TEMPORAL PREVALENCE OF *PLASMODIUM FALCIPARUM* DRUG RESISTANCE MARKERS, *PFCRT* (K76T), *PFMDR1* (N86Y), *PFDHFR* (C59R) AND *PFDHPS* (K540E) IN KANYAWEGI SUB-LOCATION, WESTERN KENYA

By

Ogari Zachary Udhili

PG/MSC/061/2010

A thesis submitted in partial fulfilment of the requirements for the award of degree of Master of Science in Medical Biotechnology in the School of Public Health and Community Development of Maseno University

July, 2014

DECLARATION

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

Ogari Zachary Udhili

PG/MSc/061/2010

Signature..... Date.....

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

Prof. Collins Ouma, (PhD)
Department of Biomedical Sciences and Technology,

Maseno University,

Maseno, Kenya.

Signature..... Date.....

Prof. Jeffrey A Bailey, (MD, PhD)
School of Medicine,

University of Massachusetts Medical School,

Worcester MA 01655.

Signature..... Date.....

Dr. Kiprotich Chelimo, (PhD)
Department of Biomedical Sciences and Technology,

Maseno University,

Maseno, Kenya

Signature…… Date……

DEDICATION

This work is sincerely dedicated to everyone who supported me in every step of this journey.

ACKNOWLEDGEMENT

First, I would like to convey my sincere gratitude to my advisors Prof. Ann Moormann and Prof. Jeffrey Bailey for the continued support of my M.Sc study and research, for their patience, motivation, enthusiasm, and immense knowledge. Their guidance helped me during the entire time of research and writing of this thesis. I could not have fathomed having better mentors for my M.Sc. study.

In addition to my advisor, I would like to wholeheartedly thank Prof. Collins Ouma for not only allowing me to run my assays in his lab but also for the vast knowledge and support he showed and shared with me in and out of the lab. My sincere thanks also goes to Dr. Chelimo for his encouragement, insightful comments and advice.

I thank my fellow lab mates in Professor Ouma's lab, Elly Munde and Winnie Okeyo for the sleepless nights we worked together before deadlines, the stimulating discussions and all the laughs and fun we shared during my stay in the lab. My grateful thanks also extend to Joslyn Foley for her advice and assistance making sure all reagents and samples got to me on time. Also I thank my friends in KEMRI: Dr. David Mulama, Emily, Sidney Ogolla, Cliff Oduor, Dennis Juma, Angela for all the support and encouragement when things were rough.

Last but not least, I would like to thank my family: my parents James Ogari and Grace Ogari for all the support and understanding throughout my life.

ACRONYMS AND ABBREVIATIONS

ACT	Artemisinin Combination Therapy
AL	Artemether Lumefantrine
AMA	Apical Membrane Antigen
CDC	Centre for Disease Control
χ^2	Chi-Square
CQ	Chloroquine
CR	Compliment Receptor
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide triphosphates
EBA	Erythrocyte Binding Antigen
gDNA	Genomic Deoxyribonucleic Acid
IBM	International Business Machines
KEMRI	Kenya Medical Research Institute
MA	Massachusetts
MDR	Multi Drug Resistance
MHC	Major Histocompatibility Complex
$MgCl_2$	Magnesium Chloride
MSP	Merozoite Surface Protein
PCR	Polymerase Chain Reaction
PF	*Plasmodium falciparum*
PFCRT	*Plasmodium Falciparum* Chloroquine Resistance Transporter
PFDHFR	*Plasmodium Falciparum* Dihydrofolate Reductase
PFDHPS	*Plasmodium Falciparum* Dihydropteroate Synthase
PFMDRI	*Plasmodium Falciparum* Multi Drug Resistance 1
RBC	Red Blood Cell
SP	Sulphadoxine Pyrimethamine
W.H.O	World Health Organization

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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. falciparum* 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 genes associated with ACT resistance in this malaria endemic population remains unknown. 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 over three time points (July 2004, August 2004 and July 2009). A total of 95 paired retrospective blood samples confirmed positive for *P. falciparum* in a previous study at Chulaimbo Health Centre in western Kenya were used. Using Polymerase Chain Reaction (PCR) and Nested PCR, the genes *pfcrt* K76T, *pfmdr1* N86Y, *pfdhfr* (C59R) and *pfdhps* (K540E) were amplified and presence of mutations determined by gel electrophoresis after Restriction Fragment Length Polymorphism (RFLP). 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% (n=0/95) in July 2004 to 2.1% (n =2/95) in August 2004 and reduced to 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). There was a negative correlation between the two SP resistant markers (r=-0.162, p=0.025) with the prevalence of *pfdhps* (K540E) increasing us *pfdhfr* (C59R) decreasing. Findings presented here suggest that resistant markers against CQ and SP have not faded and as such not recommended antimalarials in this *P.falciparum* 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 ant-malarial drug policy aimed at reducing malaria-related morbidity and mortality.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Worldwide, there are 300 to 450 million 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). 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 (Kakkilaya's, 2011). 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 processes since the 1960s when chloroquine (CQ) was the only drug recommended as a first line anti-malarial therapy in the country, replacing quinine (Mwai *et al*., 2009). Chloroquine resistant isolates of *P. falciparum* first appeared 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, chloroquine resistant isolates first emerged in 1977 and resistance steadily rose to about 70% a decade later, which 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). During the mid-2003, SP resistant parasites rapidly arose rendering this drug ineffective leading to its replacement with artemether-lumefantrine (AL) in 2004 (Amin *et al.*,

2007; Figueiredo *et al.*, 2008). Studies since 2006 (Mugittu *et al.*, 2006) have confirmed the presence of artemisinin-resistant malaria in South East Asia (Cambodia) (Phyo *et al.*, 2012) but the presence of artemisinin-resistant malaria parasites in Kanyawegi sub-location, a malaria holoendemic region in western Kenya, remains unknown.

Genetic point mutations in the homolog of the multi-drug transporter, *P. falciparum* multi-drug resistance gene 1 (*pfmdr1*) in codon 86, also known as P-glycoprotein homolog 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). In light of this, it is very important to investigate the molecular potential of *pfmdr1* in considering schemes aimed at the reversal of resistance to various anti-malarial drugs.

The *P. falciparum* chloroquine resistance transporter gene (*pfcrt)* is a key determinant in *P. falciparum* resistance to CQ (Figueiredo *et al*., 2008). Recent functional and evolutionary studies done on the *pfcrt* gene have shown it is more than a CQ resistant gene (Cooper *et al*., 2005). 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). More studies also suggest that *pfcrt* is associated with amodiaquine (AQ) resistance but to a lower level than CQ resistance (Dokomajilar *et al.*, 2006a).

Resistance to antifolates anti-malarials was a common problem in Kenya when previously used in combinations with pyrimethamine and sulphadoxine (Fansidar and Coartem) 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 have also shown that occurrence of mutation 59R in *dhfr* and 540E in *dhps* are the most strongly associated to resistance to anti-malarials (Wang *et al*., 1997). As such, the current study focused primarily on the *pfcrt*, *pfdhfr*, *pfdhps* and *pfmdr1* mutations in this malaria holoendemic region of western Kenya.

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 on to Africa. Thus monitoring this area has allowed for the presumably early detection artemisinin drug resistance (Farooq and Mahajan, 2004). Therefore it be prudent to have regular and thorough surveillance of drug resistance in Kenya as artemisinin Combination Therapy (ACT) is now widely used as the recommended therapy in the country (Lawford *et al*., 2011).

1.2 Problem Statement for Study

Plasmodium falciparum is responsible for the most virulent form of human malaria and accounts for 1.5 to 2.7 million deaths each year, with sub-Saharan Africa being the most affected (Kakkilaya's, 2011). In Kenya, malaria is responsible for over 20% of all deaths in children under 5 years (Kisia *et al.*, 2012). Drug resistance has proven to be a major problem in the country confounding individual treatment and public-health control efforts (Lawford *et al*., 2011). Drug resistance is commonly exhibited in *P. falciparum* malaria and only a few sporadic incidences of resistance have been reported in *P. vivax* (Baird, 2004).

Although the understanding of *P.falciparum* biology has significantly improved with the sequencing of the *Plasmodium* genome and the development of new technologies to study resistance and adaptability of the parasite, very few new drug targets or drugs have been clinically proven to be effective. Furthermore, development of vaccine against malaria infections has also proved to be elusive and resistance has emerged for most anti-malarial drugs generating anti-malarial drug research over the last two decades (WHO, 2012). Scientists have found out 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). Recent data also show that parasites are infecting people miles away from where the cases were first seen and are steadily growing more resistant, and that this pattern is repeating itself as was the case with CQ resistance in the 1950s when CQ resistant *P. falciparum* was first seen (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. 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 (October 2004), and 5 years after SP withdrawal as front line treatment for uncomplicated malaria infections (July 2009) in Kanyawegi sub-location in western Kenya. This data will help monitor and predict the population trends and changes in common drug resistant allele frequencies during the different stages of policy change in anti-malarial drug administration in Kenya.

1.3. Objectives

1.3.1 General Objective

To evaluate the prevalence and trajectory over time 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 temporal trajectory of the chloroquine resistance *pfcrt* (K76T) polymorphism in children presenting with *P. falciparum* malaria in a malaria holoendemic area of western Kenya.

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

iii. To determine the trends in occurrence of the antifolate drugs resistance genes *pfdhfr* (C59R) and *pfdhps* (K540E) in children presenting with *P. falciparum* malaria in a malaria holoendemic area of western Kenya.

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 temporal trajectory 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 gene 1 (*pfmdr1*) (N86Y) in children presenting with *P. falciparum* malaria in a malaria holoendemic area of western Kenya?

iii. What is the prevalence of the antifolate drugs resistance genes *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 Study Justification

Previous studies have compared drug resistance to climate change that threatens to turn common infections to untreatable diseases (WHO, 2012). In a malaria holoendemic region in western Kenya, transmission is intense and is an everyday occurrence with a parasite prevalence of up to 80% (Dent *et al.*, 2009). This causes considerable mortality and morbidity especially in the first 2-3 years of their life (Moormann *et al.*, 2012). Malaria has also been proven to be a significant burden on health system, accounting for about 60% 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 Kenya are as a result of a combination of high transmission, drug resistance to therapy and limited health services in the 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).

The current challenge in development of a vaccine to help fight malaria has left chemotherapy and insect treated bed nets (ITN) as the only practical way currently battle this disease (Kakkilaya's, 2011). These challenges include the parasite's ability to adapt to its environment, its extraordinary biological diversity, and the genetic complexity which can decrease the efficacy of control interventions (White, 1998). Recent changes that may also hamper the struggle to control malaria in this region include recent reports on ACT drug resistance in South East Asia (Alker *et al.*, 2007). The extent of the spread is not clear at the moment but if drug resistant parasites spread outside South East Asia, it would have negative implications for malaria control in malaria endemic regions of Africa reliant on ACT. Lessons of the past in regard to the spread of CQ resistance should result in effective monitoring systems of molecular markers related to drug resistance in this area to enable early detection of

increased prevalence as early as possible. The genes noted by studies to be associated to this phenomenon of resistance include *pfcrt, pfmdr1, pfdhfr* and *pfdhps* (Figueiredo *et al.,* 2008). As such, detection of the prevalence of these genes in the parasite populations over the years should support the need of molecular surveillance tools included for the detection of *P. falciparum* resistance and aid ongoing malaria control programmes.

This study aims to demonstrate that monitoring anti-malarial drug resistance using molecular methods is feasible and clinically useful within the context of malaria control and prevention strategies currently underway in Kenya. Monitoring the prevalence and incidence of drug resistance is critical in advising drug policy change to reduce malaria-related morbidity and mortality in Kenya and specifically in malaria endemic areas of western Kenya.

CHAPTER TWO

2.0 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 *Plasmodium 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. 90% of malaria deaths take place in Africa, where malaria accounts for about one in six of all childhood deaths. The disease also bestows greatly to 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. 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 this 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. 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).

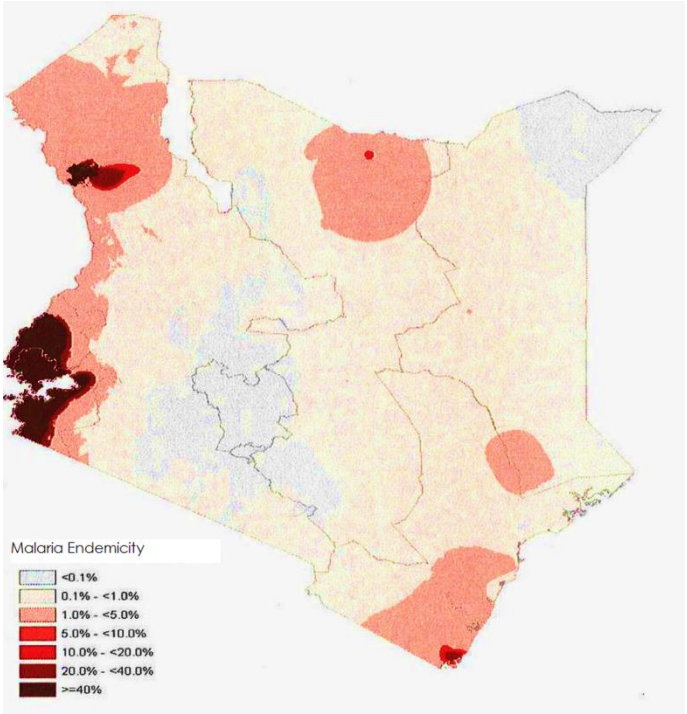

Figure 1: Map showing malaria endemicity in Kenya (Noor *et al.*, 2009)

Measures that are currently in place to control malaria are focused on malaria patients, exposed individuals and the mosquito vectors. This is performed 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 has led to the promotion of ACT as the first line drug of choice (Ponnampalam, 1981). 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 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.

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

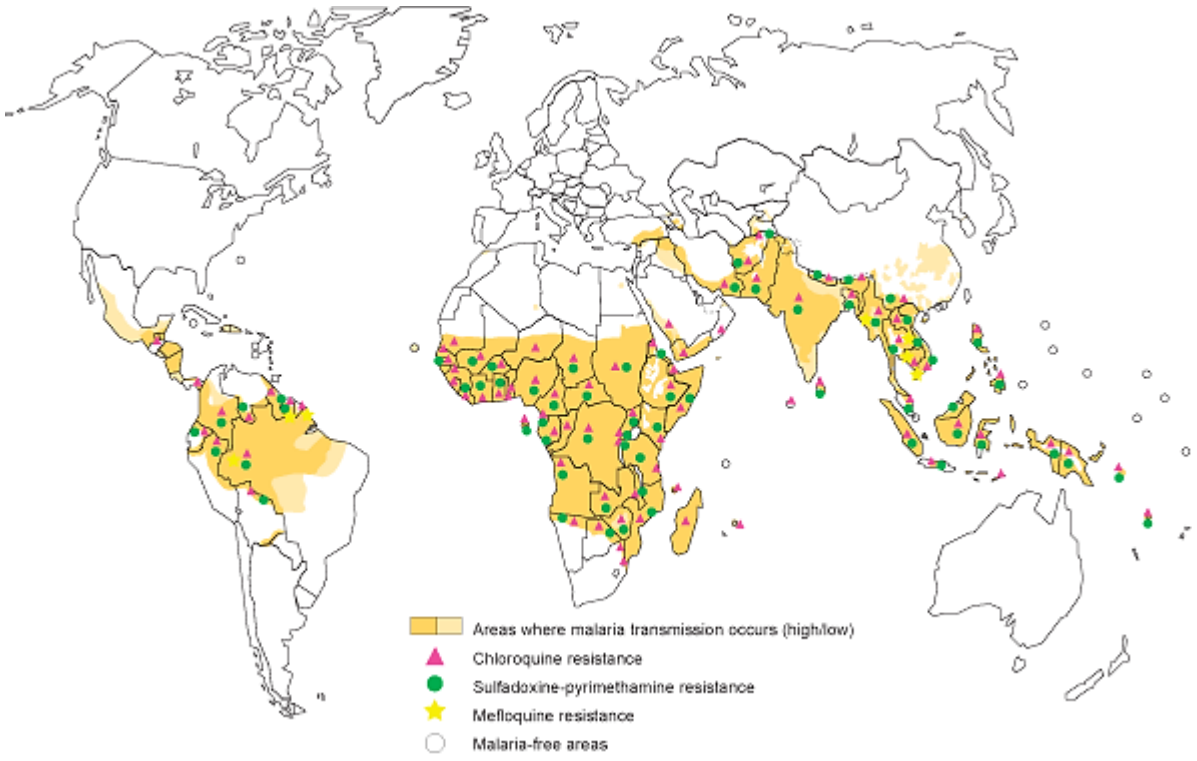

Figure 2: Drug resistance to *P. falciparum* from studies in sentinel sites, up to 2004. (WHO 2005)

In many parts of the continent, *P. falciparum* has proven resistant to most of the drug therapies introduced since CQ was first used as the first line drug of treatment for uncomplicated malaria in the late 1950's (Lopes *et al.*, 2002). Chloroquine resistant strains first appeared 50 years ago simultaneously in south America and South East Asia. Significant progress has been made to understand how CQ resistance occurred but many aspects of this problem still remain a mystery, 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 and the *P. falciparum* multi-drug resistance *1* are major players in causing resistance to chloroquine (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). Recent genetic, and pharmacological studies however show that

the gene is much more than a CQ resistant gene and affects more than one drug therapy line (Cooper *et al.*, 2005). *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 killing it (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 chloroquine drug resistance (Dokomajilar *et al.*, 2006b). These polymorphisms have been shown to vary depending on selection history and geographical location but chloroquine resistant strains have a constant wild type genotype (Dokomajilar *et al.*, 2006a). The K76T mutation looks necessary for the resistance allele to have an effect on the drug and thus is the most authentic marker of drug resistance when looking at *pfcrt* polymorphisms (Figueiredo *et al.*, 2008).

Scientists have surmised that regulation of the endogenic transporter activity related to the *pfcrt* alters the chloroquine 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). An understanding of the presence of the gene between varying time points since its withdrawal should give an idea of its prevalence and association to drug resistance and whether chloroquine resistance has faded a decade after its withdrawal from use.

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

P. falciparum multi drug resistance 1*(Pfmdr1)* also known as P-glycoprotein homolog 1 (*Pgh-1*) is a molecular marker associated with anti-malarial resistance. *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. The efflux system in *Pfmdr1* is unfortunately not drug-specific but accommodates multiple drugs and thus its significance to multi-drug resistance to date (Ferreira *et al.*, 2011).

P. falciparum multi drug resistance 1 polymorphisms have been associated with resistance to chloroquine, 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 affiliated to 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 is an ATP-Binding Cassette (ABC) 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 for implementing rational measures to overcome the drug resistance problem. This study has been able to help in predicting the resistance levels using the prevalence patterns of the gene by looking at the history of drug selection in the same population over time.

2.4 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 chloroquine 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). 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. 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. 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 heavier levels of pyrimethamine resistance than does S108N alone (Sibley *et al.*, 2001).

Consistent mutations in these two genes responsible for antifolates 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). It was thus crucial to determine the trends in occurrence of the antifolate drugs resistance genes *pfdhfr* (C59R) and *pfdhps* (K540E) as this would contribute to development of strategies for prolonging the useful therapeutic life of ACT and gain insight into how the different combinations of mutant DHFR and DHPS would affect ACT resistance.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted at Kanyawegi sub-location in Kisumu district, a malaria holoendemic area in the lowlands of western Kenya (Figure 4). Studies show that the annual entomologic inoculation rate may exceed 300 infectious bites per individual (Amek *et al.*, 2012). The area is approximately 1200 sq km in size and lies between Latitude -0.13 and Longitude 34.60. Lake Victoria is 1.3 miles to the south and its altitude is averagely 1182m above sea level. Kanyawegi sub-location has a semi-arid (0.2 - 0.5 p/pet) climate and is mostly cultivated though some natural vegetation is preserved. 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 lies between 15ºC and 30ºC (ATLAS, 2011).

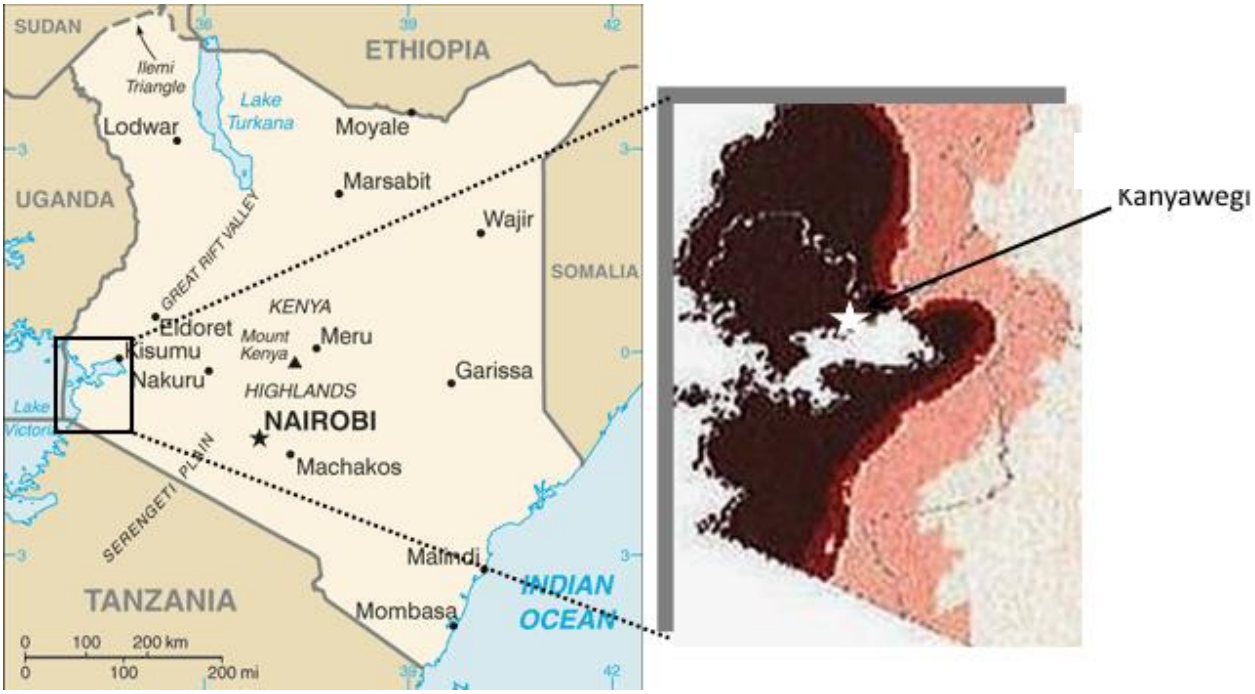

Figure 3: Map showing the Study Area

The area is mildly densely populated with 489 people per km^2. The nearest town with more than 50,000 inhabitants takes about 1:19 hour by local transportation. An estimated 1.99% of the children below 5 years are underweight, with a mortality rate of 61 per 1,000 births. Its inhabitants are predominantly of the Luo ethnic group (>96%), thus the study area has a homogeneous population. Malaria is responsible for the highest number of childhood morbidities and mortalities in the area (Piriou *et al.*, 2012). The major malaria vectors are *An. gambiae s.s.*, *An. arabiensis* and *An. funestus.* Most of malaria transmission takes place during both long and short rains.

The area houses Chulaimbo Health Centre, which is the largest and busiest health centre in the district. 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, especially among children under the age of 5 years old (White *et al.*, 2011). The study used a pair of 95 malaria positive samples collected form children under the age of years in July and October 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 as an axillary temperature >37.5C and a positive blood smear for *Plasmodium falciparum* with an Hgb^{-1} >5.0 gm/dl. 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. 89 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 or adult has not been a residence of the study area since 1999, 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 positive. Individuals who were generally unwell due to other unconfirmed health conditions were also excluded in the study.

3.3 Sample Size Calculation

The study provided a comprehensive temporal characterization of *P. falciparum* resistance at 3 time points in western Kenya. 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 5% was arrived at using the formula;

$$n = \frac{N \times z^2 \times p \times (1-p)}{d^2 \times (N-1) + z^2 \times p \times (1-p)} \text{(WHO, 2011)}$$

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.

$$\frac{285 * 1.96^2 * 0.7 * (1-0.7)}{0.02^2 * (217-1) + 1.96^2 * 0.7 * (1-0.7)} = 95$$

3.4 Parasite DNA Extraction and Species Identification

The retrospective blood samples used has 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 patient's blood specimen. 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 microscope at *100 magnification. The malaria parasites were discerned by their physical features and the appearance of the RBC's they affected. Parasite DNA was isolated from whole blood of *P. falciparum* positive individuals using QIAamp Kits (Qiagen, Inc., Valencia, CA, USA) according to manufactures instruction.

3.5 Molecular Detection of Mutations in Drug Targets

Mutations in the *pfcrt* (K76T), *pfmdr1* (N86Y), *pfdhfr* (C59R) and *pfdhps* (K540E) genes were detected using a PCR-restriction fragment length polymorphism (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. The PCR primers and conditions used for the four genes were as shown 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 as shown in appendix 1 for the four genes. The second PCR products were resolved by electrophoresis on 2.5% agarose gel and visualized by staining with Phenix *GelRed* nucleic acid stain.

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. Various restriction enzymes were used on the PCR products for RFLP following the 2nd round of PCR. For identification of the *pfdhfr* mutation, the PCR products were digested with *Xmn1* 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 1* was used to digest the *pfcrt* products at codon 76. Digestions were done in 20 µL 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) transillumination.

3.7 Data Management and Analysis

Gel electrophoresis results obtained after RFLP were entered into a Microsoft Excel spreadsheet, with cleaning and validation of the data done using SPSS® statistical software package version 20.0 (IBM SPSS Inc., Chicago, IL, USA). The frequencies and prevalences 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 $P \leq 0.05$.

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 (SSC) (See Appendix 5) and the University of Massachusetts medical school center of bioethics.

CHAPTER FOUR

4.0 RESULTS

4.1 *PfCRT* Genotypes

Of the 95 pair of *P. falciparum* isolates genotyped, each from the same patient in July 2004 and August 2004 and 17 isolates from July 2009, 19 isolates were successfully detected of the *pfcrt* haplotypes 76 T, K and TK. Four (4.2%) of the 95 July 2004 samples, three (3.2%) of the 95 August 2004 samples and two (11.8%) of the 17, 2009 samples carried the mutant 76T haplotype. Only two (2.1%) of the July 2004 samples carried the wild type 76K haplotype while it only occurred in 2 (2.1%) of the August 2004 samples and 1(5.9%) in the July 2009, 17 successfully amplified samples. Mixed haplotype 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 was not significant between July 2004 and August 2004 (p = 0.754). The frequency between August 2004 and July 2009 even though not statistically significant (p = 0.138) was lower than the subsequent period.

These results indicate that the prevalence of the 76T haplotype increased and remained high in 2009.

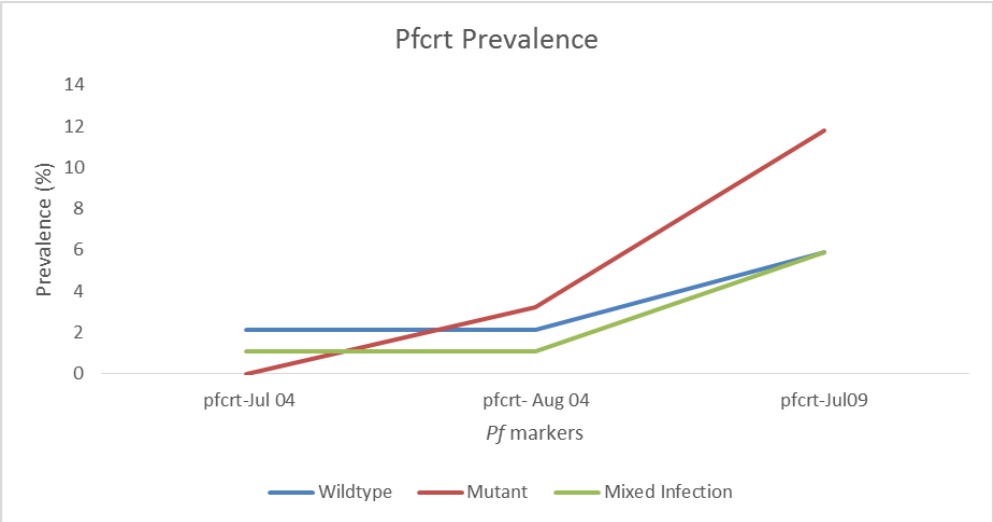

Figure 4: Line chart showing trends overtime of pfcrt polymorphisms from July 2004 to July 2009

4.2 *PfMDR1* Genotypes

All the 207 samples from the three time points were genotyped for *pfmdr1* at codon 86. No mutant haplotype 86Y were found in any 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. As for the mixed infections 86*NY* , three (3.2%) was found in July 2004, Only one (1.1%) sample carried the mixed haplotype infection in August 2004 while no mixed mutation was found in 2009. These results indicate that the overall prevalence of the *pfmdr1* mutation remains low 5 years after introduction of ACT. There was no statistical significance in the prevalence of the mutations between the three time points ($p = 0.223$ & $p = 0.759$)

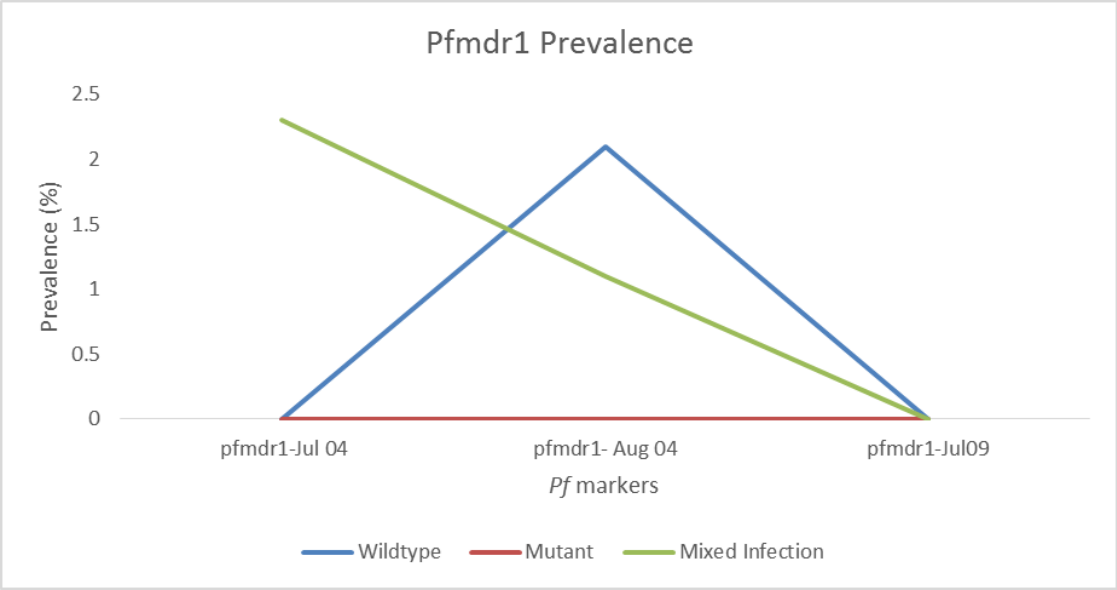

Figure 5: Line chart showing trends overtime of pfmdr1 polymorphisms from July 2004 to July 2009

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 one (1.1%) isolate in October 2004. The prevalence of the mutant haplotype 59R was 17.9% (17 of 95) in July 2004, 9.5% (9 of 95) and 52.9% (9 of 17) in 2009. 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 October 2004 and 5/17 (29.4%) in 2009. There was no statistical significance (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 (p<0.05).

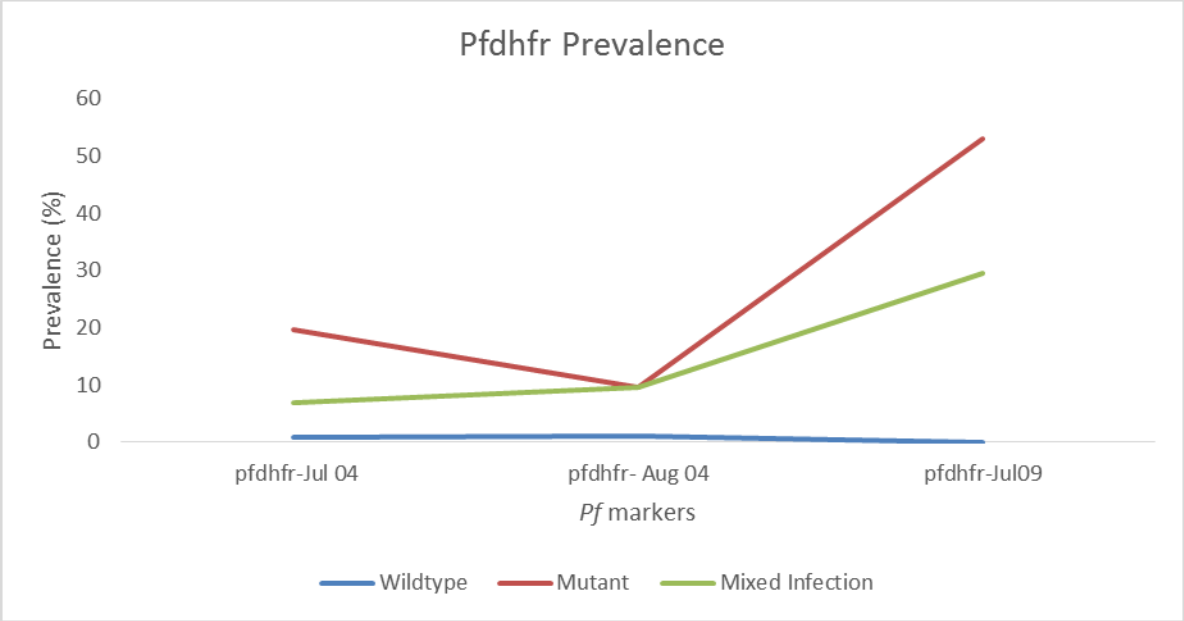

Figure 6: Line chart showing trends overtime of pfdhfr polymorphisms from July 2004 to July 2009

4.4 *Pfdhps* Genotypes

Ninety five isolates from July 2004, the same number from October 2004 and 17 isolates from 2009 were successfully genotyped for the *dhps* mutation at the 540 codon. Forty five (47.4%) of the

genotyped isolates carried the mutant 540E haplotype in July 2004, Nine (9.5%) carried the mutant codon in October 2004 and only one (5.9%) of the 2009 isolates had the polymorphism. As for the wild type codon 540K, fourteen (14.7%) showed the haplotype in isolates from July 2004, Fifty one (53.7%) in October 2004 and 16 (94.1%) in 2009. There was a significance in the prevalence of the *pfdhps* mutants between July and August of 2004 ($p < 0.05$) and August 2004 and July 2009 ($p = 0.015$).

Forty six isolates were successfully genotyped for both *dhfr* and *dhps* mutations. The prevalence of the combined mutant haplotype was eight (8.42%) in July 2004, only one (1.1%) had the combined mutant haplotype in October 2004 and none of this haplotype was seen in in July 2009. The mixed mutant haplotype 59CR/540EK remained highly prevalent at 16.8% (July 2004), 13.6% (October 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 October 2004 and 0% among the isolates collected in 2009.

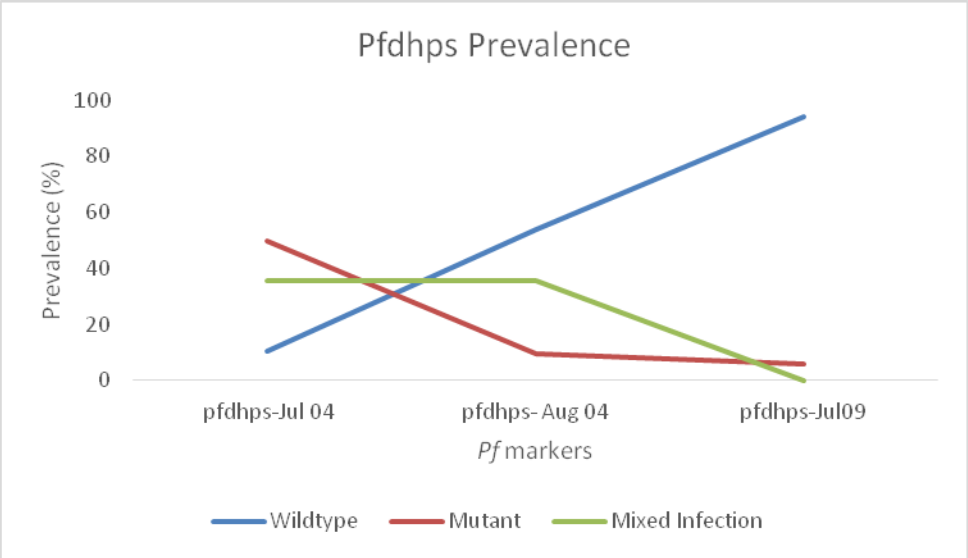

Figure 7: Line chart showing trends overtime of pfdhps polymorphisms from July 2004 to July 2009

4.5 Association between Antifolate Drug Resistant Markers.

Pearson's Correlation Co-efficient was used to determine the association between the antifolate drug resistant genotypes through the time points. Statistical significance was considered at $P \leq 0.05$. There

was generally a negative correlation between the two SP resistant markers through the years (Table 1) with a positive correlation evident only in the prevalence of the *pfdhps* (K540E) in July 2004 and *pfdhfr* (C59R) in July 2009 ($r = 0.878$, $p < 0.05$).

	pfdhfr-Jul 04	***pfdhps-Jul 04***	***pfdhfr- Aug 04***	***pfdhps- Aug 04***	***pfdhfr-Jul09***	***pfdhps-Jul09***
pfdhfr-Jul 04	1					
pfdhps-Jul 04	-0.466327764	1				
pfdhfr-Aug 04	0.984260616	-0.576299185	1			
pfdhps-Aug 04	-0.82094561	-0.114219096	-0.721636	1		
pfdhfr-Jul 09	0.012243126	**0.878041897**	-0.12538307	-0.575695203	1	
pfdhps-Jul 09	-0.509429707	-0.404458466	-0.45917084	0.779007303	-0.71171	1

Table 1: Pearson's Correlation Co-efficient table showing association between the Antifolate Drug Resistant Markers

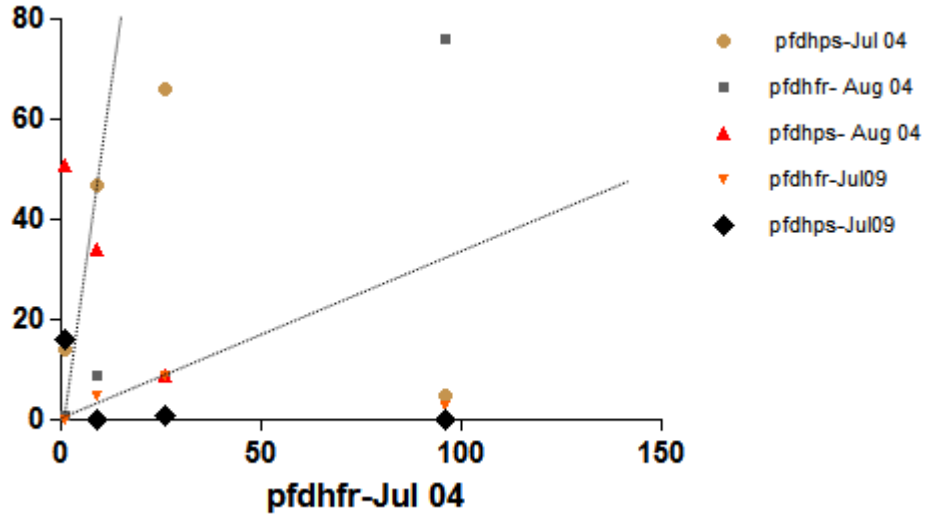

Figure 8: Chart showing association between Antifolate Drug Resistant Markers through the study.

CHAPTER FIVE

5.0 DISCUSSION

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

Malaria remains a major concern in western Kenya especially the Lake Region despite reports of significant reduction in malaria related morbidity and mortality by approximately <44% in areas around 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 ant-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 cross-sectional study was performed in order to determine the prevalence of common drug-resistant mutations within the study population residing in western Kenya and to determine if the prevalence of these molecular markers changed over the period 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 chloroquine 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 was not statistically significant in this study. The frequency of the mutant *pfcrt* haplotype remained less than 11% but seemingly there was a trend of increase of the mutant from the 4% in 2004 to 12% in 2009 (Figure 4).

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* T76 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 chloroquine usage in Kenya. These findings are in contrast to other observations from other endemic areas in which the mutant allele significantly reduced in the population after a reduction chloroquine 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 endemic places in East Africa (Mwai *et al.*, 2009; Hand and Meshnick, 2011).

The persistence of the *pfcrt* K76T gene in isolates from a highly endemic area such as Kanyawegi (Figure 4) indicates that selection for the mutant codon is in progress. Despite changes in government ant-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 (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 reported (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 not widespread in this area or most probably a reflection of the formal policy of complete removal of CQ in Kenya.

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 these 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). For the *dhfr* gene analysed, a total of 17/95 samples was successfully sequenced for positions 59R in July 2004 and 9/95 in August of the same year showing a decrease (p=0.144) just after the fourth week of follow up, but then, significantly recovered (p=0.0001) after 5 years (52.9%).

In contrast to the *pfdhfr* mutation, the prevalence of mutations at the *dhps* codon (540E) analysed decreased dramatically from 47.4% in July 2004 to 5.9% in July 2009 (p=0.0001). It would appear 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%).

In the current study, mixed *pfdhps* genotypes were consistent 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* haplotypes 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 (October, 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 data also indicates that the drugs selection acted differently on the resistant alleles of *dhps* and *dhfr*, as evidenced by their prevalences over this study period. This clearly further showed that SP and ACT do not select for multidrug resistant parasites in areas with high endemicity as other studies in this area have shown (McCollum *et al.*, 2012). Notably, the data 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 process 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 area.

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 (0.0%) 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* haplotype remained less than 11% but seemingly there was trend of increase of the mutant from the 4% in 2004 to 12% in 2009 (p=0.138) (Figure 4). 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 endemic places in East Africa (Mwai *et al.*, 2009; Hand and Meshnick, 2011). The persistence of the *pfcrt* K76T gene in isolates from a highly endemic area such as Kanyawegi (Figure 4) 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 6

6.0 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* (K76T) mutation. Results demonstrated that the prevalence of the mutant gene 76T, associated with increased resistance to CQ was not statistically significant in this study. The prevalence in frequency was not significant between July 2004 and October 2004 (p = 0.754) while the frequency between August 2004 and July 2009 even though not statistically significant (p = 0.138) was lower than the subsequent period. 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 analyzed, a total of 17/95 samples were successfully sequenced for positions 59R in July 2004 and 9/95 in August of the same year showing a decrease (p=0.144) just after the fourth week of follow up, but then, significantly recovered (p=0.0001) 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 (p=0.0001), mixed *pfdhps* genotypes were consistent 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* haplotypes 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* N86Y 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* haplotype remained less than 11% but seemingly there was trend of increase of the mutant from the

4% in 2004 to 12% in 2009 (p=0.138).

6.2 CONCLUSIONS

1. The prevalence of the K76T mutation was persistent in isolates from this highly endemic area indicating that selection for the mutant codon is in progress. Despite changes in government ant-malarial policy in 2010, studies show that CQ was still used to treat malaria in 37% of households surveyed in Kisumu as compared to 32% that used ACT. 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 contain these 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 & 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 chloroquine is still compromised, but further studies are required to assess the clinical relevance of this observation. These findings will serve as a baseline for further monitoring of drug-resistant *P. falciparum* malaria in Kenya.

6.3 RECOMMENDATIONS

1. The high prevalence of *P. falciparum* mutant *pfcrt* codon K76T indicates that efficacy of chloroquine is still compromised, but further studies are required to assess the clinical relevance of this observation. These findings will serve as a baseline for further monitoring of drug-resistant *P. falciparum* malaria in Kenya.

2. The complexity of this observations compared with other studies around Africa, stresses the relevance of more focused clinical and molecular studies on the *in vitro P. falciparum* susceptibility to anti malarials in this region especially on other *pfmdr1* polymorphisms besides the N86Y haplotype. The other mutations have been associated with ACT resistance in other parts of the world. It would be of interest to study them in this area under increasing ACT pressure.

3. More research needs to be done on the impact of the mutations on the efficacy of SP in significant numbers of patients especially in 2009, as the results raise concern especially the higher prevalence of the mutations seen than previously detected. Genetic analysis of parasites from a larger survey in the area should be better for understanding the general development of anti-malarial drug resistance. This study contrasts with other studies 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 & 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 could arise that would also impact malaria prevention and control efforts. Therefore future studies should include whole genome analysis in clinically drug resistant isolates in order to characterize other relevant mutations.

2. The sample size was limited to ongoing studies and therefore was limited in power to detect minor changes in allele frequencies within the population. It would be of interest if the study was conducted with a larger sample size.

3. Alternative *pfmdr1* haplotypes 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 haplotypes 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 at 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 haplotype 86Y associated with artemisinin sensitivity, however the lack of the gene is not likely due to selective pressure induced by ACT as completion of the follow up study preceded

widespread use of ACT . The prevalence of alternative *pfmdr1* mutations under increasing ACT pressure in this area would be of interest to future studies.

4. The increase of pfdhfr mutations has been previously noted from Tanzania in cross sectional studies during the era of SP and after the adoption of ACT us the drug of choice. The potential of this haplotype as a single marker of drug efficacy for SP should be investigated.

5. 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. It would be of interest to see what the data yields in this case to find out whether the immune status of an individual affects the mutation prevalence

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APPENDICES

APPENDIX 1: Mutation sites, primer and primer sequences and PCR reaction conditions of Polymorphic genes investigated.

Gene and PCR round	PCR Primers (5' → 3')	PCR Conditions
DHFR 59 1st Round	A1: 5' – TTTATATTTTCTCCTTTTTA (20bp) A2: 5' – CATTTTATTATTCGTTTTCT (20bp)	Initial denaturation 94˚C x 3m PCR 5 cycles 94˚C x 45s, 54˚C x 45s, 72˚C x 1.5m 35 cycles 94˚C x 30s, 56˚C x 30s, 72˚C x 45s Final Elongation 72˚C x 10m Hold @ 4˚C
DHFR 59 2nd Round	A1: 5' – TTTATATTTTCTCCTTTTTA (20bp) A2: 5' – CATTTTATTATTCGTTTTCT (20bp)	Initial denaturation 94˚C x 3m PCR 5 cycles 94˚C x 45s, 54˚C x 45s, 72˚C x 1.5m 35 cycles 94˚C x 30s, 56˚C x 30s, 72˚C x 45s Final Elongation 72˚C x 10m Hold @ 4˚C
DHPS 540 1st round	R1: 5' – AACCTAAACGTGCTGTTCAA (20bp) R2: 5' – AATTGTGTGATTTGTCCACAA (21bp)	Initial denaturation 94˚C x 3m PCR 40 cycles 94˚C x 1m, 45˚C x 45s, 72˚C x 1m Final Elongation 72˚C x 10m Hold @ 4˚C
DHPS 540 2nd round	K: 5' – TGCTAGTGTTATAGATATAGG**at**GAG**c**ATC (30bp) K1:5' – CTATAACGAGGTATTgCATTTAATgCAAGAA (31bp)	Initial denaturation 94˚C x 3m PCR 5 cycles 94˚C x 45s, 54˚C x 45s, 72˚C x 1.5m 35 cycles 94˚C x 30s, 56˚C x 30s, 72˚C x 45s Final Elongation 72˚C x 10m Hold @ 4˚C
PfCRT 76 1st round	CRT-1: (21bp) GACGAGCGTTATAGAGAATTAA CRT-2: (20bp) CCAGTAGTTCTTGTAAGACC	Initial denaturation 94˚C x 3m PCR 40cycles; 94˚C x 30s, 47˚C x 1m, 72˚C x 1.5m Final Elongation 72˚C x 3m Hold @ 4˚C
PfCRT 76	CQR-A: (21bp) TGTGCTCATGTGTTTAAACTT	Initial denaturation 94˚C x 5m

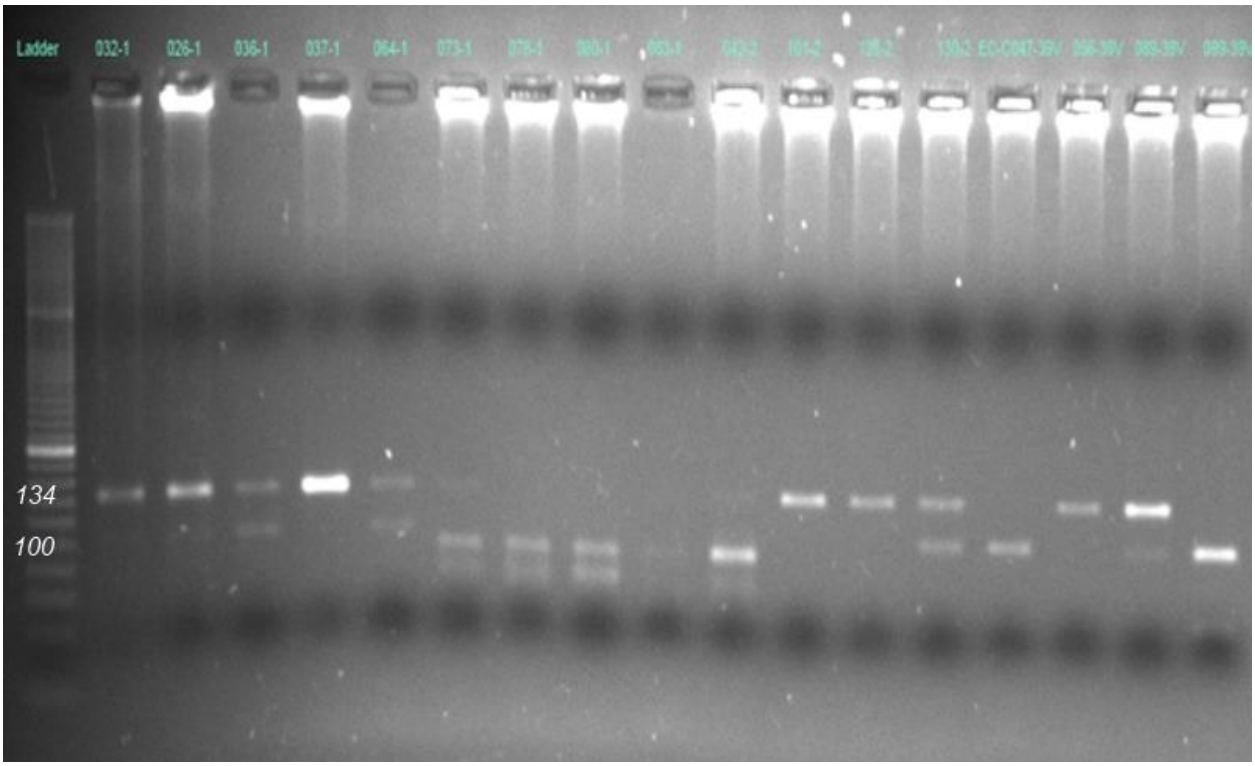
2nd round	CQR-B: (23bp) CAAAACTATAGTTACCAATTTTG	PCR 30 cycles 94˚C x 30s, 52˚C x 1m, 72˚C x 1m Final Elongation 72˚C x 3m Hold @ 4˚C
PfMDR1 86 1st round	MDR-A1: (30bp) TGTTGAAAGATGGGTAAAGAGCAGAAAGAG MDR-A3: (33bp) TACTTTCTTATTACATATGACACCACAAACA	Initial denaturation 94˚C x 2m PCR 30 cycles; 94˚C x 1m, 45˚C x 1m, 72˚C x 45s Final Elongation 72˚C x 5m Hold @ 4˚C
PfMDR1 86 2nd round	MDR-A4: (30bp) AAAGATGGTAACCTCAGTATCAAAGAAGAG MDR-A2: (33bp) GTCAAACGTGCATTTTTTATTAATGACCAttTA	Initial denaturation 94˚C x 2m PCR 30 cycles 94˚C x 1m, 52˚C x 1m, 72˚C x 45s Final Elongation 72˚C x 5m Hold @ 4˚C

APPENDIX 2: Control DNA and fragment size expected of Polymorphic genes investigated.

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

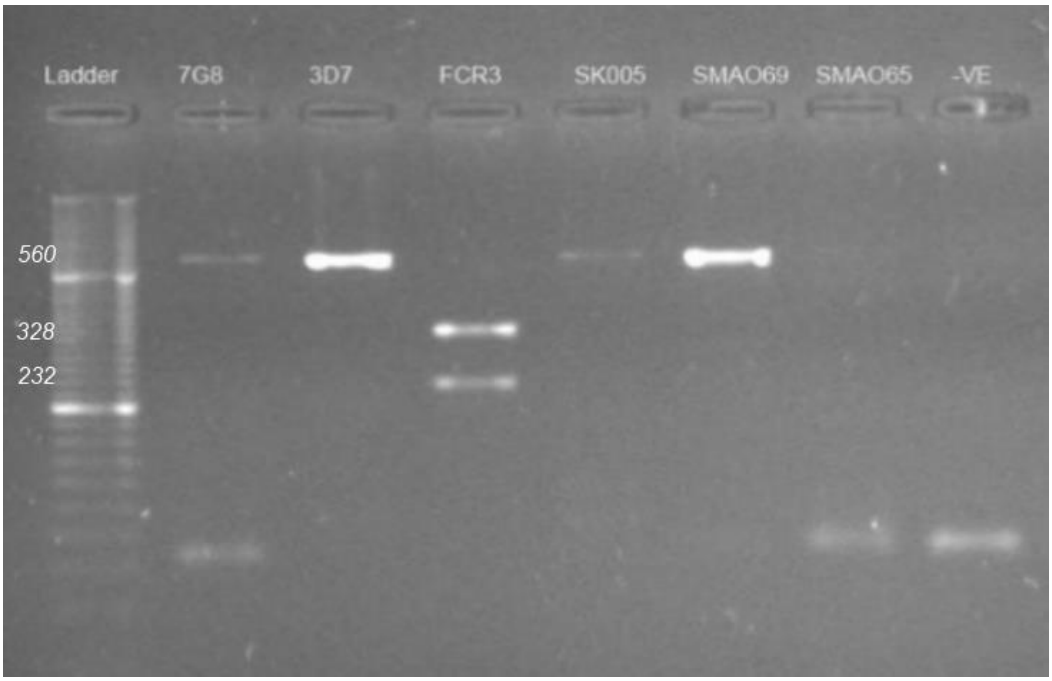
APPENDIX 3: RFLP analysis of the *Plasmodium falciparum* polymorphisms

3A. Agarose gel of the restriction digests of the Pfcrt PCR Product



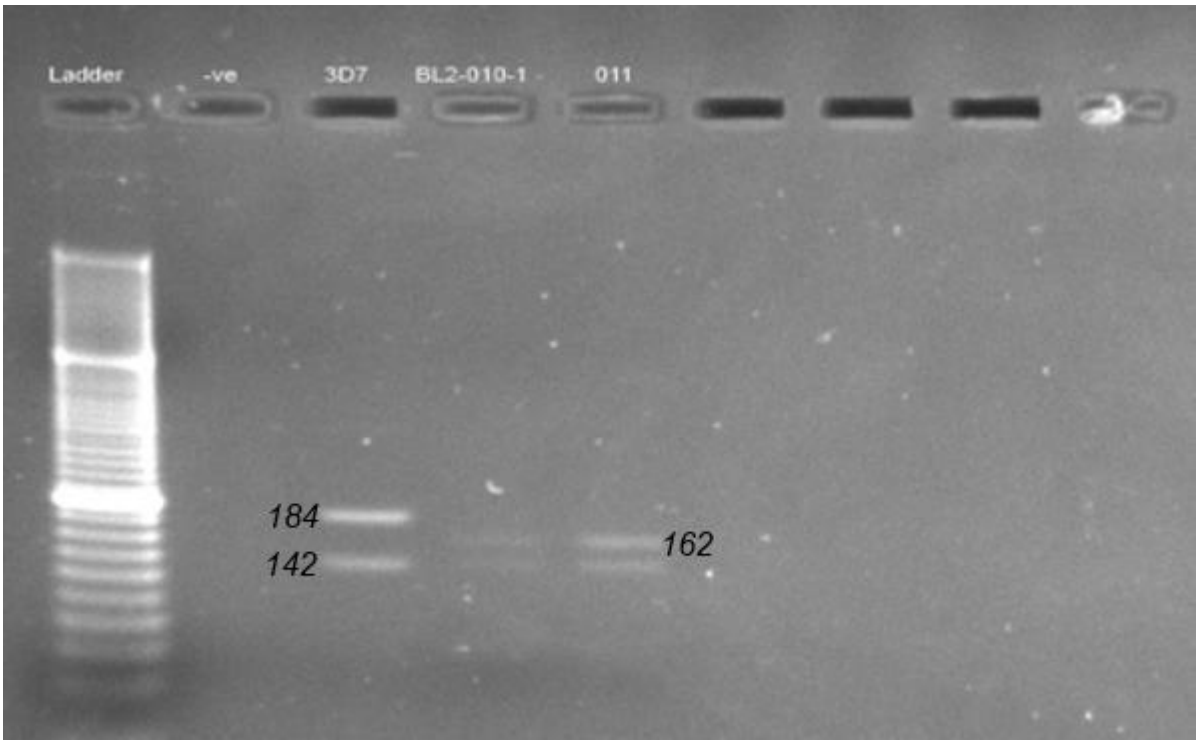
Agarose Gel Products of the restriction enzyme of the *pfcrt* PCR products. Fragment sizes are in bp (base pair). The fragment encompassing *pfcrt* at codon 76 was amplified and digested with *Apo I*, which cleaves the wild type into 134 and 100 bp fragments while the mutant remains intact at 134 bp.

3B. Agarose gel of the restriction digests of the *Pfmdr1* PCR Product



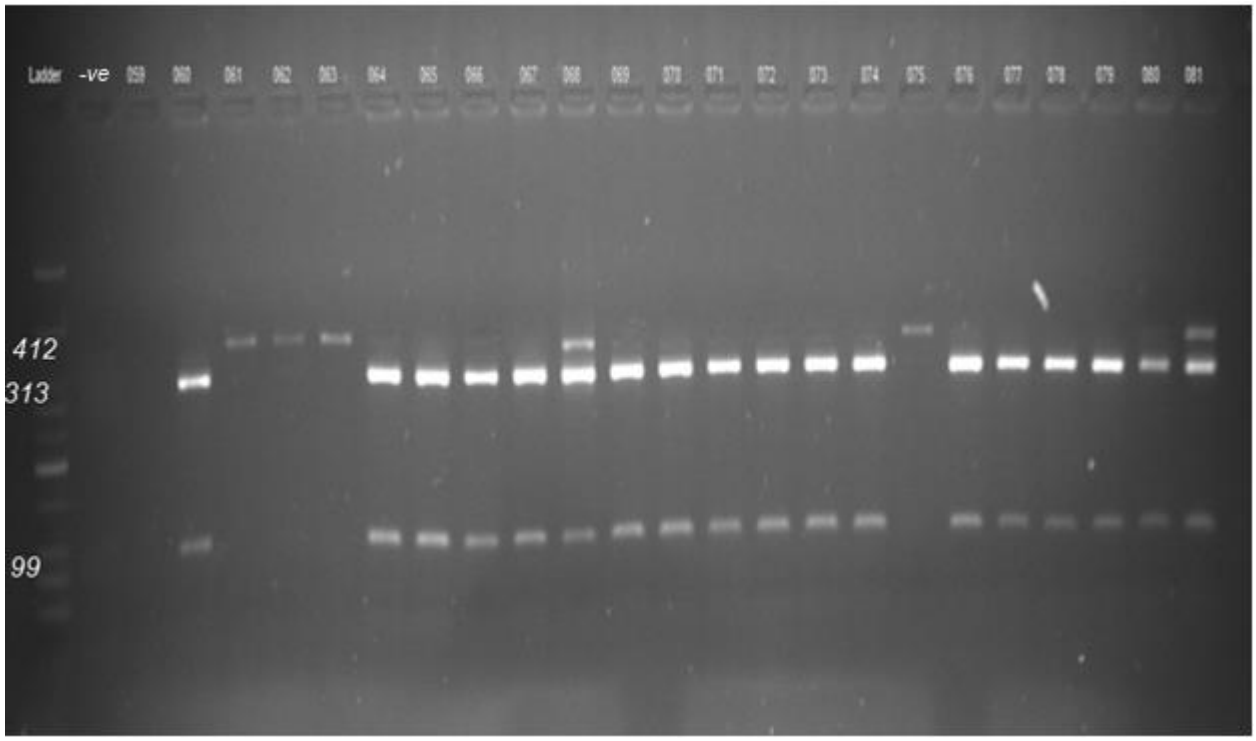
Agarose Gel Products of the restriction enzyme of the *pfmdr1* PCR products. Fragment sizes are in bp (base pair). The fragment encompassing *pfmdr1* at codon 86 was amplified and digested with *Afl III*, which cleaves the mutant into 232 and 328 bp fragments while the wild type remains intact at 560 bp.

3C. Agarose gel of the restriction digests of the *Pfdhfr* PCR Product



Agarose Gel Products of the restriction enzyme of the *pfdhfr* PCR products. Fragment sizes are in bp (base pair). The fragment encompassing *pfdhfr* at codon 59 was amplified and digested with *XmnI*, which cleaves the wild type into 142 and 184 bp fragments while the mutant is cleaved at 22, 142 and 162 bp. The 22 bp fragment is too faint to be seen using electrophoresis.

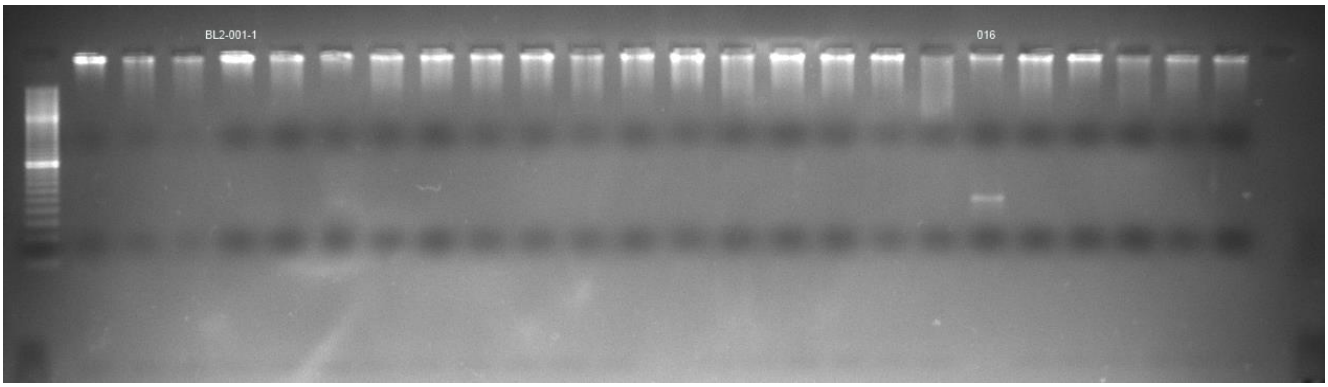
3D. Agarose gel of the restriction digests of the *Pfdhps* PCR Product

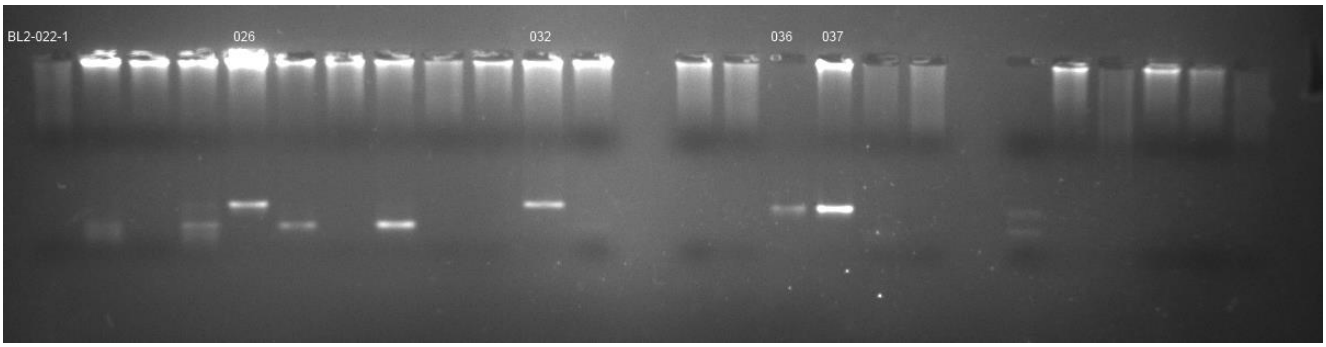


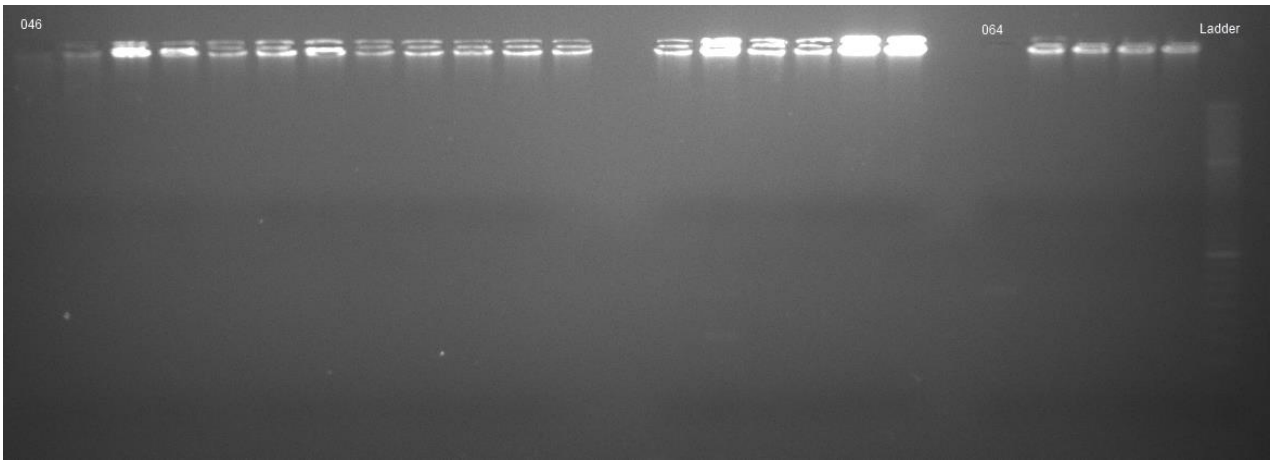
Agarose Gel Products of the restriction enzyme of the *pfdhps* PCR products. Fragment sizes are in bp (base pair). The fragment encompassing *pfdhps* at codon 540 was amplified and digested with *Fok 1*, which cleaves the wild type into 7, 19 and 412 bp fragments while the mutant is cleaved at 7, 19, 99 and 313 base pairs. The 7 and 19 bp fragments are too faint to be seen using electrophoresis.

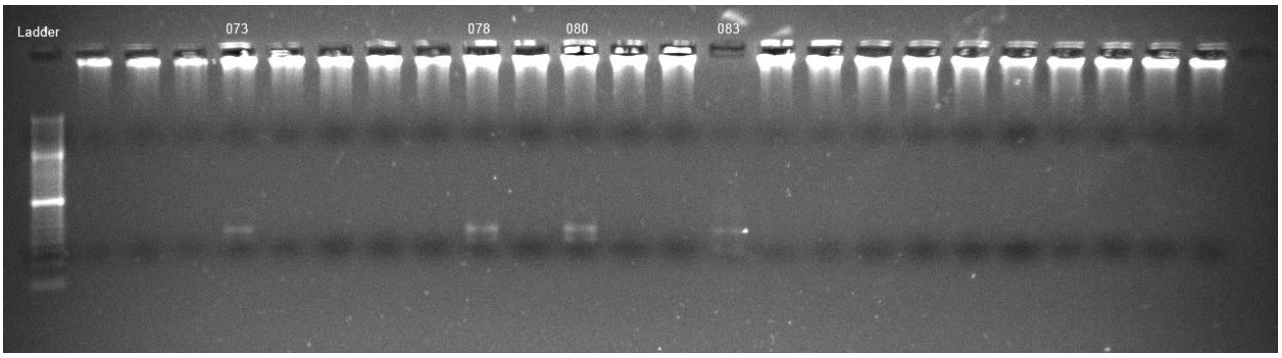
APPENDIX 4: Gel Electrophoresis Photos of Round 1 & 2 PCR products of the *Plasmodium falciparum* drug resistance markers.

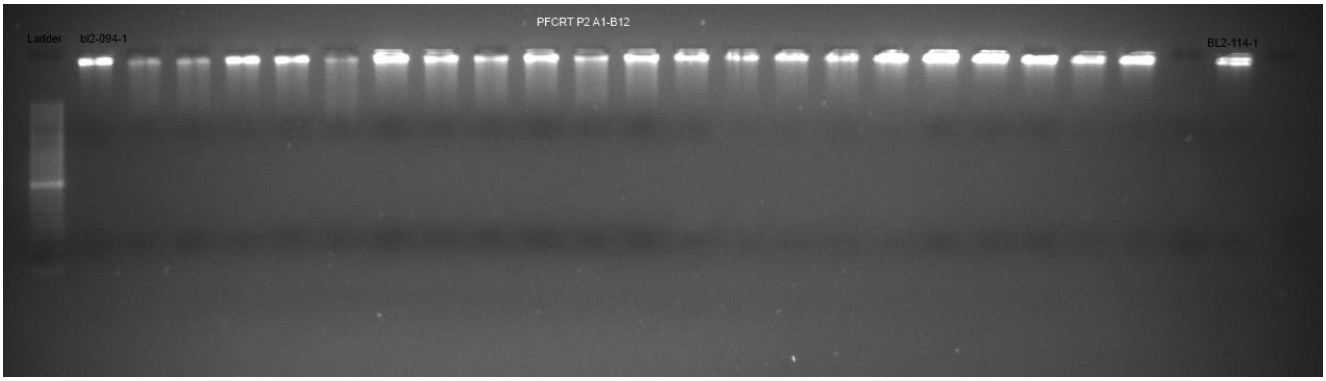
4A. Gel Electrophoresis Photos of Round 1 & 2 Pfcrt PCR products.

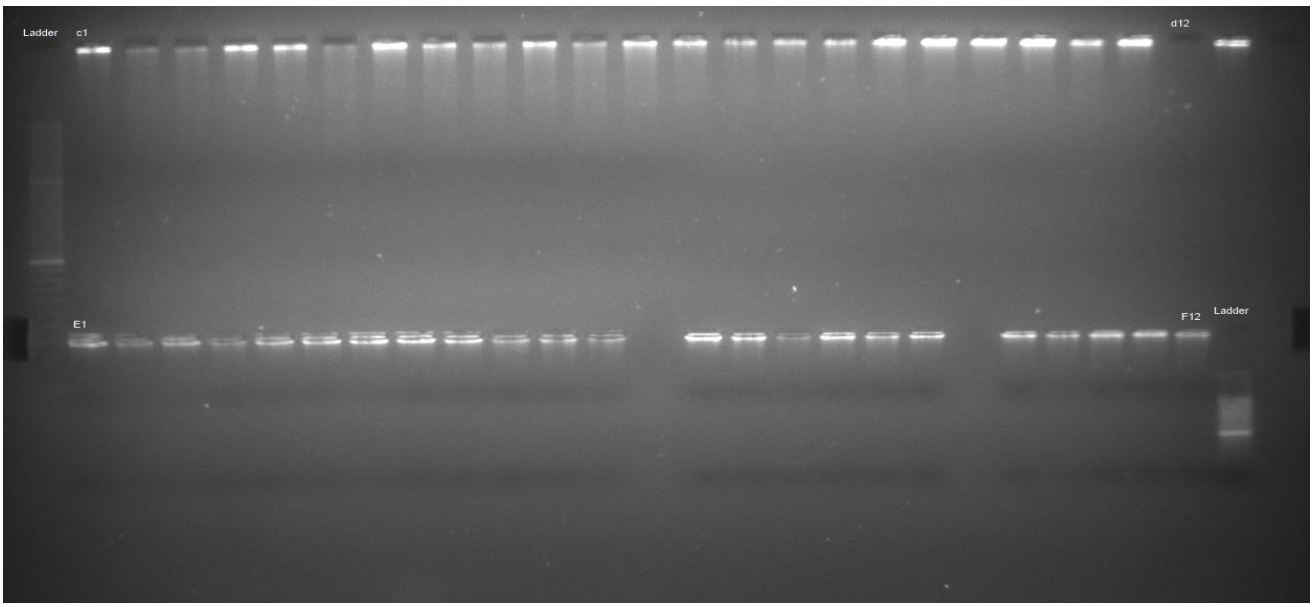




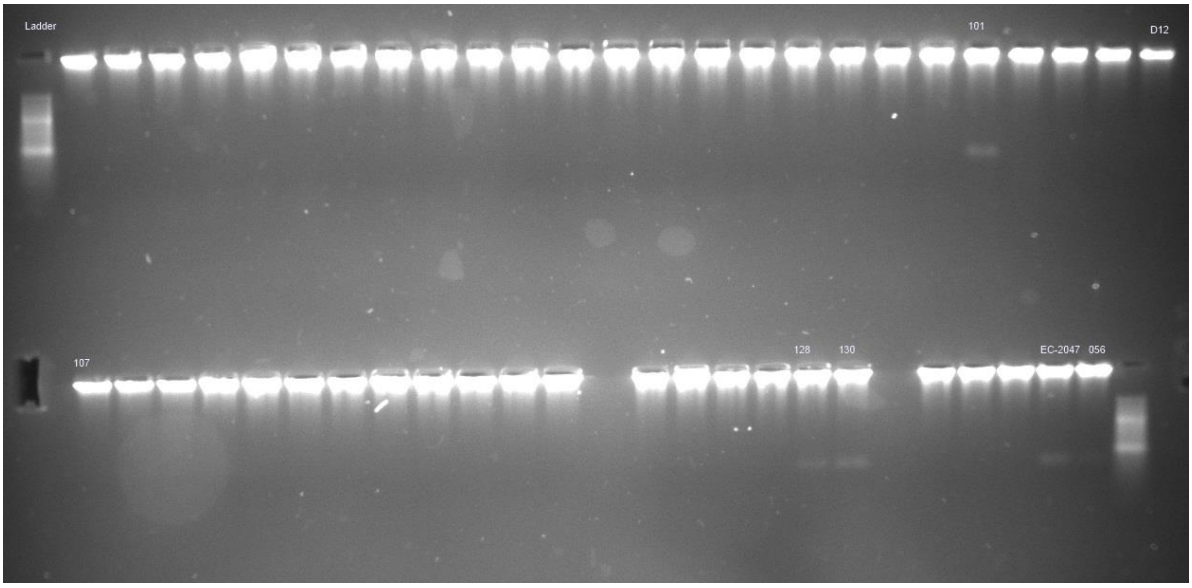


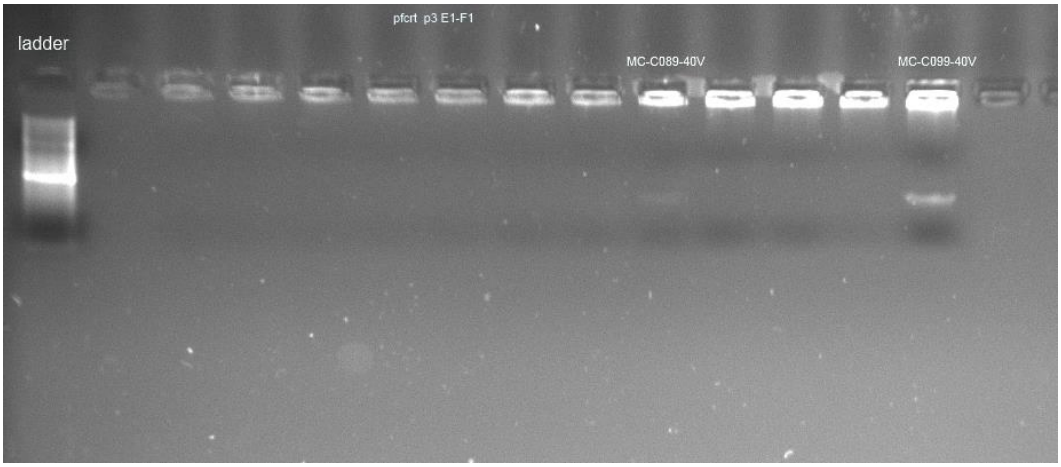

Ladder
073
078
080
083


PFCRT P2 A1-B12
Ladder
bl2-094-1
BL2-114-1

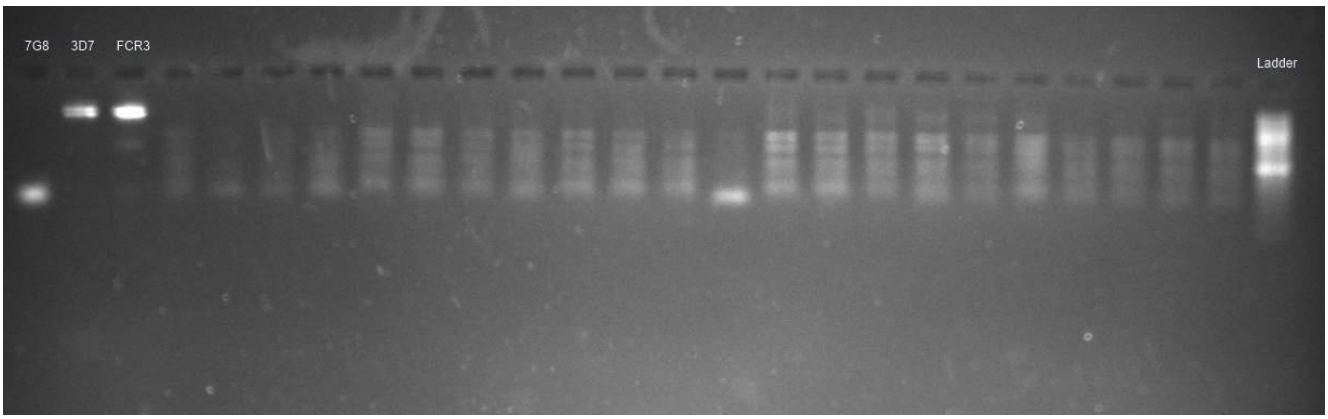

Ladder
c1
d12
E1
F12
Ladder

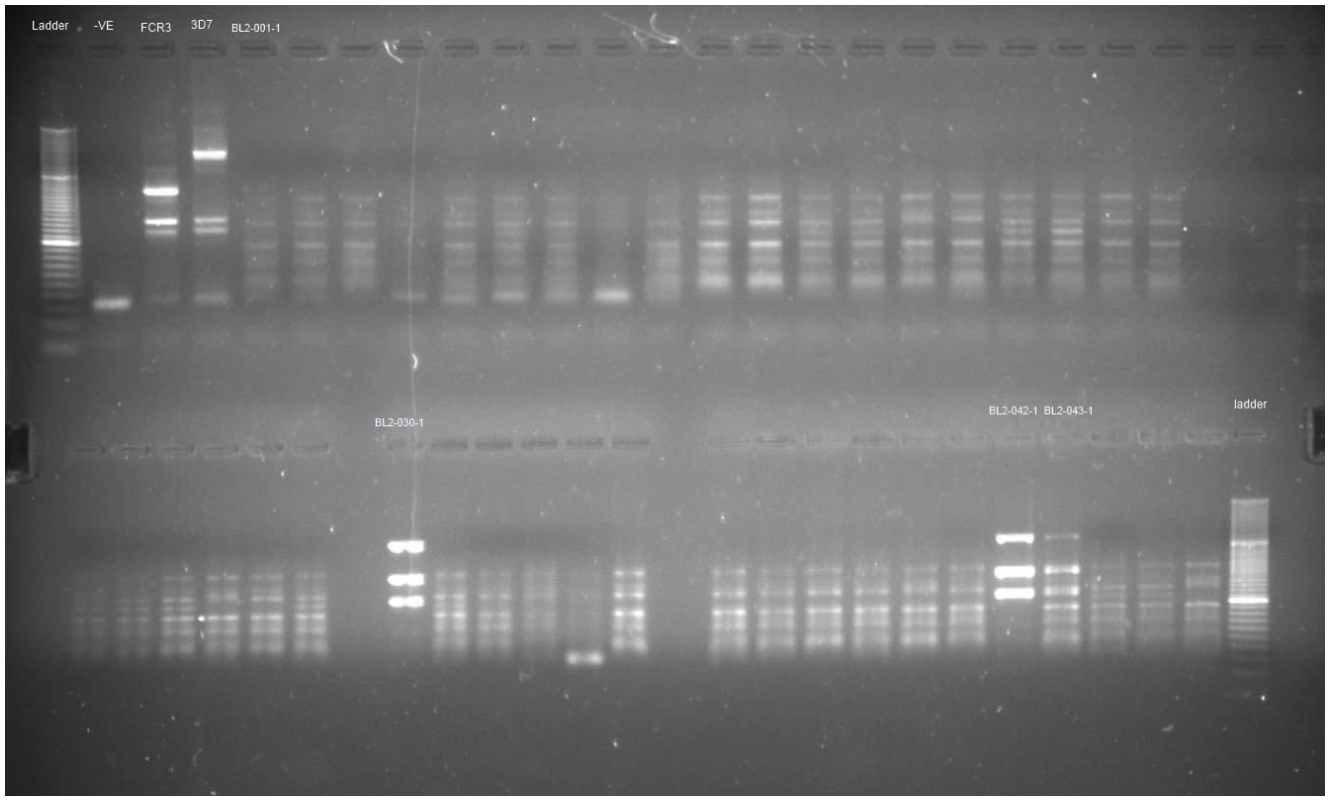
Master Mix 2

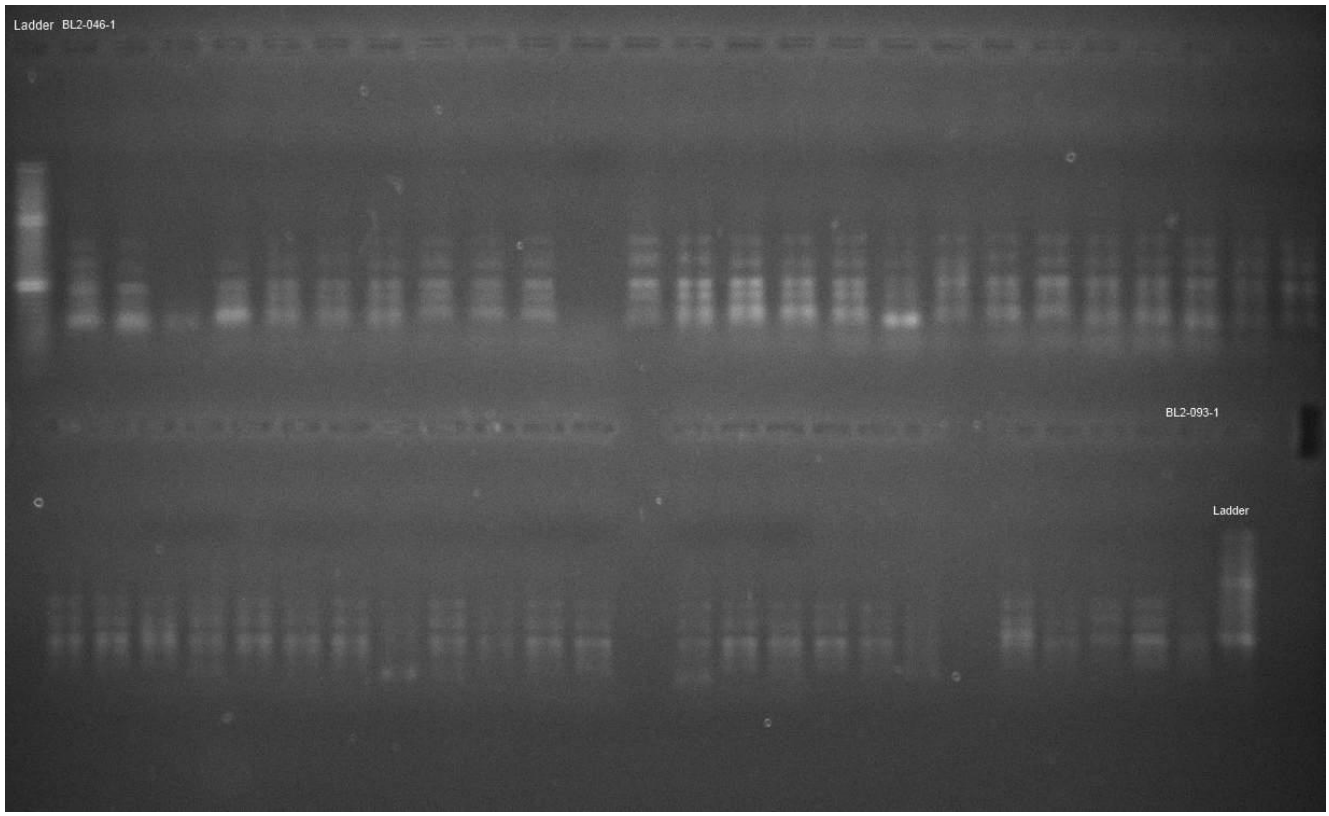


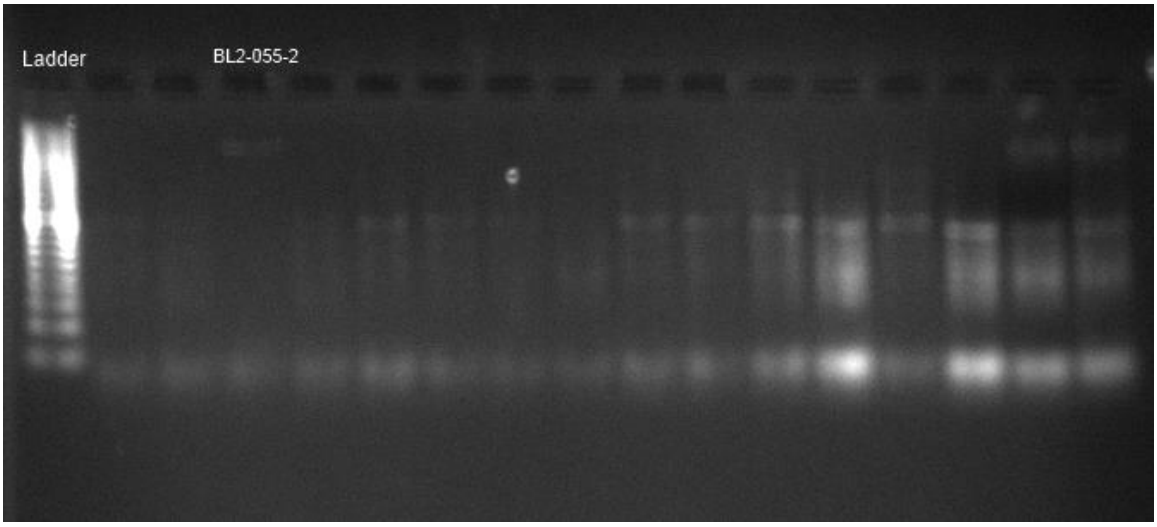


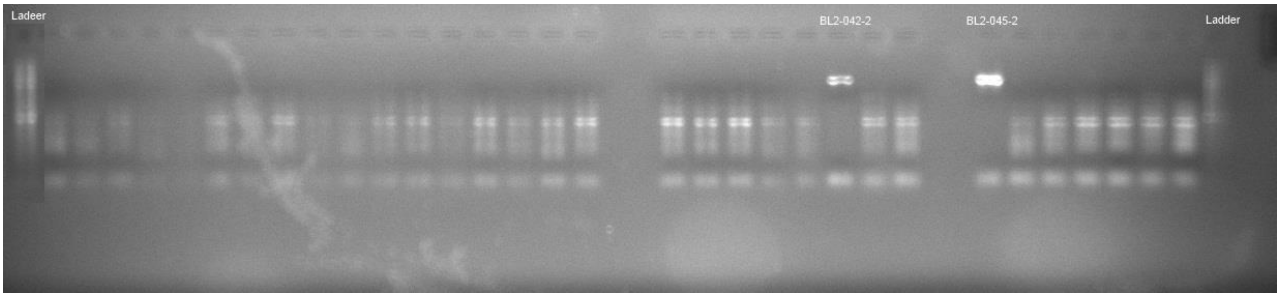
4B. Gel Electrophoresis Photos of Round 1 & 2 Pfmdr1 PCR products.

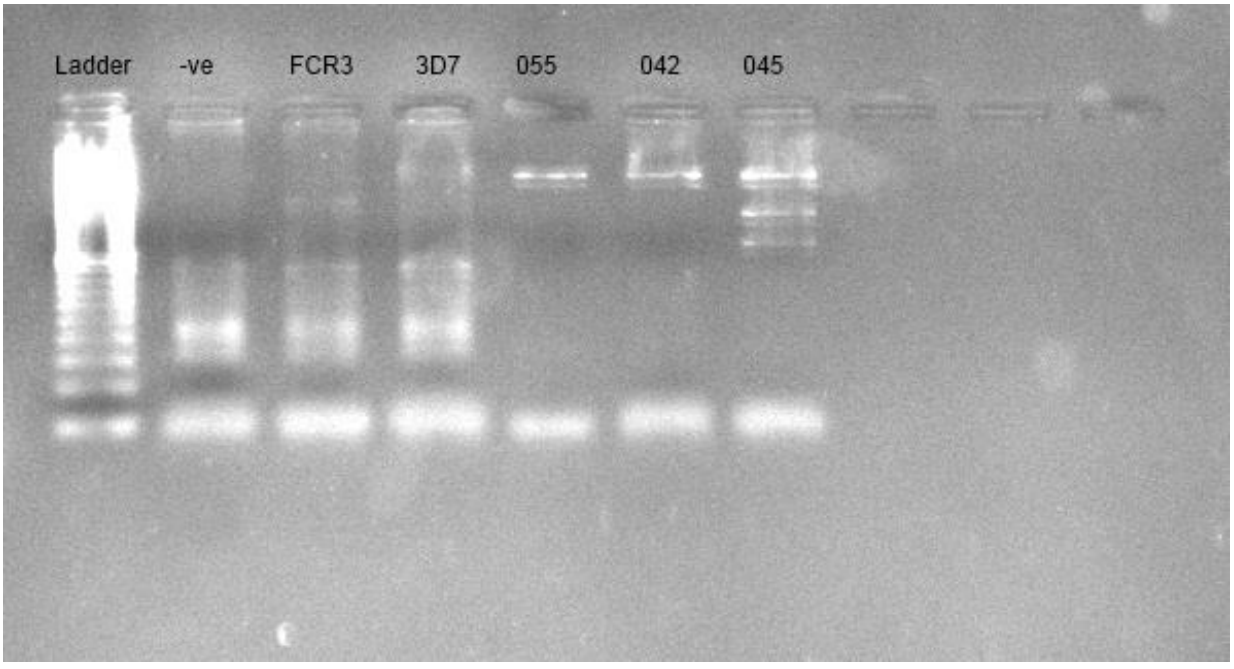



Ladder
-VE
FCR3
3D7
BL2-001-1
BL2-030-1
BL2-042-1
BL2-043-1
ladder

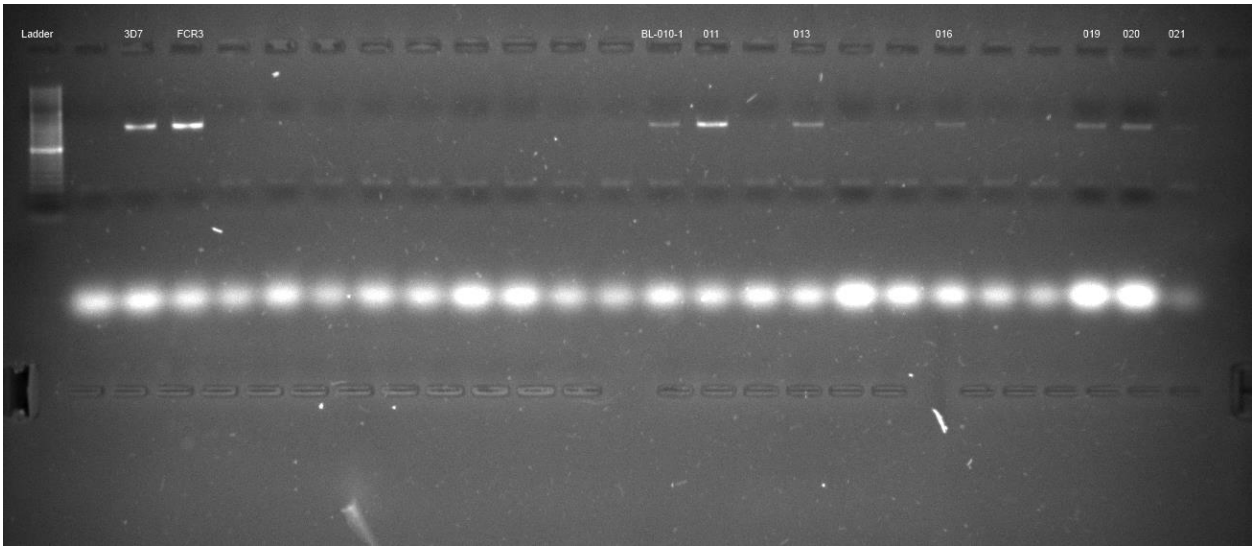

Ladder
BL2-046-1
BL2-093-1
Ladder

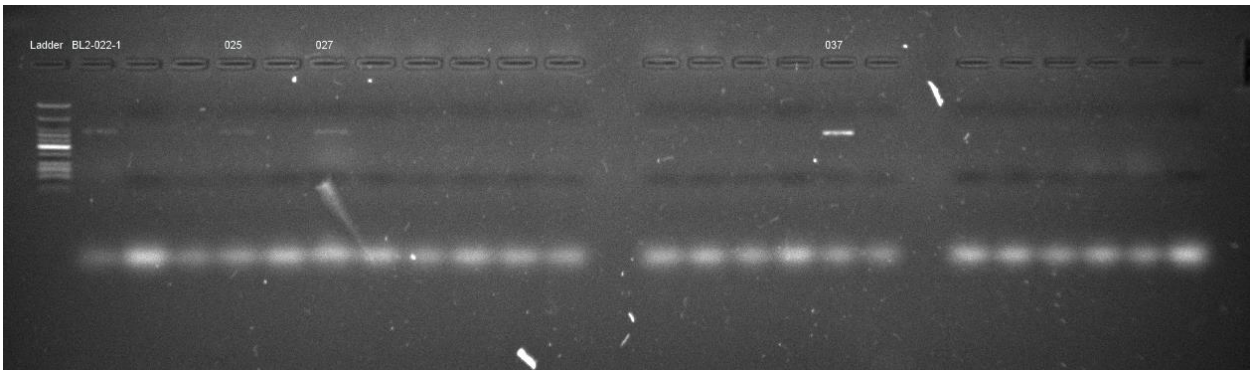

Ladder
BL2-055-2

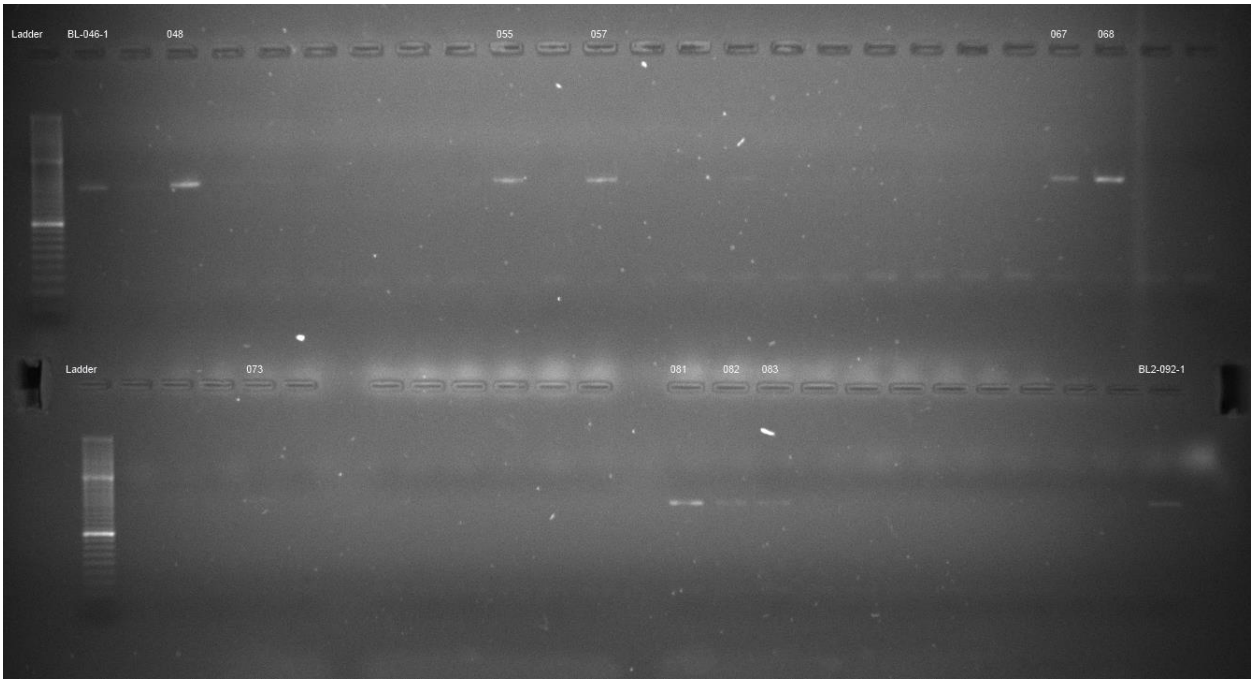

Ladeer
BL2-042-2
BL2-045-2
Ladder

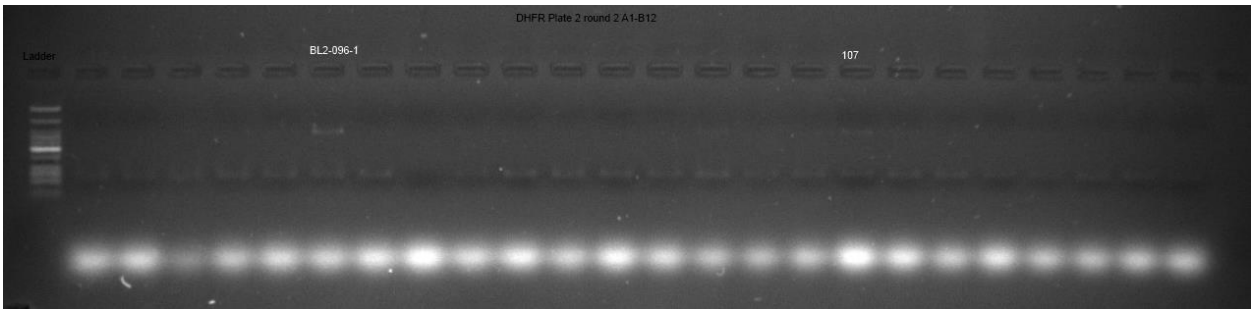

Ladder
-ve
FCR3
3D7
055
042
045

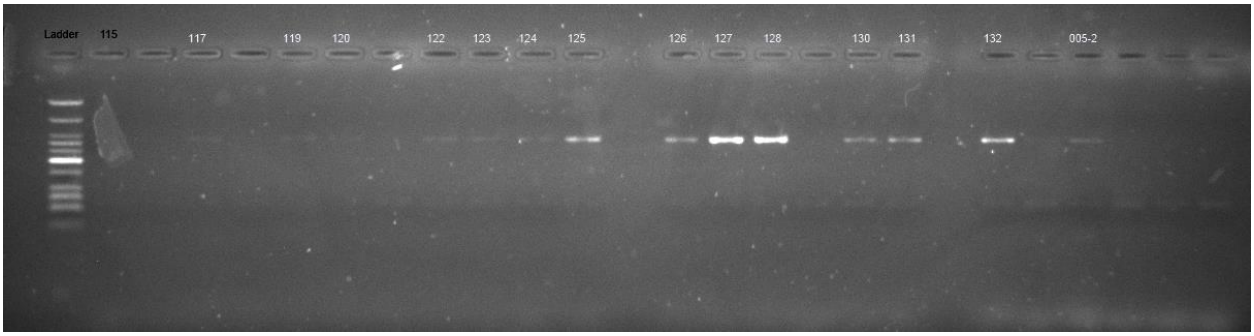
4C. Gel Electrophoresis Photos of Round 1 & 2 Pfdhfr PCR products.

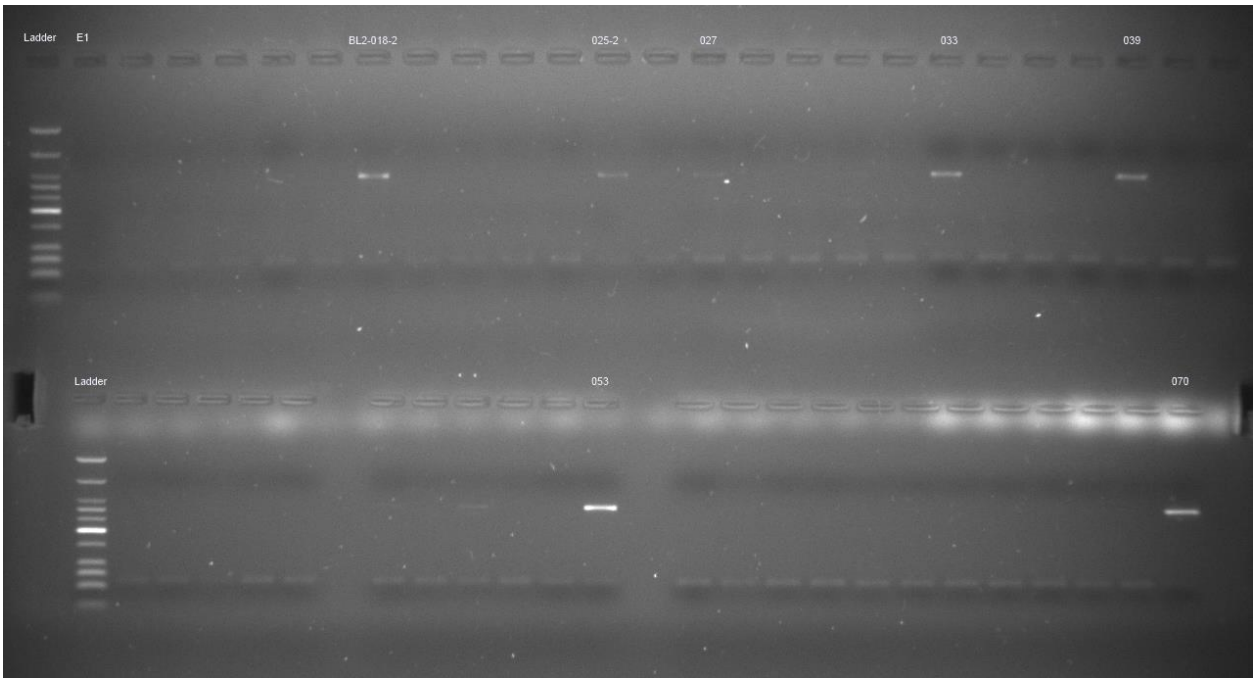


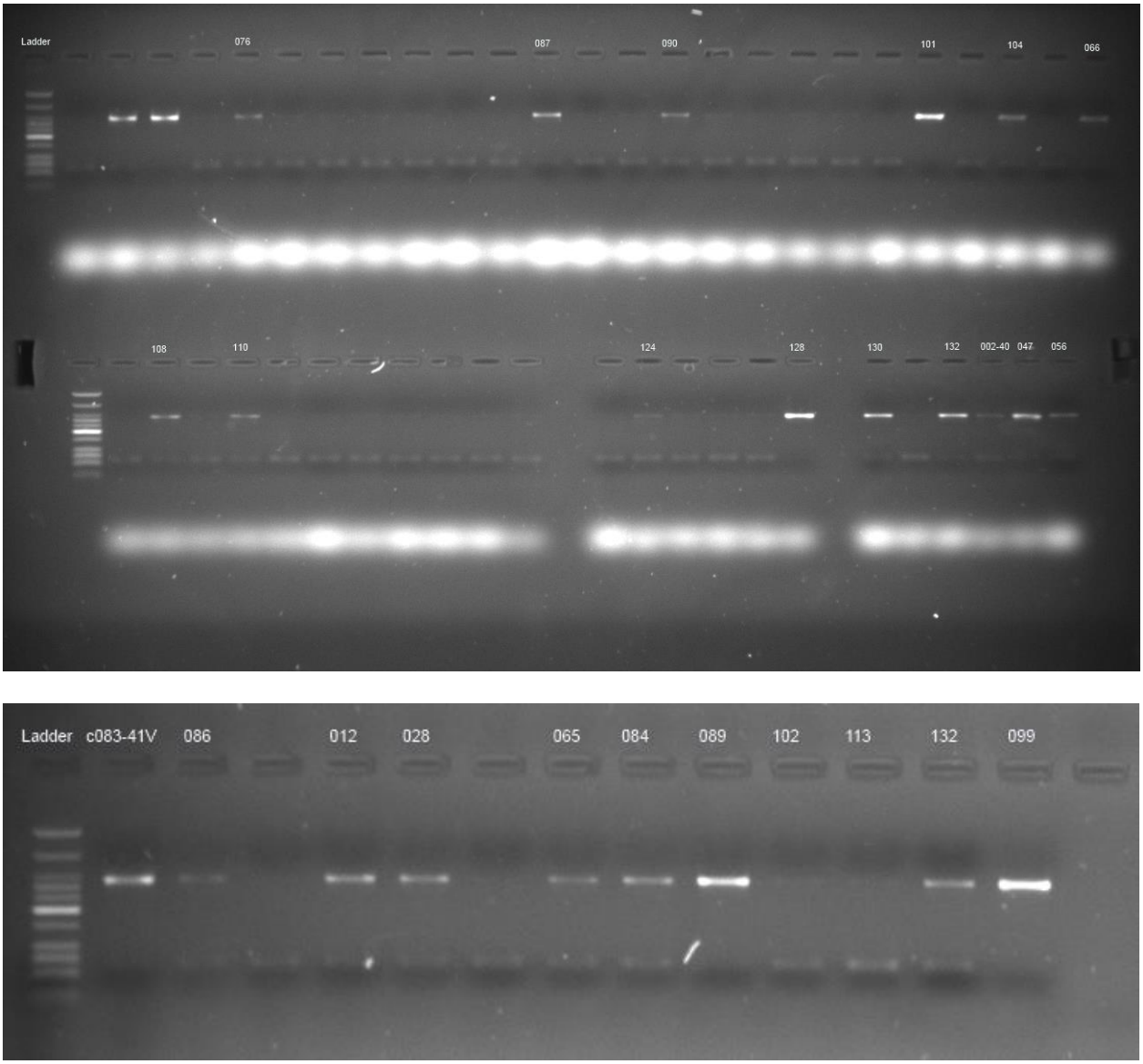




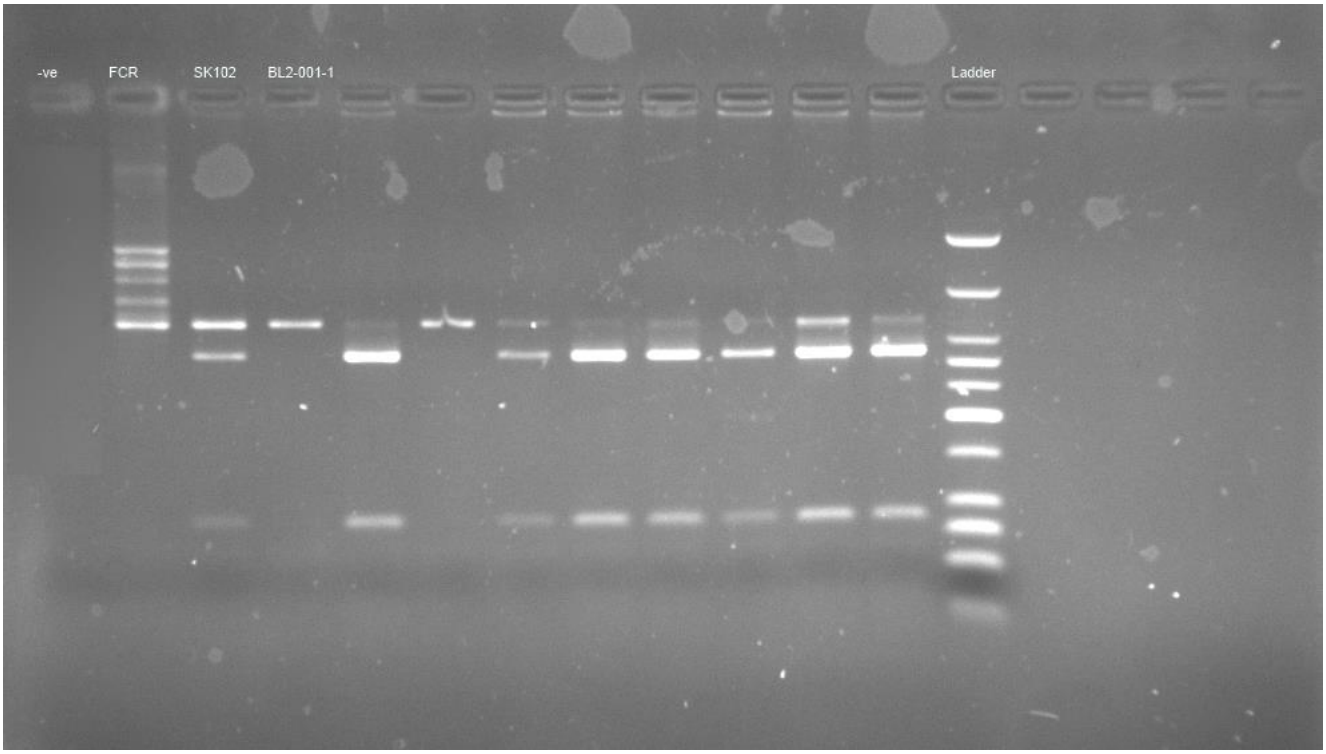

DHFR Plate 2 round 2 A1-B12
Ladder
BL2-096-1
107

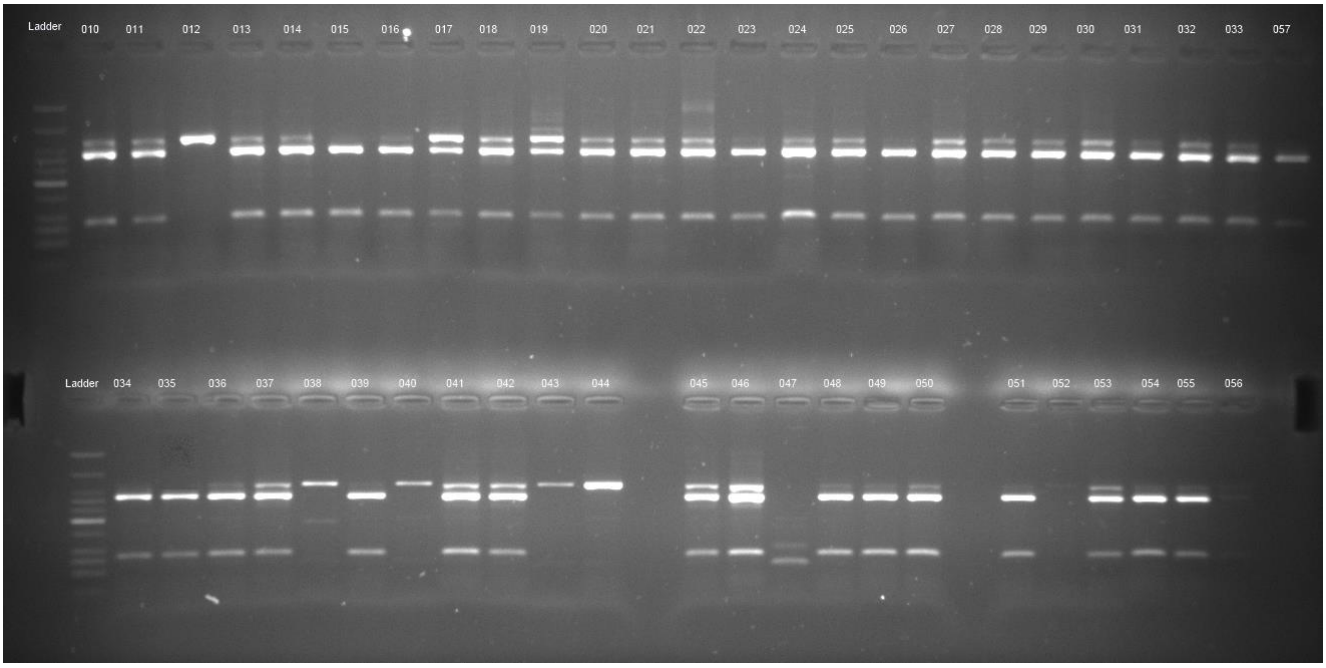

Ladder
115
117
119
120
122
123
124
125
126
127
128
130
131
132
005-2

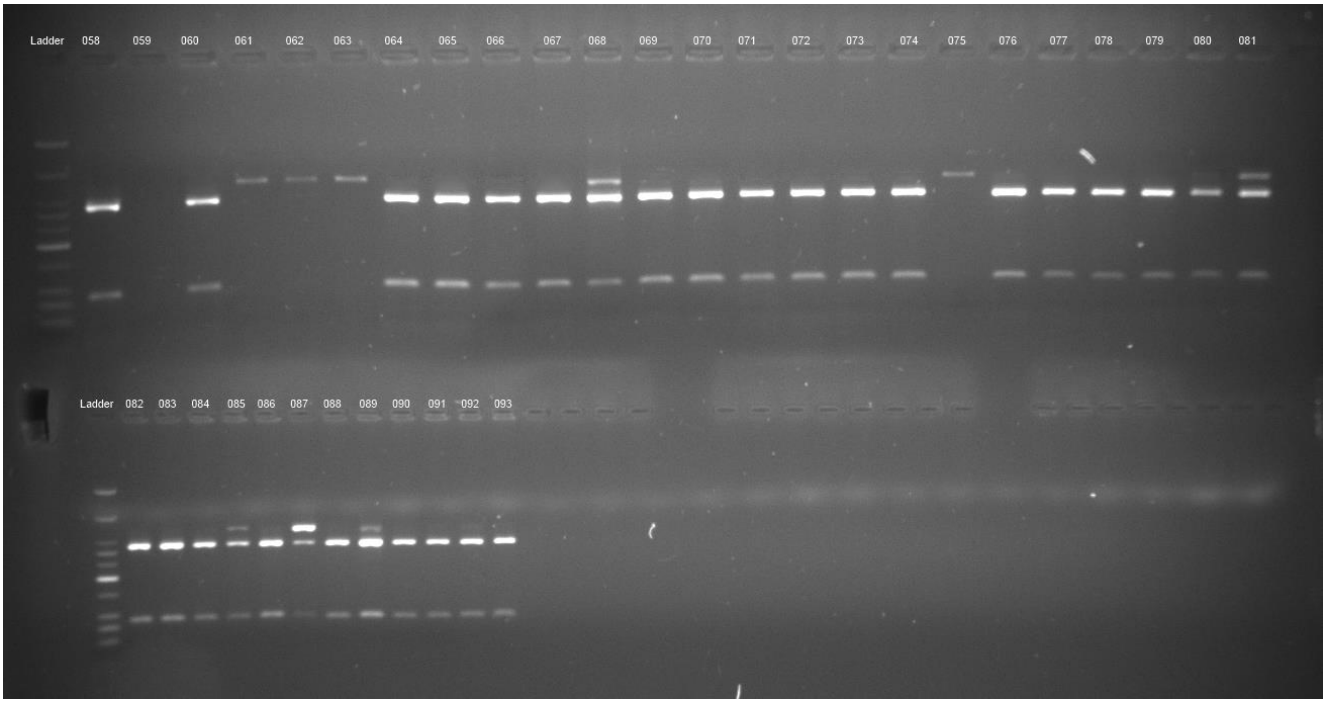

Ladder
E1
BL2-018-2
025-2
027
033
039
Ladder
053
070



4D. Gel Electrophoresis Photos of Round 1 & 2 Pfdhps PCR products.


-ve
FCR
SK102
BL2-001-1
Ladder


Ladder 010 011 012 013 014 015 016 017 018 019 020 021 022 023 024 025 026 027 028 029 030 031 032 033 057
Ladder 034 035 036 037 038 039 040 041 042 043 044 045 046 047 048 049 050 051 052 053 054 055 056


Ladder 058 059 060 061 062 063 064 065 066 067 068 069 070 071 072 073 074 075 076 077 078 079 080 081
Ladder 082 083 084 085 086 087 088 089 090 091 092 093

APPENDIX 5: RESEARCH APPROVAL FOR THE STUDY

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840, Tel: (02) 722541,Fax(02) 720030, KEMRI, NAIROBI, Kenya, E-mail: Kemrilib @ken.healthnet.org

12/8/02

KEMRI/RES/7/3/1

7th August, 2002

Dr. A. M. Moormann
CVBCR

FORWARDED
DIRECTOR
VECTOR BIOLOGY & CONTROL RESEARCH CENTRE

Thro'
Director
CVBCR
KISUMU

12/8/2002

Dear Sir,

RE: SSC PROTOCOL NO. 666 "Immunologic studies investigating the association between malaria, Epstein Barr Virus and the prevalence of endemic Burkitt's lymphoma" by A. M. Moormann et al

This is to inform you that during the 96th Ethical Review Committee meeting held on 6th August, 2002, the above protocol was discussed and granted approval for you to embark on your studies.

G.A.O.SEKO
FOR: SECRETARY
KEMRI/NATIONAL ETHICAL REVIEW COMMITTTEE