tetrahydrofolate result in reduced conversion of glycine to serine, decreased methionine metabolism and lower thymidylate levels with a subsequent halt of DNA replication (Gregson and Plowe, 2005).

Studies show that the change of isoleucine to leucine at codon 59 has an adverse effect on pyrimethamine binding and similarly addition of DHFR N51I and C164R polymorphisms confer higher levels of pyrimethamine resistance than does S108N alone (Sibley *et al.*, 2001).

Consistent mutations in these two genes responsible for antifolate treatment failure has not been well established and it is thought that their frequency and mutations differ across regions (Mberu *et al.*, 2000). Other studies, however show a relationship between the two genes in conferring

resistance to anti-malarial drugs in samples taken from the same patient before and after treatment (Wang *et al.*, 1997; Figueiredo *et al.*, 2008). It was thus crucial to determine the trends in occurrence of the antifolate drug resistance genes *pfdhfr* (C59R) and *pfdhps* (K540E) as this would contribute to development of strategies for prolonging the therapeutic life of ACT and gain insight into how the different combinations of mutant DHFR and DHPS would affect ACT resistance.

CHAPTER THREE : MATERIALS AND METHODS

3.1 Study Area

The study was conducted at Kanyawegi sub-location in Kisumu County, a malaria holoendemic area in the lowlands of western Kenya (Figure 2.1). Studies show that the annual entomologic inoculation rate in Kanyawegi may exceed 300 infectious bites per individual (Amek *et al.*, 2012). The area is approximately 1200 km^2 in size and lies between Latitude -0.13S and Longitude 34.60E. Lake Victoria is 3 kms to the south and its altitude is approximately 1182 m above the sea level. Kanyawegi sub-location has a semi-arid (0.2 - 0.5 p/pet) climate and is mostly cultivated. The landscape is mostly covered with mosaic croplands/vegetation. The climate is classified as a tropical wet, with a subtropical moist forest biozone. The average temperature is between 15°C and 30°C all over the year.

The area is mildly densely populated with 489 people per km^2 (Moormann *et al.*, 2012). An estimated 3% of children below 5 years are underweight, with a mortality rate of 61 per 1,000 births (Moormann *et al.*, 2012). Its inhabitants are predominantly of the Luo ethnic group (>96%), thus the study area has a homogeneous population suitable for genetic studies. Malaria is responsible for the highest number of childhood morbidities and mortalities in the area (Piriou *et al.*, 2012). Most of malaria transmission takes place during both long and short rains (Piriou *et al.*, 2012).

Chulaimbo Health Centre is the largest and busiest health centre in the district (Moormann *et al.*, 2012). It is located in Kisumu East Division where the mortality of children under 5 years of age is 18.4% (Gerald, 2007).

3.2 Study Population

Kanyawegi area has a high incidence and prevalence of malaria (Figure 1), especially among children under the age of 5 years (White *et al.*, 2011). The study used 95 malaria positive samples in July 2004, 95 samples in August of 2004 and 17 samples from the same area in July 2009. The set of samples used were part of those used in earlier studies done from 2001 by the University of Massachusetts Medical School in collaboration with KEMRI.

3.2.1 Inclusion criteria

Children presenting with febrile illness were recruited at the Chulaimbo Rural Training Center (CRTC) in July 2004. Thick and thin Geimsa stained blood films were prepared from finger prick samples. Acute malaria infection was defined at an axillary temperature >37.5C and a positive blood smear for *P. falciparum* with a haemoglobin level of >5.0 gm/dl. The haemoglobin test measuring the amount of haemoglobin in the blood was done using the HemoCue® Hb 201^{+} System.

After obtaining signed informed consent from the parent, whole blood was collected by venipuncture from each child prior to treatment with a 6-dose regimen of CoArtem. Ninety five children were included in this analysis, mean age 25 months (range 1 – 66 months), none were HIV^{+}. A second venous blood sample was collected from each participant 4 weeks later.

3.2.2 Exclusion criteria

Patient were excluded if the child had not been a residence of the Kanyawegi area, and had a haemoglobin level of less than 5.0 gm/dL, parasitaemia without fever or evidence of another etiology of fever at time of recovery based on clinical officer's examination. The patient was also excluded from the study if he/she was HIV^{+}. Individuals who were generally unwell due to other unconfirmed health conditions were also excluded from the study.

3.3 Sample Size Calculation

The venous blood samples had already been collected over the years from a previous study and stored at -80°C. The sample size of 95 samples for the study with a 95% confidence interval and precision level of 2% was arrived at using the formula (Zignol *et al.*, 2012).

$$n = \frac{N \times z \times p \times (1 - p)}{d^2 \times (N - 1) + z^2 \times p \times (1 - p)}$$

Where N is the number of confirmed new malaria cases in Chulaimbo per year (KNBS, 2010), z is the value that corresponds to the desired confidence level of 95%. p is the expected prevalence of the *P. falciparum* drug resistance gene *pfcrt* from available data (Zhong *et al.*, 2008), d is absolute precision value.

$$\frac{205 * 1.96 * 0.7 *(1-0.7)}{0.02^2 * (205-1) + 1.96^2 * 0.7 *(1-0.7)} = 95$$

3.4 Parasite DNA Extraction and Species Identification

The archived blood samples used had been confirmed of *P. falciparum* infection by microscopic observation of thin and thick Giemsa-Stained blood films. A drop of the blood was collected from the patients' blood sample. The drop of blood was spread on the glass slide and dipped in a Giemsa stain solution (eosin, methylene blue and azure B) that stained the malaria parasites and examined under a micıoscope at X100 magnification. The malaria parasites were discerned by their physical features and the appearance of the RBC's they infected. Parasite DNA was isolated from whole blood of *P. falciparum* positive individuals using QIAamp Kits according to the manufacturer's instructions (Qiagen, Inc., Valencia, CA, USA) by use of anionic detergent and enzymic proteolysis to liberate the DNA, followed by purification on caesium chloride gradients.

3.5 Molecular Detection of Mutations in Drug Targets

Detection of mutations of the malaria drug resistant markers by nested PCR and digestion of the 2nd round PCR products in restriction fragment length polymorphism (RFLP) were done following protocols authored by Dokomajilar (2004). Mutations in the *pfcrt* (K76T), *pfmdr1* (N86Y), *pfdhfr* (C59R) and *pfdhps* (K540E) genes were detected using a PCR- RFLP method. Nested PCR was performed for all the codons conferring the mutations in four genes. All reactions were carried out in 25µl reaction mixtures containing 1.5 – 2 mM $MgCl_2$, 200µM dNTP mixture (Invitrogen, USA), 1 unit of Taq polymerase (Invitrogen, USA), and a pair of primers (0.25µM each). For the four mutations 5ul of DNA was used as template in the first and second PCR reaction. Further details about the PCR primers and conditions used for the four genes are provided in Appendix 1.

Purified genomic DNA from *P. falciparum* clones were used as positive controls and the expected fragment size used to identify the polymorphisms for the 4 genes is as shown in Appendix 1.The second PCR products were resolved by electrophoresis on 2.5% agarose gel and visualized by staining with Phenix *GelRed* nucleic acid stain according to protocols by Dokomajilar (2004).

3.6 Restriction Fragment Length Polymorphism – PCR of *pfdhfr, pfdhps, pfmdr1* and *pfcrt*

Mutation specific restriction endonuclease digestion was used to detect SNPs in *pfdhfr* at codon 59, *pfdhps* at 540, *pfmdr1* at codon 86 and *pfcrt* at position 76. For identification of the *pfdhfr* mutation, the PCR products were digested with *Xmn*1 to determine the polymorphism at position 59. The enzyme *Fok* 1 was used to detect wild and mutant *pfdhps* allele at position 540. The *pfmdr1* mutations at codon 86 were identified using *Afl* III restriction enzyme. The enzyme *Apo I* was used to digest the *pfcrt* products at codon 76 (see Appendix 3). Digestions were done in 20μL reactions containing 10μL of the PCR fragments according to the manufacturer's instructions (New England Biolab, Beverly, MA, USA). Digested products were separated through electrophoresis on 2.5% agarose gel, and visualized by ultraviolet (UV) trans illumination

Table 3.1: Control DNA and fragment size expected of Polymorphic genes investigated after Restriction Fragment Length Polymorphism.

Gene	Wild type controls	Mutant controls	Wild type fragments (bp)	Mutant fragments (bp)
PfMDR1 86	HB3	FCR3	560	232, 328
PfCRT 76	HB3	DD2	34, 100	134
DHPS 540	FCR3	Peru	7, 19, 412	7, 19, 99, 313
DHFR 59	3D7	V1/S	142, 184	22, 142, 162

3.7 Data Analysis

The frequencies and prevalence of the point mutations in the four genes was determined using Chi square analysis. Pearson's Correlation Co-efficient was used to determine the association between the genotypes and drug failures. Statistical significance was considered at $P \leq 0.05$. All the statistical tests were performed in SPSS® statistical software package version 20.0 (IBM SPSS Inc., Chicago, IL, USA)(Norman H. Nie, 2009).

3.8 Ethical Considerations

The study approval was obtained from the Ethical Review Board for the Kenya Medical Research Institute and its Scientific Steering Committee (See Appendix 5).

3.9 Limitation of the study

In 2009 there was a government policy of mass administration of antimalarial drugs and treated bed nets in malaria prone areas of western Kenya. Due to these interventions there was significant reduction of malaria cases during that period hence over the period of sample collection, very few samples were malaria positive. That is why this study only captured a total of 17 samples in 2009.

CHAPTER FOUR: RESULTS

4.1 *PfCRT* Genotypes

In this study, 95 pair of *P. falciparum* isolates were genotyped, each from the same patient in July 2004 and August 2004 and 17 isolates from July 2009, 4 (4.2%) of the 95 July 2004 samples, three (3.2%) of the 95 August 2004 samples and 2 (11.8%) of the 17, 2009 samples carried the mutant 76T genotype. Only two (2.1%) of the July 2004 samples carried the wild type 76K genotype while it only occurred in 2 (2.1%) of the August 2004 samples and 1(5.9%) in the July 2009. Mixed genotype infections (76 K/T) were found in 3(3.2%) of the July 2004 samples, 1(1.1%) of the 95 August 2004 samples and 1(5.9%) of the July 2009, 17 samples. The prevalence in frequency of the mutant markers was not significantly different between July 2004 and August 2004 (x^2 = 0.0054, p = 0.754). Similarly the frequency between August 2004 and July 2009 was not statistically significant ($x^2 = 0.5264, p = 0.138$), however the change overtime significantly increased over the years for wild type only ($x^2 = 0.298, p = 0.0054$) (Table 4.1).

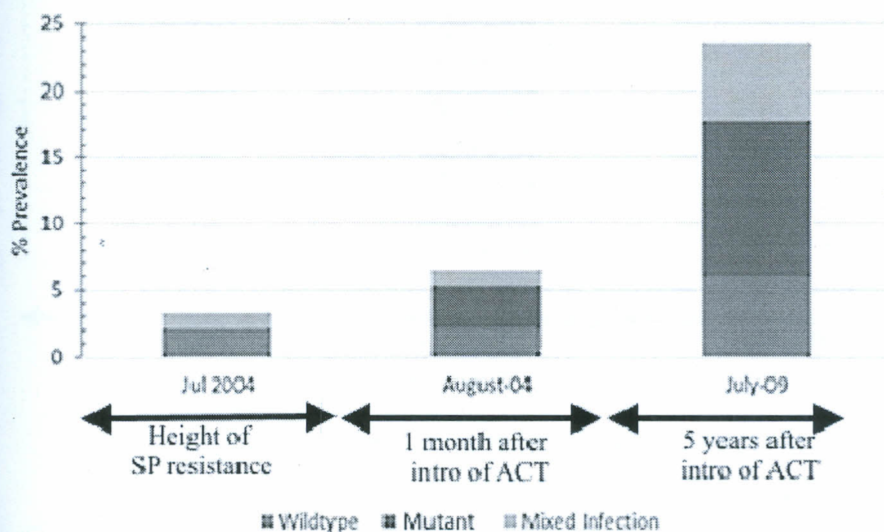

Figure 4.1: Bar Graphs showing prevalence of *pfcrt* polymorphisms during the 3 time points. The results indicate low frequency in the *pfcrt* polymorphisms in the 3 time points and subsequently no change overtime.

4.2 *PfMDR1* Genotypes

All the 207 samples from the three time points were genotyped for *pfmdr1* at codon 86. No mutant genotype 86Y was found in the isolates. Prevalence of the wild type *pfmdr1-86N* was n=0 in July 2004, n = 2 (2.1%) in August 2004 and no mutant (0.0%) was seen in the samples genotyped five years later in 2009 (Figure 4.2). As for the mixed infections (86N/Y), three (3.2%) was found in July 2004. Only one (1.1%) sample carried the mixed genotype infection in August 2004 while no mixed mutation was found in 2009. The prevalence in frequency of the mixed markers was not significantly different between July 2004 and August 2004 (x^2 = 0.3966, p = 0.223). Similarly the frequency in the wild type markers between July 2004 and August 2004 was not statistically significant (x^2 = 0.5264, p = 0.2329). Lack of the mutant markers meant there was no change over time over the years (Table 4.1).

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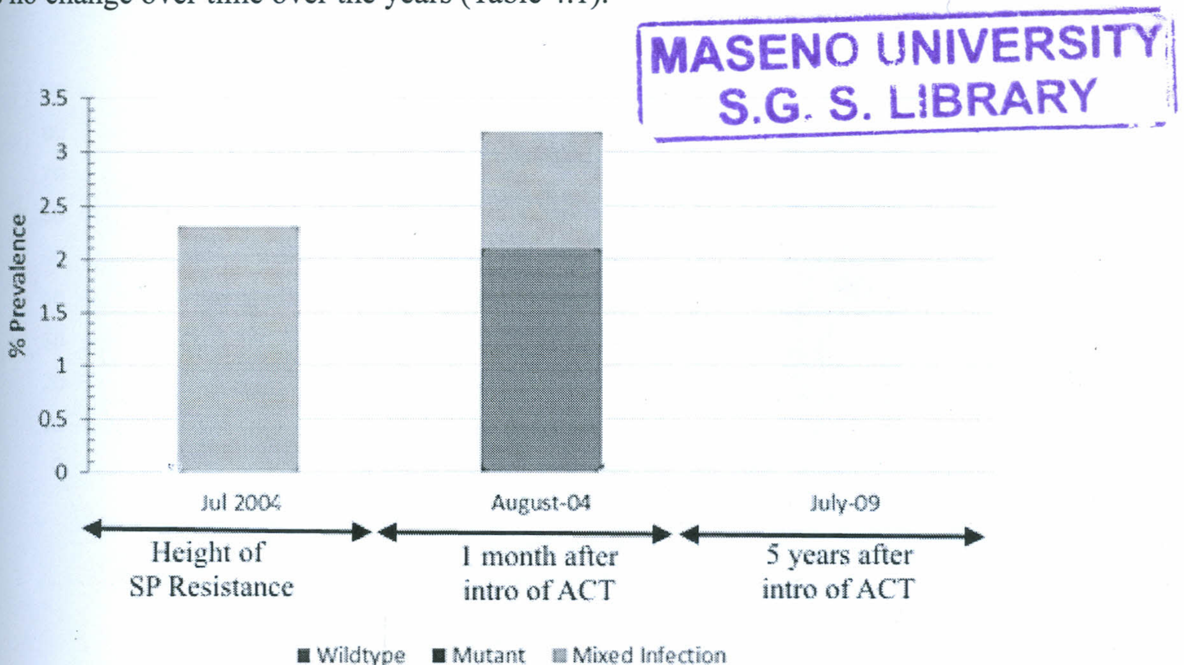

Figure 4.2: Bar Graphs showing prevalence of *pfmdr1* polymorphisms during the 3 time points. The results indicate lack of the mutant polymorphism in all the time points. There is also a decrease in the frequency of the mixed infection (86N/Y) and the subsequently lack of it in July 2009.

4.3 *Pfdhfr* Genotypes

The July 2004 isolates (n=95) were all successfully genotyped for the *pfdhfr* mutation at codon 59. The wild type 59C was relatively rare occurring only in 1 isolate (1.1%) in August 2004. The prevalence of the mutant genotype 59R was 17.9% (17 of 95) in July 2004, 9.5% (9 of 95) and 52.9% (9 of 17) in 2009 (Figure 4.3). Mixed polymorphisms 59C/R was found in 4/95 (4.2%) of the isolates genotypes in July 2004, 9/95 (9.5%) of those genotyped in August 2004 and 5/17 (29.4%) in 2009. There was no statistically significant difference (x^2 = 0.91, p = 0.144) in the prevalence of the *pfdhfr* mutations between July and August of 2004. The prevalence between August 2004 and July 2009 was however strongly significant (x^2 = 0. 1023, p<0.05) (Figure 4.3) however there was a no significant reduction in the trend from 2004 to 2009 (Table 4.1).

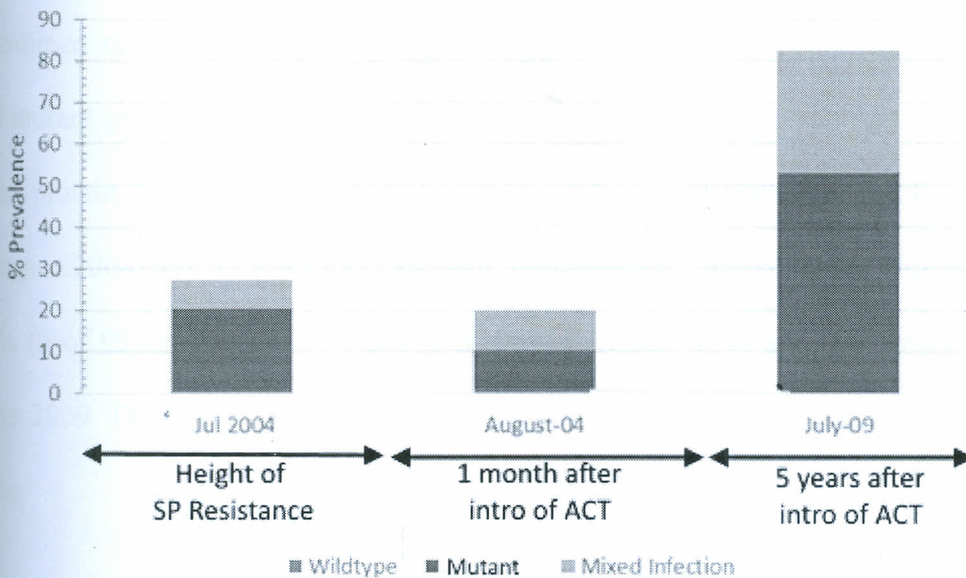

Figure 4.3: Bar Graphs showing prevalence of *pfdhfr* polymorphisms during the 3 time points. Results indicate presence of the mutant marker in all the timepoints and low frequency of the wiltype marker in all the timepoints.

4.4 *Pfdhps* Genotypes

Ninety five isolates from July 2004, the same number from August 2004 and 17 isolates from 2009 were successfully genotyped for the *dhps* mutation at the 540 codon. Forty five of the genotyped isolates carried the mutant 540E genotype in July 2004(47.4%), 9 (9.5%) carried the mutant codon in August 2004 and 1 (5.9%) of the 2009 isolates had the polymorphism. As for the wild type codon 540K, 14 (14.7%) showed the genotype in isolates from July 2004, 51 (53.7%) in August 2004 and 16 (94.1%) in 2009. There was a significant increase in the prevalence of the *pfdhps* wild type between July and August of 2004 ($p < 0.05$) and August 2004 and July 2009 ($p = 0.015$), and also depicted change overtime between 2004 and 2009 ($p = 0.0001$) (Figure 4.4).

Forty six isolates were successfully genotyped for both *dhfr* and *dhps* mutations. The prevalence of the combined mutant genotype was eight (8.42%) in July 2004, only one (1.1%) had the combined mutant genotype in August 2004 and none of this genotype was seen in in July 2009. The mixed mutant genotype 59CR/540EK remained highly prevalent at 16.8% (July 2004), 13.6% (August 2004) and 82.3% five years later. Prevalence of the combined wild type mutation was 3.15% (3 of 95) in July 2004, 5.2% (5 of 95) in August 2004 and 0% among the isolates collected in 2009. The change overtime significantly increased over the years for all the markers (Table 4.1).

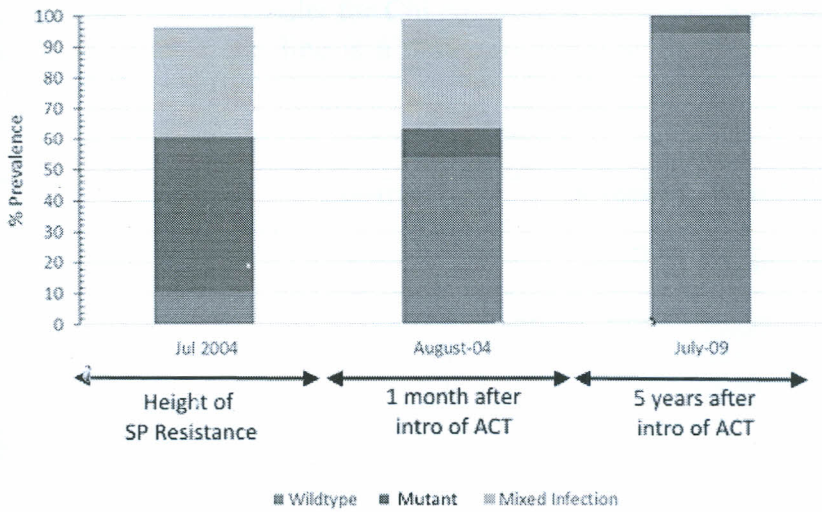

Figure 4.4: Bar Graphs showing prevalence of *Pfdhps* polymorphisms during the 3 time points. Results indicate rise in the frequency of the Wildtype while there is a decrease in the mutant polymorphism.

Table 4.1: Table showing p-value results for Chi square test for trend. Significant result in bold suggests that the slope of the trend line is a non-zero and thus there was significance in the trajectory of the markers ($p<=0.05$)

Gene	Genotype		Chi-square for slope (linear trend)
			p-value
pfcrt 76	76T	mutant	**0.0054**
	76K	wild-type	0.5264
	76K/T	mixed	0.298
pfmdr1 86	86Y	mutant	N/a
	86N	wild-type	0.3966
	86N/Y	mixed	0.2328
pfdhfr	59R	mutant	0.91
	59C	wild-type	0.7817
	59C/R	mixed	0.1023
pfdhps	540E	mutant	**<0.001**
	540K	wild-type	**<0.001**
	540E/K	mixed	**0.0002**

CHAPTER FIVE: DISCUSSION

5.1 Prevalence and Temporal Trajectory of the Chloroquine Resistance *pfcrt* (K76T)

Malaria remains a major health concern in western Kenya despite reports of significant reduction in malaria related morbidity and mortality by approximately <44% in other areas within the country (WHO, 2012). The selection of effective and affordable anti-malarials for efficient treatment and control remain very vital in this period of emergence of *P. falciparum* resistance to various anti-malarials. Chloroquine failure in Kenya was initially reported in 1977 (Terlouw et al., 2003). Three and a half decades later, Kenya has gone through two major changes of anti-malarial drug policies and currently there is concern that the effectiveness of the third drug policy, artemisinin-based combination therapy (ACT) as the best treatment against malaria may be in jeopardy. This retrospective study was performed in order to determine the prevalence of common drug-resistant mutations within a population residing in western Kenya and to determine if the prevalence of these molecular markers changed over the period of time when ACT was first introduced as the first line antimalarial and SP resistance was at its peak. The study sought to determine whether *P. falciparum* remained susceptible to the SP and CQ drugs after reports suggesting that the drug resistance fades after withdrawal from use (Hand and Meshnick, 2011). Results demonstrated that the prevalence of the mutant gene 76T, associated with increased resistance to CQ did not significantly change over time within this study population.

Resistance to CQ is largely determined by the *pfcrt* K76T polymorphism. The increase in *pfcrt* resistance in the current study is in line with other studies in which the prevalence of the mutant *pfcrt* codon 76 showed an increase in prevalence over the same time period in which there was an antimalarial policy change from SP to ACT (Severini *et al.*, 2006; Afoakwah *et al.*, 2014). In

spite of the decline in the prevalence of the *Pfcrt* 76K mutation since the antimalarial policy change in 1993, the 12% prevalence recorded in this study is considered high after sixteen years of the abolishment of CQ usage in Kenya. These findings are in contrast to other observations from other holoendemic areas in which the mutant allele significantly reduced in the population after a reduction in CQ use (Mwai *et al.*, 2009; Mbogo *et al.*, 2014). Taken together, these findings show that the K76T mutation does not seem to be abating in this area, as it did in the coastal region of Kenya and other malaria holoendemic places in East Africa (Mwai *et al.*, 2009; Hand and Meshnick, 2011).

The persistence of the *pfcrt* K76T gene in isolates from a highly holoendemic area such as Kanyawegi indicates that selection for the mutant codon is in progress. Despite changes in government anti-malarial policy (in 2010), CQ was still used to treat malaria in 37% of households surveyed in Kisumu as compared to 32% that used ACT by 2010 (Watsierah *et al.*, 2010). It thus appears that widespread use of CQ in the private sector may be high enough to exert selective pressure on the parasite population and thus the prevailing prevalence of the *pfcrt* K76T mutation over the 5 years.

5.2 Prevalence and Change of the Polymorphism in *P. falciparum* Multi-drug Resistant Gene 1 (*pfmdr1*) (N86Y)

The prevalence of the clinically relevant *pfmdr1* N86Y polymorphism was studied to analyse its association with ACT treatment failure (Lim *et al.*, 2009) and reports of decline in efficacy of ACT in Kenya (Borrmann *et al.*, 2011). The current study showed an absence of the mutant 86Y gene in isolates from both the baseline and the follow up isolates suggesting that the parasites harbouring this mutation are either absent or not widespread in this area or most probably a reflection of the formal policy of complete removal of CQ in Kenya. These results indicate that

the overall prevalence of the *pfmdr1* mutation remains low 5 years after introduction of ACT. This is expected since the precise time ACT was introduced in western Kenya, 5 years after change of treatment policy to ACT, *pfmdr1* genotypes were still not seen in the successfully tested samples. This observation is consistent with other reports from Africa (Dokomajilar *et al.*, 2006b; Happi *et al.*, 2009) and generally depicts the high efficacy of the ACT therapy and further suggests that the artemisinin resistance has not spread to the current holoendemic area of western Kenya, thus assuring that the global efforts to contain ACT resistance remains effective.

5.3 Prevalence and Change of the Polymorphism in Antifolate Drugs Resistance Genes *pfdhfr* (C59R) and *pfdhps* (K540E)

Results obtained from this study revealed presence of both the antifolate drugs resistance genes *pfdhfr* (C59R) and *pfdhps* (K540E). It appears that a significant proportion of isolates carried the *pfdhps* mutation prior to 2004, and use of ACT as the first line treatment for malaria in Kanyawegi resulted in the development of highly mutant *pfdhfr* (52.9%). Further results from the study showed that mixed *pfdhps* genotypes were consistent in frequency during the initial year (33.7% and 35.8%) and were not seen five years later (0.0%). This was not the case with mixed *pfdhfr* genotypes in which case there was an increase in prevalence between the baseline and follow up studies (from 4.2% to 29.4%). It would be of interest to note that the wild type allele 59C was not present during the baseline study and in subsequent follow ups contrary to *pfdhps* in which there was a significant increase just after ACT implementation (August, 2004). This observation is in line with other findings showing the high prevalence of SP mutant-resistant markers 59C and 540E in western Kenya (Carter *et al.*, 2005). The results also suggest that the drugs selection acted differently on the resistant alleles of *dhps* and *dhfr*, as evidenced by their prevalence over this study period. Notably, the results presented here shows that ACT use in

Kenya is not just associated with the shrink of K540E but also the progressive decrease of mutations in *pfdhfr* at codon 59. This drug pressure seems to be fast and effective as demonstrated by the drastic decrease of SP-resistant mutations in the short time between the baseline and four weeks follow-up. The complexity of this observation stresses the relevance of more population-based studies that can evaluate the effects of drug selection on malaria parasite populations.

The switch from SP to ACT as the drug of choice for combating uncomplicated malaria in 2004 was expected to decrease SP use in the country, possibly leading to restoration of SP and CQ sensitive parasites. In the current study, it is clear that discontinuing use of SP led to the reversion of codon 540 to wild type over a five year period, and SP sensitivity *in vivo* could be restored. It would be of value to further monitor the SP efficacy and post-treatment selection of *pfdhfr* and *pfdhps* alleles *in vivo* to inform future treatment guidelines in this study population.

5.4 Comparison of the Prevalence of Alleles within *pfcrt* K76T and *pfmdr1* N86Y Pre- and Post-Adoption of Artemether Lumefantrine

There was absence of the *pfmdr1* N86Y isolates from both the baseline and the follow up isolates suggesting that the parasites harbouring this mutation are not widespread in this area. The 86Y has been associated with increased resistance to artemisinin (Alker *et al.*, 2007), therefore absence of this mutation may be a good indicator for the *in vivo* efficacy of ACT in this region. This would be expected since this was the precise time ACT was introduced in western Kenya, 5 years after change of treatment policy to ACT, *pfmdr1* genotypes were not seen in the successfully tested samples. This is consistent with other reports from Africa (Dokomajilar *et al.*, 2006b; Happi *et al.*, 2009). Resistance to CQ is largely determined by the mutant gene *pfcrt* 76T, however in the current study, the frequency of the mutant *pfcrt* genotype remained less than 11%

but seemingly there was trend of increase of the mutant from the 4% in 2004 to 12% in 2009. This is concordant with other studies where prevalence of the mutant *pfcrt* codon 76 showed an increase in prevalence over the same time period after antimalarial policy replaced SP with ACT (Severini *et al.*, 2006; Afoakwah *et al.*, 2014). This result goes to show that the K76T mutation does not seem to be letting up in this area, as it is in the coastal region of Kenya and other malaria holoendemic places in East Africa (Mwai *et al.*, 2009; Hand and Meshnick, 2011). The persistence of the *pfcrt* K76T gene in isolates from a highly holoendemic area such as Kanyawegi indicates that selection for the mutant codon is current. Despite changes in government ant-malarial policy, as late as 2010, CQ was still used to treat malaria in 37% of households surveyed in Kisumu, compared to 32% that used ACT (Watsierah *et al.*, 2010) and this would be the reason for the prevailing prevalence of the *pfcrt* K76T mutation.

CHAPTER SIX: SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY OF FINDINGS

Ninety five (95) *P. falciparum* isolates were genotyped to demonstrate the prevalence and temporal trajectory of the chloroquine resistance *pfcrt* mutation. Results demonstrated that the prevalence of the mutant gene 76T, associated with increased resistance to CQ did not change significantly overtime in this study population. The prevalence was not statistically significant between July 2004 and August 2004 while the frequency between August 2004 and July 2009, was also not statistically significant. The current study showed an absence of the mutant *P. falciparum* multi-drug resistant gene 86Y gene in isolates from both the baseline and the follow up time points. Results obtained from this study revealed presence of both the antifolate drugs resistance genes *pfdhfr* (C59R) and *pfdhps* (K540E). For the *dhfr* gene samples sequenced for positions 59R in July 2004 and August of the same year showed an increase in the frequency just after the fourth week of follow up, but then, significantly recovered after 5 years (52.9%). In contrast to the *pfdhfr* mutation, the prevalence of mutations at the *dhps* codon (540E) analyzed decreased dramatically from 47.4% in July 2004 to 5.9% in July 2009 prevalence of mixed *pfdhps* genotypes were comparable during the initial year (33.7% and 35.8%) and were not seen five years later (0.0%). This was not the case with mixed *pfdhfr* genotypes in which case there was an increase in prevalence between the baseline and follow up studies (from 4.2% to 29.4%). There was 0% presence of the *pfmdr1* 86Y isolates from both the baseline and subsequent time points August 2004 and July 2009. As for the mutant gene *pfcrt* 76T, a large determinant to resistance to CQ, the current study showed that the frequency of the mutant *pfcrt* genotype remained less than 11% but seemingly there was trend of increase of the mutant from the 4% in

2004 to 12% in 2009.

6.2 CONCLUSIONS

1. The increase in prevalence and change over time of the K76T mutation in this highly holoendemic area indicate that selection for the mutant codon is in progress. It thus appears that widespread use of CQ in the private sector may be high enough to exert selective pressure on the parasite population and thus the prevailing prevalence of the *pfcrt* K76T mutation over the 5 years.
2. The results of the study show the absence of the *pfmdr1* N86Y isolates from both the baseline and the follow up isolates suggesting that the parasites harbouring this mutation are not widespread in this area. This result generally depicts the high efficacy of the ACT therapy and further suggests that the artemisinin resistance has not spread to the current holoendemic area of western Kenya, thus assuring that the global efforts to control *pfmdr1* drug resistance by remains effective.
3. Trends in occurrence of the antifolate drugs resistance genes *pfdhfr* (C59R) and *pfdhps* (K540E from the study indicate that the continued rise in the prevalence of the mutations associated with SP resistance and the prevalence of those linked with CQ resistance indicates that a continued drug pressure, from either SP or CQ use is preventing the restoration of SP and CQ susceptible parasites in Kanyawegi
4. Comparison between the prevalence of alleles within *pfcrt* K76T and *pfmdr1* N86Y pre- and post-adoption of artemether lumefantrine as first line of treatment against malaria in western Kenya show that there is no association between the two. The *pfmdr1* N86Y was not prevalent at the baseline part of the study and this trend continued to July 2009. This was not the case with *pfcrt* K76T genotype whose prevalence was constant through the study. The persistent trend of *P. falciparum* mutant *pfcrt* codon K76T indicates that

efficacy of CQ is still compromised, but further studies are required to assess the clinical relevance of this observation.

6.3 RECOMMENDATIONS FROM STUDY

1. The high prevalence of *P. falciparum* mutant *pfcrt* codon K76T indicates that efficacy of CQ is still compromised, but further studies are required to assess the clinical relevance of this observation in light of the fact that several African countries e.g. Mozambique, Senegal and Tanzania show that malaria parasites are succumbing to the formerly used drug chloroquine.
2. Alternative *pfmdr1* genotypes have been linked to susceptibility to artemisinin, a component of the current anti-malarial first line therapy. *In vivo,* treatment with Artemisinin Lumefantrine selects for the N86Y, D1246 and Y184F genotypes and *in vitro* the genotype combination D1246Y/N1042D/S1034C is linked with increased artemisinin susceptibility (Chavchich *et al.*, 2010). In this study, the prevalence of the N86Y polymorphism in chromosome 5 was singled out for this study as it was thought to be the most authentic marker of drug resistance when looking at *pfmdr1* polymorphism (Ferreira *et al.*, 2011). There was absence of the mutant genotype 86Y associated with artemisinin sensitivity. The prevalence of alternative *pfmdr1* mutations under increasing ACT pressure in this area would be of interest in future studies.
3. More research needs to be done on the impact of the mutations on the efficacy of SP with a larger sample size especially in 2009, as the sample size at this time point was limited. This study contrasts with other studies (Mwai *et al.*, 2009; Okombo *et al.*, 2014) conducted over the same period and thus highlights the importance of surveillance and the heterogeneity in drug resistance that may occur within Kenya.

4. The continued rise in the prevalence of the mutations associated with SP resistance and the prevalence of those linked with CQ resistance indicates that a continued drug pressure, from either SP or CQ use is preventing the restoration of SP and CQ susceptible parasites in Kanyawegi. These findings again contrast with other finding done in the country and thus stress the need for continued surveillance and the heterogeneity in drug resistance that seems to be occurring in the country.

6.4 RECOMMENDATIONS FOR FUTURE RESEARCH

1. The study is limited to the detection of known mutant alleles. Novel drug resistant mutations have arisen e.g. the K13-propeller gene that would also impact malaria prevention and control efforts. Therefore it would be of interest if future studies looked at these new markers.
2. The Traditional PCR method used in this study is very time consuming and results are based on size discrimination. Although expensive it would be of interest to conduct experiments with newer high-throughput methods like the 5' nuclease real time PCR which have an increased dynamic range of detection and no post pcr processing.
3. The increase of *pfdhfr* mutations has been previously noted in Tanzania in cross-sectional studies during the era of SP and after the adoption of ACT as the drug of choice. The potential of this genotype as a single marker of drug efficacy for SP should be investigated.
4. Analysis of mutation in relation to the clinical status (symptomatic or asymptomatic) and according to age (younger or older than 10 years) has shown interesting results in related studies (Zignol *et al.*, 2012). It would be of interest to see what the data yields when comparing the prevalence of the markers between symptomatic and asymptomatic adults

to find out whether the immune status of an individual affects the mutation prevalence

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