MOLECULAR MARKERS ASSOCIATED WITH AMODIAQUINE RESISTANCE IN RODENT MALARIA PARASITE *Plasmodium berghei* ANKA

LOISE GATHIGIA NDUNG'U

MB300-0010/15

A THESIS SUBMITTED TO PAN AFRICAN UNIVERSITY INSTITUTE OF SCIENCE, TECHNOLOGY AND INNOVATION IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY OF THE PAN AFRICAN UNIVERSITY.

2017

DECLARATION

This thesis is my original work and has not been submitted or presented for examination or any other award in any other institution.

Loise Ndung'u **Reg No: MB300-0010/15**

Signature …… Date ……

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

1. **Dr. Daniel Kiboi**

   Department of Biochemistry, JKUAT

   Signature……. Date……

2. **Dr. Beatrice Irungu**

   Centre for Traditional Medicine and Drug Research, KEMRI, NAIROBI

   Signature …….. Date ……

3. **Prof. Esther Magiri**

   Department of Biochemistry, JKUAT.

   Signature…… Date……

DEDICATION

This thesis is dedicated to my lovely husband Sammy, my beautiful daughter Tiffany and the entire Ndung'u family.

ACKNOWLEDGEMENT

Throughout my entire Msc training period, I have met quite a number of people who have been very instrumental in the successful completion of this thesis. Thanks you all for your endless support that you extended to me. I feel greatly indebted to you all.

Special appreciation goes out to:

My supervisors: **Prof. Magiri:** Working on this research project under your intellectual guidance has been a delightful learning experience. Your criticism, insights and encouragement ensured the successful completion of this work. **Dr. Kiboi:** I was greatly humble to work with in this project. You introduced me to this exciting world of research and have constantly mentored me. Your selfless sacrifice especially during the molecular part cannot go unmentioned. Your unwavering support, encouragement, criticism and promptness when called upon ensured the successful completion of this work. **Dr. Beatrice:** Your guidance, criticism and endless support ensured the successful completion of this work. God bless.

PAUISTI, AU and **JICA**- This work would not have been done without your financial support. Feel appreciated.

KEMRI-CTMDR staff: From the center director; **Dr. Mwitari,** to the laboratory and technical staff. You made my stay at KEMRI awesome.

KEMRI –Animal house staff: From **Mr Lukas**, **Mr. Steve** and **Ms Lucy**. Thank you for ensuring constant supply of the mice. God bless.

My husband and daughter; you were the cheering squad that kept me on going. Love you all. My mum, dad and siblings: your encouragement and prayers can't go unmentioned. God bless you

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin Combined Therapy
AL	Artemether lumefantrine
AO	Acridine Orange
AQ	Amodiaquine
AQr	Amodiaquine resistant
AQs	Amodiaquine sensitive
CDC	Centre for Disease Control and Prevention
CRT	Chloroquine Resistance Transporter
CNS	Central Nervous System
CQ	Chloroquine
CTMDR	Centre for Traditional Medicine and Drug Research
DEAQ	Desethylamodiaquine
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribose Nucleic Acid
DT	Day Test

ED	Effective Dose
IFA	Immuno Fluorescence Antibody
ITN	Insect Treated Nets
KEMRI	Kenya Medical Research Institute
MDR	Multi Drug Resistance
MQ	Mefloquine
NHE	Na^+/H^+ Exchanger
PCR	Polymerase Chain Reaction
PQ	Piperaquine
PRBC	Parasitized Red Blood Cells
PRQ	Primaquine
QBC	Quantitative Buffy Coat
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
SP	Sulphadoxine Pyrimethamine
UBP	Deubiquitinating Protease
VP2	V-type/H^+ Pyrophosphatase-2
WHO	World Health Organization

ABSTRACT

Due to the high genetic plasticity of *Plasmodium falciparum* to evolve resistance to single drug, W.H.O recommended the use of Artemisinin combination therapies (ACT) for treatment of uncomplicated *P. falciparum* malaria in Sub- Saharan Africa. To date, the combination of amodiaquine-artesunate is the first or second line of choice for treatment of uncomplicated malaria in Africa. Mode of action of amodiaquine (AQ) is predicted to be similar to that of chloroquine (CQ), thus they may share similar resistance mechanisms. The main objective of this study was to investigate the markers associated with AQ resistance in *P.berghei*. To this effect, resistant parasites had to be selected. The study used rodent malaria parasite *Plasmodium berghei* ANKA as a surrogate model to *P.falciparum*. The study interrogated mechanism of AQ resistance four ways: i) resubmission of parasite to AQ pressure for a further 16 passages, determined the resistance levels after every 4 passages and the stability of the resistant lines by storing at -80 degrees for a month. ii) determined cross resistance profiles of the resistant line against chemically and mechanistically related and unrelated antimalarial drugs iii) assess quinolone resistance suspect gene multi-drug resistance gene-1 (*mdr1*), chloroquine resistance transporter (*crt*), deubiquitinating protease 1 (*ubp*) and *kelch13* by PCR and sequencing iv) evaluated the expression profiles of putative transporter V-type/H^+ pyrophosphatase-2 (*vp2*), *mdr1*, Ca^{2+}/H^+antiporter (*cvx1*) and Na^+/H^+ exchanger (*nhe1*) by qRT – PCR. From the results obtained, the effective dosage that reduced 99% of parasitaemia (ED_{99}) of sensitive line and resistant line were 5.05mg/kg and 20.73mg/kg respectively. After freezing at -80 degree for at least one month, the resistant parasite remained stable with an ED_{99} of 18.22. This study further shows that AQ resistant phenotypes are cross resistant to chloroquine (6 fold), artemether (10 fold), primaquine (5 fold), piperaquine (2 fold) and lumefantrine (3 fold), suggesting they are

‘’multidrug resistant phenotype’’. Sequence analysis for single nucleotide polymorphisms (SNPs) in *P. berghei chloroquine resistant transporter* (*Pbcrt*), *P. berghei multidrug resistance gene 1* (*Pbmdr1*), *P. berghei deubiquitinating enzyme 1* (*Pbubp1*) and *P. berghei Kelch13 domain* (*Pbkelch13*) however revealed no SNPs. Polymorphisms in these genes is associated with quinoline and artemisinin resistance suggesting AQ resistant phenotype is controlled by other unknown variants. This study further provides, evidence that AQ resistance is associated with high mRNA transcripts in resistance compensatory and modulatory genes; *Pbmdr1*, Ca^{2+}/H^{+} antiporter (*vcx1*), *V-type H+ pumping pyrophosphatase 2* (*vp2*) and *sodium hydrogen ion exchanger1* (*nhe1*). In conclusion, this study was able to develop stable multidrug resistant *P.berghei* by continuous submission of parasites to AQ drug pressure for 36 passages. The study further, reveals that amodiaquine is associated with cross resistance in LM, CQ. PQ, PMQ and ATM. Increased transcript level of *mdr1, vp2, cvx1* and *nhe1* is associated with amodiaquine resistance in *P.berghei* ANKA. The study further reveals that *mdr1, crt, ubp1* and *kelch 13* domain are not associated with amodiaquine resistance in *P. berghei*. This study therefore recommends that drug selection of the parasite should be continued to acquire a highly stable resistant *P.berghei*. The study further recommends more cross resistant studies using different classes of antimalarial drugs to increase the knowledge of resistance mechanism among different antimalarial drugs. A whole genome sequencing and transcriptome profiling of AQ resistant *P.berghei* should be carried out. This will reveal any novel mutation in the unknown causal gene that may be associated with amodiaquine resistance. To further the understanding of amodiaquine resistance, the validation of the suspected genes should be done using PlasmoGEM resources as well as CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats).

CHAPTER ONE: INTRODUCTION

Drug resistance remains a bottleneck in elimination of malaria. To date, the human malaria parasite *Plasmodium falciparum* has evolved mechanisms for evasion of drug action to all available antimalarial drugs (Miotto *et al.,* 2015). From the 1940s up to the 1990s, chloroquine (CQ) was the mainstay of malaria therapy worldwide (Randall 2014). The emergence of *Plasmodium falciparum* resistant isolates was first reported in South East Asia and south America in the 1950s (Peters, 1971; Fidock *et al*., 2000) and by 1970s, CQ was no longer effective in these parts of the world. In Africa, CQ resistant isolates only emerged in the 1970s. However, within 10years, the level of resistance to CQ had risen rapidly (Peters, 1971; Fidock *et al*., 2000), both in Southern and Eastern Africa (Bloland *et al*., 1999). In 1993, Malawi was the first African country to replace CQ as the first line treatment of uncomplicated malaria with the antifolate combination sulphadoxine/ pyrimethamine (SP) (Kublin *et al*., 2003). In 1999, Kenya also replaced CQ with Sulphadoxine/Pyrimethamine. Other countries such as Uganda and Tanzania followed suit soon after (Eriksen *et al*., 2005; Kamya *et al*., 2002). However, the *P. falciparum* soon developed resistance to SP in regions such as Cambodia border, S. Asia (Kublin *et al*., 2003).

Due to the rapid emergence of resistance to single drug treatment, WHO recommended the use of Artemisinin combination Therapy (ACT) as the first line treatment for *Plasmodium falciparum* malaria in all endemic regions in sub Saharan Africa (WHO 2006). The artemisinin based combination therapies recommended for the treatment of *P.falciparum* malaria include: artemether–lumefantrine (AL), amodiaquine-artesunate (AQ-ASN), artesunate-mefloquine (MQ-

ASN), artesunate-sulfadoxine-pyrimethamine (ASN-SP) and dihydroartemisinin-piperaquine (WHO 2006).

Amodiaquine (AQ) in combination with artesunate has been reintroduced as a first line treatment of uncomplicated malaria in countries such as Nigeria, Burundi, and Chad among others (Eastman *et al*., 2009). The rationale behind this combination is the rapid reduction of parasite biomass by artesunate which is a short acting drug while the AQ stays longer to clear the remaining parasites (WHO 2006). In high malaria transmission settings, AQ will be the primary drug exerting selection pressure, thus higher risk to development of resistance especially in presence of high CQ resistant parasites.

To understand mechanisms of resistance, AQ resistant parasites need to be established. One way of selecting AQ resistance line is to employ *in vitro* method using *P. falciparum.* However, this approach is expensive and difficult to establish stable resistant parasite for genotyping (Nzila and Mwai, 2010). Alternatively, establishing drug resistant parasite using rodent malaria *in vivo* is relatively simple and straight forward to attain stable resistant lines (Carlton *et al*., 2001). Indeed genes associated with the mechanisms of resistance to many antimalarials in *P. falciparum* have correlated with those in *P. berghei* (Carlton *et al*., 2001). Furthermore, atovaquone, SP and mefloquine resistance in *P. berghei* has shown correlation to resistance in *P. falciparum* (Gervais *et al*., 1999; Carlton *et al*., 2001). *P.berghei* was thus used as surrogate model to *P.falciparum*.

Initial studies by Kiboi, (2008) and Langat, (2010) initiated selection of AQ resistance using a rodent malaria parasite *P. berghei* ANKA however resistance was low and thus the markers were not investigated. The mechanism of AQ resistance since its use as a therapeutic antimalarial drug has not been controversial. This study thus investigated the role of chloroquine (and other

quinolone drugs) resistance and compensatory genes in conferring resistance to AQ in *P. berghei*. These genes are: *mdr-1* (Sisowath *et al*., 2007), *crt* (Mwai *et al*., 2009), *ubp1 (*Hunt *et al.*, 2007*), kelch 13 (*Miotto *et al.*, 2015*), nhe1*, *vp2* and *cvx1* (Jiang *et al*., 2008).

1.0 Problem Statement

Rapid emergence of antimalarial drug resistance has hindered the malaria elimination strategies. In 2015, there were an estimated 212 million cases of malaria worldwide (range 148 –304 million) and an estimated 429 000 deaths (range 235 000–639 000) (WHO 2016). 92% of all malaria deaths occur in Africa. Moreover, an estimated 292 000 African children died before their fifth birthday due to malaria (WHO 2016). Due to the emergence of *P. falciparum* resistance to monotherapy, WHO has recommended artemisinin combination therapy (ACT) as first line or second line treatment of uncomplicated malaria (WHO 2006). Among the ACTs is amodiaquine artesunate combination (AQ-ASN). However, the mismatches in pharmacokinetic in AQ-ASN combination means that the selection pressure will primarily be exerted by AQ. Consequently, emergence of AQ resistance would render AQ-ASN ineffective and thus complicate elimination of malaria using chemotherapy. To date the full definition of AQ resistance remains to be understood or controversial. Thus there is need to understand the markers associated with AQ resistance.

1.1 Justification of The Study

There is clear correlation between the use of ineffective antimalarial drug and malaria associated mortality especially in endemic countries (Guerin *et al*., 2002). By uncovering the markers associated with resistance, it would be possible for monitoring the emergence of resistance and thus directly impacting on policy and use of effective drug to cure malaria. To counter this

resistance problem the molecular targets as well as the genetic determinants of resistance to the existing drugs need to be fully understood. Such understanding can then provide a basis for tracking resistance mutations in natural infections, and eventually for designing new combination therapies. Furthermore, defining the mechanisms of action of existing drugs by finding their targets will facilitate the design of new drugs.

1.2 Objectives of The Study

1.2.1 General Objective

To investigate the markers associated with amodiaquine resistance in *P. berghei* ANKA.

1.2.2 Specific Objectives

1. To determine the ED_{50} and ED_{99} of amodiaquine against resistant and sensitive *P. berghei* ANKA.
2. To evaluate the cross resistance profile of amodiaquine resistant *P. berghei* ANKA against chemically and mechanistically related and unrelated antimalarial drugs.
3. To assess *Pbmdr1, Pbubp1*. *Pbkelch13* and *Pbcrt* genes for point mutations.
4. To analyse expression levels of resistance modulatory and compensatory genes, *Pbnhe1*, *Pbmdr1*, *Pbvp2* and *Pbcvx1* genes.

CHAPTER TWO: LITERATURE REVIEW

2.1 Global incidences of malaria

Malaria remains a major global health problem throughout Africa, Oceania, Asia and Latin America, covering over 90 countries (WHO 2012). In 2015, there were an estimated 212 million cases of malaria worldwide (range 148 –304 million) and an estimated 429 000 deaths (range 235 000–639 000 (WHO 2015: CDC 2015).

The geographical distribution of the four human parasites species is variable and dependent on season, endemicity and vector distribution. Majority of infections and almost all deaths in Africa are caused by *P. falciparum*, the most dangerous of the four human malaria parasites (WHO 2010). The most effective malaria vector, the mosquito *Anopheles gambiae* is widespread in Africa and difficult to control (Marianne *et al*., 2010; WHO 2006).

The malaria incidences are further increased by parasite resistance to available anti-malaria drugs. The sulfadoxine-Pyrimethamine and chloroquine drugs that were largely used have almost been rendered ineffective by resistance to *P. falciparum* (Kublin *et al*., 2003; Bloland *et al*., 1999).

2.2 Human malaria species and Diseases

The species infecting humans are: *P. falciparum, P. vivax, P. ovale and P. malaria*. *P. falciparum* is found worldwide in tropical and subtropical areas, and especially in Africa where this species predominates (Marianne *et al*., 2010). *P. vivax* is found mostly in Asia, Latin America, and in some parts of Africa. Because of the population densities especially in Asia it is probably the most prevalent human malaria parasite. *P. ovale* is found mostly in Africa

(especially West Africa) and the islands of the western Pacific. *P. malariae*, is found worldwide, and is the only human malaria parasite species that has a quartan cycle (three-day cycle). (The three other species have a tertian, two-day cycle).

Plasmodium falciparum can cause severe malaria because it multiples rapidly in the blood, and can thus cause anemia (Marketa *et al*., 2015). *P. vivax* (as well as *P. ovale*) has dormant liver stages ("hypnozoites") that can activate and invade the blood ("relapse") several months or years after the infecting mosquito bite (Winstanley *et al*., 2004). P. ovale is biologically and morphologically very similar to *P. vivax*. However, though different from *P. vivax*, it can infect individuals who are negative for the Duffy blood group, which is the case for many residents of sub-Saharan Africa. This explains the greater prevalence of *P. ovale* (rather than *P. vivax*) in most of Africa**.** If untreated, *P. malariae* causes a long-lasting, chronic infection that in some cases can last a lifetime. In some chronically infected patients *P. malariae* can cause serious complications such as the nephrotic syndrome. (Winstanley *et al*., 2004).

2.3 Lifecycle of Plasmodium

The malaria parasite life cycle involves two hosts: human host and *Anopheles* mosquito. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host. Sporozoites then infect liver cells and mature into schizonts, which then rupture and release merozoites into the bloodstream. However, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later. After this initial replication in the liver (exo-erythrocytic schizogony), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony). Merozoites infect red blood cells. The ring stage trophozoites mature into schizonts, which

rupture releasing merozoites. At this stage, some parasites differentiate into sexual erythrocytic stages (gametocytes). Blood stage parasites are responsible for the clinical manifestations of the disease (Gabrielle and Manuel 2015).

Then the gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal and they replicate in the mosquito. This multiplication of the parasites in the mosquito is known as the sporogonic cycle. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes. The zygotes in turn become motile and elongated forming ookinetes which invade the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (Fig 2.1) (CDC 2016).

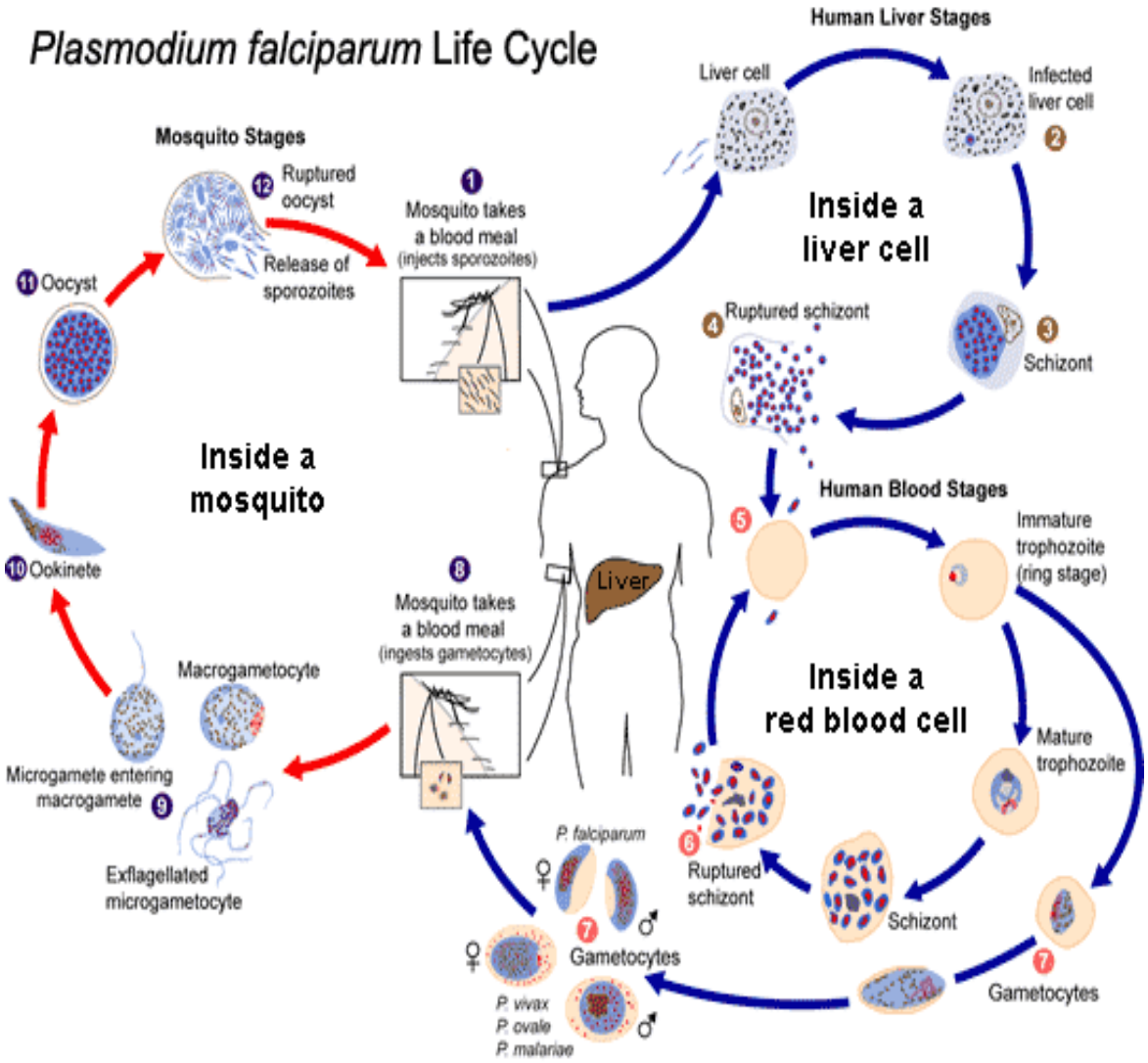


Fig 2.1: Lifecycle of *Plasmodium falciparum* (CDC 2016)

2.4 Malaria Control

Malaria control strategies are based on three approaches, the basic principles of malaria control focus on measures to prevent or reduce the anopheles mosquito.

2.4.1 Insecticide Treated Nets (ITNs)

The use of nets for protection against nuisance insects started in historical times (Lengeler, 2004). ITNs are a low cost and highly effective method of reducing the incidence of malaria (D'Alessadro, 2001). In Sub- Saharan Africa, several studies show that ITNs reduce morbidity

and mortality in pregnant women (Gamble *et al*., 2007). Insecticide Treated Nets (ITNs) aims at interrupting human-vector contact. They also kill vectors and reduce local transmission. They have also been shown to substantially reduce child mortality in malaria endemic areas of Africa (WHO, 2010). The ITNs thus reduces the pressure on antimalarials drugs which is very important in view of increasing drug resistant falciparum malaria parasites (Lengeler, 2004).

2.4.2 Vector Control by use of insecticides and repellants.

Vector control aims at reducing the anopheles breeding using insecticides and repellant application thus reducing the level of transmission. The use of chemical has not been successful across Africa and Latin America (Breman *et al*., 2006) due to the emergence of mosquito strains resistant to commonly used insecticides. While in S. E. Asia, there has been successful use of insecticides (Breman *et al*., 2006).

2.4.3 Vaccine Development

The development of an effective vaccine has remained elusive (Webster and Hill, 2003). The diversity of parasites' antigens expressed at different stages of the life cycle and immune evasion by intrinsic antigenic variation has hindered development of a vaccine (Florens *et al*., 2002). Advances in genomics have aided proteomics research in identifying potential vaccine candidates such as var genes encoding *P. falciparum* erythrocyte membrane protein (PfEMP), and rifin, stevor and clag gene products given their prominent role in malarial pathogenesis (Hoffman *et al*., 2002; Webster and Hill, 2003). A promising subunit vaccine, RTS, S/AS02A based on pre-erythrocytic antigen also induces partial protection in young African children against falciparum infection and a range of clinical illness (Alonso *et al*., 2004). Although the generation of initial vaccines will reduce the malaria burden, it will not render other control measures such as chemotherapy redundant (Moorthy *et al*., 2004). Moreover, with a safe,

effective and affordable vaccine being several years away (Greenwood, 2005), chemotherapy remains the most important means of controlling malaria (Ridley, 2002; White, 2004).

2.5 Diagnosis

2.5.1 Light Microscopic Examination

Simple light microscopic examination of Giemsa stained blood films is the most widely practiced and useful method for definitive malaria diagnosis (Warhurst *et al*., 1996). It is the gold standard method for definitive malaria diagnosis with advantages of differentiation between species, quantification of the parasite density and ability to distinguish clinically important asexual parasite stages (CDC 2016). However, this method relies on electricity thus may not be applicable in areas where there is no electricity. The laboratory technician also requires some training (WHO 2001).

2.5.2 Quantitative Buffy Coat Technique

Quantitative Buffy Coat (QBC) technique was designed to enhance microscopic detection of parasites and simplify malaria diagnosis (Clendennen *et al*., 1995). This method involves stain of parasite DNA in micro-hematocrit cubes with fluorescent dye e.g. acridine orange (AO) and its subsequent detection by epi-fluorescent microscopy. The QBC technique is a rapid and a sensitive test for the diagnosis of malaria (Adeoye *et al*., 2007; Barman *et al*., 2003; Salako *et al*., 1999; Pornsilapatip *et al*., 1990). Although QBC is simple, and reliable, it requires specialized instrumentation, is costly than convectional light microscopy and is inefficient in determining species and parasite numbers.

2.5.3 Antigen Detection Tests

This method is also called rapid or dipstick test. It uses rapid immuno-chromatographic techniques which have advantages in that no special equipment is required, minimal training needed, no electricity needed and the reagents are stable at ambient temperatures (Bell *et al*., 2006). However, this method has high pretest cost, does not allow one to differentiate between species, quantify the parasite density or even distinguish clinically important asexual parasite stages (Endeshaw *et al*., 2008). Furthermore, detectable antigen can persist for days after adequate treatment and cure; therefore, the test cannot adequately distinguish a resolving infection from treatment failure due to drug resistance, especially early after treatment (Ratmbosoa *et al*., 2008).

2.5.4 Serological Technique

Diagnosis of malaria using serological method is usually based on the detection of antibodies against asexual blood stages malaria parasites (She *et al*., 2007; Reesing 2005). One of the serological methods is Immuno-Fluorescence Antibody Testing (IFA). This method is a very reliable serological test for malaria since its very sensitive and specific (Sulzer *et al*., 1969; Reesing 2005). However, it is a very time consuming and subjective method. This method is useful in epidemiological surveys, for screening potential blood donors (Mungai *et al*., 2001).

2.5.5 Molecular Techniques

2.5.5.1 Polymerase Chain Reaction (PCR)

Detection of parasite genetic material through molecular techniques such as polymerase-chain reaction (PCR) techniques is becoming a more frequently used tool in the diagnosis of malaria, as well as the diagnosis and surveillance of drug resistance in malaria. Specific primers have

been developed for each of the four species of human malaria. One important use of this new technology is in detecting mixed infections or differentiating between infecting species when microscopic examination is inconclusive (Beck. 1999). Primary disadvantages to these methods are overall high cost, high degree of training required, need for special equipment, absolute requirement for electricity, and potential for cross contamination between samples (WHO 2001; Warkhust *et al*., 1996).

2.5.5.2 Loop Mediated isothermal amplification (LAMP) Techniques

With the LAMP method, unlike with *PCR*, there is no need for heat denaturation of the double stranded DNA into a single strand. It is a simple and inexpensive molecular malaria diagnostic test that detects the conserved 18S ribosome RNA gene of *P.falciparum* (Poon *et al*., 2006). It is characterized by the use of 4 different primers specifically designed to recognize 6 distinct regions on the target gene and the reaction process proceeds at a constant temperature using strand displacement reaction. Amplification and detection of gene can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 65°C). It provides high amplification efficiency, with DNA being amplified 10^9-10^{10} times in 15-60 minutes. Because of its high specificity, the presence of amplified product can indicate the presence of target gene. It also has high sensitivity and specificity to *P. vivax, P.ovale* and *P. malariae* (Han *et al*., 2007; Aonuma *et al*., 2008). Thus this method is easy, sensitive, quick and lower in cost compared to PCR. However, reagents require cold storage.

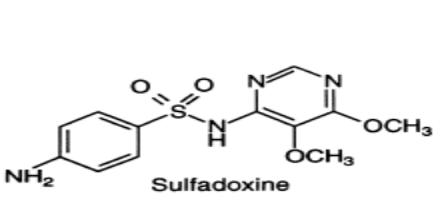
2.6 Malaria Chemotherapy

Antimalarial agents can be classified according to their modes of action against the different life cycle stages of the parasites and according to their chemical structure (Winstanley *et al*., 2004).

The main life cycle targets are the trophozoites and schizonts in the red blood cells, the schizonts in the liver and finally the gametocytes in red blood cells (Chiang *et al*., 2006; Robert *et al*., 2001). The anti-trophozoite/schizont drugs are mefloquine, chloroquine, quinine, halofantrine, sulfadoxine, amodiaquine and artemisinin (Robert *et al*., 2001). The liver schizont drugs are primaquine, lumefantrine and pyrimethamine while the anti-gametocyte drugs are chloroquine, artemisinin, amodiaquine, and quinine (Robert *et al*., 2001; Vangapandu *et al*., 2006).

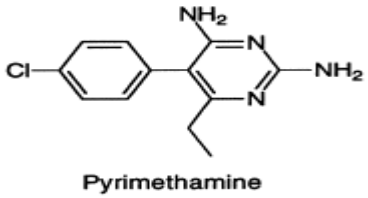
2.6.1 Antifolate

Antifolates are antimalarial drugs that inhibit the synthesis of parasitic pyrimidines and thus parasitic DNA (Robert *et al*., 2001). They attack all growing stages of the malaria parasite (Chiang *et al*., 2006). The only useful combinations of antifolate drugs are synergistic mixtures that act against parasite-specific enzymes. Type 1 antifolate such as sulfadoxine (Fig 2.2a) and dapsone (Fig 2.2c) inhibits dihydropteroate synthetase (Nzila, 2006). While type-2 antifolates; such as pyrimethamine (Fig 2.2b) and proguanil (Fig 2.2d) inhibit dihydrofolate reductase (Nzila, 2006). These compounds inhibit the synthesis of tetrahydrofolate co-factors essential in the synthesis of the pyrimidine deoxythymidylate for parasitic DNA. Due to a marked synergistic effect, a drug of the first group (type 1) is usually used in combination with a drug of the second one (type 2) such as SP (Sulphonamides-Pyrimethamine) (Nzila, 2006).



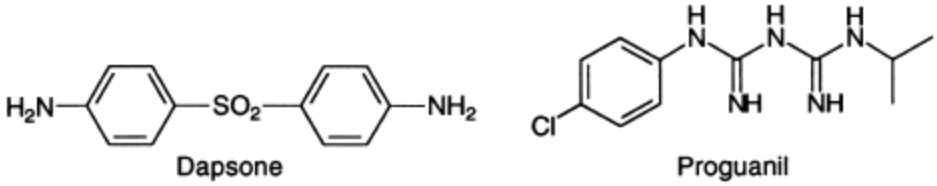
Sulfadoxine

(a)



Pyrimethamine

(b)

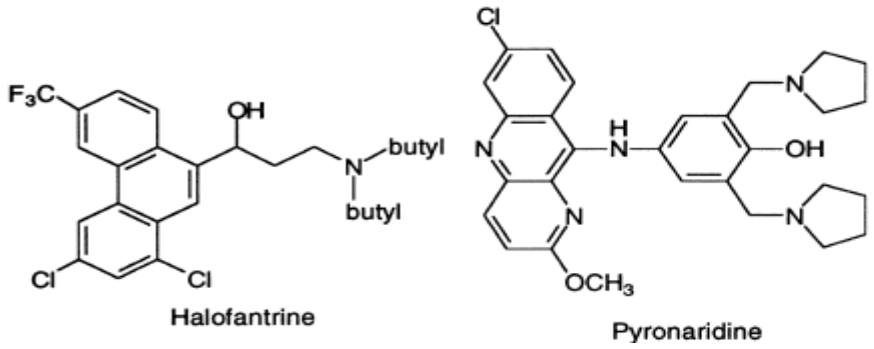


(c) (d)

Fig 2.2: chemical structures of sulfadoxine, Pyrimethamine, Dapsone and Proguanil (Robert *et al*., 2001)

2.6.2 Aryalcohols

The common aryalcohols include halofantrine (Fig 2.3a), pyronaridine and lumefantrine. Halofantrine, which is a blood schizonticide active against all malaria parasites, is effective against chloroquine-resistant malaria but cardiotoxicity has limited its use as a therapeutic agent (Robert *et al*., 2001). Lumefantrine has an elimination life of up to six days in malarial patients. Pyronaridine is an acridine derivative and a synthetic drug (Fig 2.3b) widely used in China (Robert *et al*., 2001). The Chinese oral formulation is reported to be effective and well tolerated but has a low oral bioavailability contributing to high cost of the treatment (WHO, 2006). Despite differences in the ring structure and side-chain substituents aryl-alcohols share the basic chemical characteristic, a hydroxyl group near the ring hypothesized to confer the antimalarial activity.



(a) (b)

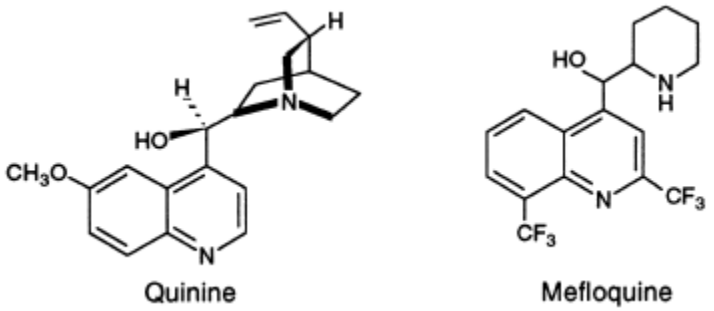
Fig 2.3: chemical structures of Halofantrine and Pyronaridine (Robert *et al*., 2001)

2.6.3. Quinoline Methanol

Quinoline antimalarials and related aryl alcohols owe their origins to quinine (Fig 2.4a). Quinine the active ingredient of cinchona bark has the longest period of effective use but there is a decrease in clinical response against *P. falciparum* in some areas (Robert *et al*., 2001). Moreover, quinine is associated with toxicity (such as nausea, and dizziness) and the three daily dosage administration over 7 days required, results in poor compliance (Baird, 2005).

Mefloquine (Fig 2.4b) is structurally related to quinine and its long half-life of 14–21 days has probably contributed to the rapid development of resistance (Robert *et al*., 2001). Mefloquine emerged as a successor to CQ in the 1980s but resistance emerged at the border between Thailand and Cambodia within a few years owing to widespread use of quinine (Duraisingh and Cowman, 2005). It has small therapeutic range and is less potent than chloroquine owing to relatively weak interaction with free heme (Winstanley *et al*., 2004).

Mefloquine remains a drug of choice for prophylaxis before traveling to malaria areas where chloroquine-resistant *P. falciparum* exists (Baird, 2005). Mefloquine is a potent long acting drug against falciparum resistant to 4-aminoquinolines and sulfa-pyrimethamine combinations.



(a) (b)

Fig 2.4: chemical structures of Quinine and Mefloquine (Robert *et al*., 2001).

2.6.4. Bisquinolines

Bisquinolines are compounds with two quinoline nuclei bound by a covalent aliphatic or aromatic link (Davis *et al*., 2005). Piperaquine is a potent bisquinoline antimalarial available as piperaquine base (PQ) or as its water-soluble tetra-phosphate salt, piperaquine phosphate (PQP) (Davis *et al*., 2005). PQP was first synthesized in 1960s by Shanghai Pharmaceutical Industry Research Institute in China and Rhone Poulenc in France (Hung *et al*., 2004). In China PQ replaced CQ as the first-line treatment for *P. falciparum* malaria from 1978 until the emergence of resistance in the 1990s (Tarning *et al*., 2005).

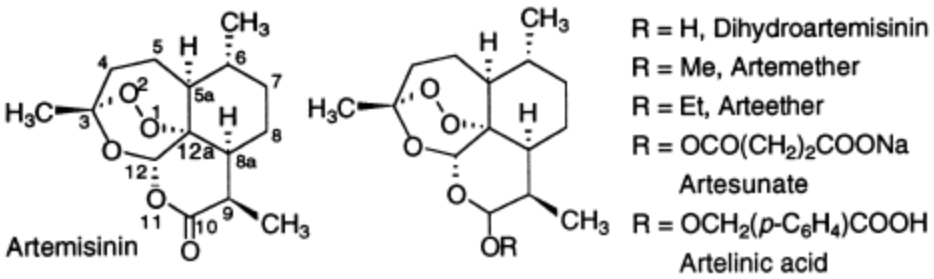
PQ is active mainly on late stage trophozoites and its mechanism of action is similar to 4-aminoquinolines mainly interfering with the heme polymerization. The drug has a long half-life of 17–25 days (Tarning *et al*., 2004). Because of its relatively low cost and good tolerability, PQ has enjoyed resurgence in clinical use as a coformulation with dihydroartemisinin in the product

Artekin ® (Holleykin Pharmaceuticals Co. Ltd, Guangzhou, China) (Basco and Ringwald, 2003; WHO, 2006).

2.6.5. Artemisinin and its derivatives

Artemisinin (Fig 2.5a) and its derivatives artesunate, arteether, artelinic acid, and dihydroartemisinin (active metabolite) (Fig 2.5b) are more potent blood schizonticidal activity, well tolerated and the most rapidly effective antimalarial drugs known (Robert *et al*., 2001). Artemisinin and its derivatives are toxic to malaria parasites *in vitro* at nanomolar concentrations, whereas micromolar concentrations are required for toxicity to mammalian cells. One reason for this selectivity is the enhanced uptake of the drug by the parasite (Ridley 2002; Robert *et al*., 2001). *P. falciparum*-infected erythrocytes concentrate dihydroartemisinin and artemisinin to more than 100-fold higher concentration than do uninfected erythrocytes. They are thought to exert their activity through interaction with heme preventing detoxification of heme by polymerization into hemozoin (O'Neill and Posner, 2011). Artemisinin and its derivatives thus, appear to be the best alternative for the treatment of severe malaria and artemether has been included in the WHO List of Essential Drugs for the treatment of severe multi drug resistant malaria (WHO, 2006).

Most patients show clinical improvement within 1-3 days after treatment (Robert *et al.*, 2001). Artemisinin and its derivatives are limited by poor oral bioavailability, high recrudescence due to their short half-life (3–5 h). When used in monotherapy, a treatment as long as 5 days is required for complete elimination of the parasites (Ridley, 2002). They are then preferentially used in combination with other antimalarial agents such as sulfadoxine pyrimethamine, lumefantrine, AQ, PQ, Pyronaridine or mefloquine to increase cure rates and to shorten the duration of therapy in order to minimize the emergence of resistant parasites (Robert *et al*., 2001).



(a) (b)

Fig 2.5: chemical structures of Artemisinin and its derivatives (Robert *et al*., 2001).

2.7.6. Aminoquinolines

2.6.6.1 Chloroquine

Chloroquine (Fig 2.6a) is a 4-aminoquinoline derivative that had been the drug of choice for treatment of non-severe malaria until *P.falciparum* species developed resistance against CQ. Chloroquine enters the red blood cells inhabited by the parasite and accumulates in high concentration within the food vacuole. Only inside the acidic food vacuole, chloroquine is protonated to CQ^{2+}. Resistant parasites seem unable to produce haemozoin, but they are still able to digest haemoglobin. In non-resistant forms, most of the ferriprotoporphyrin IX is sequestered in haemozoin.

The enzyme heme polymerase located in the food vacuole uses ferriprotoporphyrin IX released from haemoglobin digestion as substrate for hemozoin biosynthesis. In chloroquine sensitive malaria parasite, the drug is taken up into food vacuoles and it is proposed that here it competes with the haembinder for the ferriprotoporphyrin IX. The chloroquine caps heme molecules to form ferriprotoporphyrin-chloroquine complex. The complex formed is highly toxic and readily disrupts the parasite's cell membrane causing cell lysis to form a destructive compound. The

accumulated toxic heme may also be exported to the membrane and cause parasite death due to the permeabilization of membranes to ions.

Chloroquine was the mainstay of malarial therapy in the 1940. However, *P. falciparum* soon developed resistance to CQ rendering it ineffective in the treatment of uncomplicated malaria. This was widely as a result of using it as monotherapy thus increasing the selection pressure of resistant parasites.

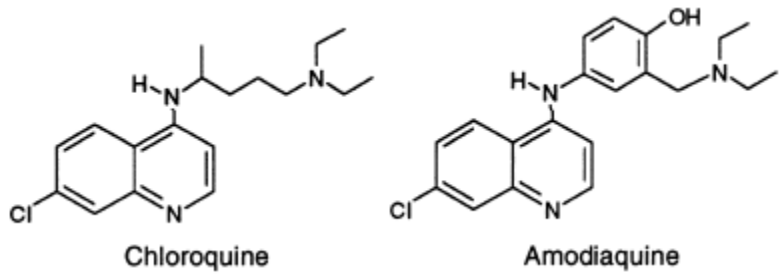
2.6.6.2 Amodiaquine

Amodiaquine (AQ) (Fig 2.6b) is a synthetic antimalarial compound with a pharmacokinetic properties and mode of action similar to that of CQ (Li *et al*., 2002; Warhurst *et al*., 2003). The global use of AQ has declined owing to its association with hepatotoxicity and occurrence of agranulocytosis (Biagini *et al*., 2005). However, some countries have continued to use AQ in the therapeutic management of uncomplicated malaria with no reports of severe adverse effects (WHO, 2001). Amodiaquine is therefore recommended for treatment and not for prophylaxis (Livertox.nih).

AQ is pro-drug which is rapidly metabolized in the liver to N-desethylamodiaquine (DEAQ) within 6–12 hrs. DEAQ has a higher concentration time profile and remains in the plasma for 10–14 days and is responsible for most of the antimalarial activity (Li *et al*., 2002; Mariga *et al*., 2004). AQ is more potent than chloroquine *in vitro* reflecting increased potential for complexation with heme (Winstanley *et al*., 2004). Since AQ retains a high degree of efficacy against all but the most highly chloroquine-resistant strains, there has been a recent increase in its use (Li *et al*., 2002; WHO, 2006). Although cross resistance with CQ exist, the utility of AQ in

combination with other antimalarial drugs is being studied in parts of Africa (Winstanley *et al*., 2004).

AQ is still widely used as monotherapy, providing continued selection pressure for resistance and may continue to worsen despite deployment of the corresponding ACTs (WHO, 2006). In addition, the presence of low-level AQ resistance in the East Africa region provides a clear warning of the existing dangers in widespread use of AQ monotherapy.



(a) (b)

Fig 2.6: chemical structures of Chloroquine and Amodiaquine (Robert *et al*., 2001)

2.7 Antimalarial Drug Resistance

Parasite resistance is the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within limits of tolerance (D'Alessandro, 2001). This definition was subsequently modified to specify that 'the drug must gain access to the parasite or infected red blood cell for the duration of the time necessary for the normal action of the drug (D'Alessandro, 2001). Resistance has also been used when referring to therapeutic failure after administration of a standard dose of a drug. This definition is used in WHO standard *in vivo* test protocol. However, in this *in vivo* test, serum drug levels are not normally measured thus the observed

therapeutic failure might be due to mel-absorption, rapid or abnormal metabolism or the presence of latent infections other than malaria.

Resistance is also thought of as a shift to the right of the drug response curve, thus requiring higher drug concentrations to achieve the same parasite clearance and its emergence also depends on the level of host immunity (White, 2004). Resistance emerge *de novo* through spontaneous gene mutations or duplications, exposure of parasite to sub therapeutic drug levels, and elimination of half-life and reduction in antimalarial susceptibility (White, 2004).

2.7.1 Monitoring Drug Resistance in Malaria

The need for monitoring antimalarial drug resistance has increased due to its rapid spread over the last few decades. Tracking of evolving resistance patterns is essential for proper management of clinical cases and for determining thresholds for revising national malaria treatment policies.

The available testing procedures include *in vivo* tests, *in vitro* sensitivity assays and studies of gene mutations (molecular markers).

2.7.1.1 *In vivo* Techniques

Therapeutic efficacy tests are the gold standard tests that form the basis for any antimalarial drug policy decision. The first standardized test system of *in vivo* drug response was developed in 1965 following reports of chloroquine resistance in *P.falciparum* using mice and rats as animal models.

2.7.1.2 *In vitro* Techniques

In vitro drug tests can be used to assess patterns of cross resistance of different drugs, assess the baseline susceptibility of drugs to be introduced and to temporally and geographically monitor parasite susceptibility to drugs.

Temporal and geographical monitoring of parasite susceptibility through *in vitro* testing procedures provides an early warning of impending resistance before it becomes clinically apparent. These tests are also useful in monitoring changes in susceptibility to a drug that has already been withdrawn. In cases where a drug combination is used where it is not possible to carry out *in vivo* tests for each component of the combination, *in vitro* tests can monitor susceptibility to each drug (WHO, 2001).

2.7.1.3 Molecular markers

Drug resistance molecular markers have potential for predicting therapeutic efficacy on a large scale. Major advantage is in the fact that collection, storage, transport of samples for molecular analysis is easier than for *in vitro* testing. They are however limited in that molecular markers of resistance are available for a short time and thus valid for *P.falciparum* SP, cycloguanil and CQ while for other drugs are yet to be determined (Ridley, 2002).

Molecular studies of resistance could provide an early warning system or can help target therapeutic efficacy tests. This can be useful in monitoring the prevalence of molecular markers where the drug has already been withdrawn or where a drug combination is in use. However prediction accuracy of molecular markers can differ substantially in different epidemiological settings (White, 2004).

2.7.2 Mechanisms of drug resistance in malaria

The occurrence of drug resistance normally involves two phases. Phase 1, a genetic event occur in the parasites that makes them resistant to that particular drug. Phase 2, involves the selection of the resistant parasites by the drug via drug pressure after which they multiply (White 2004).

This is only possible if the genetic events are not deleterious to the survival or reproduction of the parasite.

These genetic events are mutations or changes in the copy number of the genes encoding or relating to the drug's parasite target or influx/efflux pumps that affect intra-parasitic concentrations of the drug. The genes suspected to cause drug resistance to AQ include:

2.7.2.1 *Chloroquine resistance transporter gene (crt)*

Chloroquine resistance transporter (*crt*), is a gene that has 13 exons and is located on chromosome 7. *Crt* encodes a putative transporter CRT protein with 424 a.a and 48.6kDa in molecular weight (Fidock *et al*., 2000). This protein contains ten predicted transmembrane domains localized on the membrane of the digestive vacuole and is involved in drug influx and/or PH regulation (Valderramos and Fidock, 2006).

Chloroquine resistance transporter (crt) has been identified as a key determinant to antimalarials drug resistance (Valderramos and Fidock, 2006). Lys76Thr mutation in the *Pfcrt* gene, the most common marker for CQ resistance has also been strongly associated with 4-aminoquinoline resistance such as AQ. AQ is still effective against some CQ resistant strains of *P. falciparum.* Recent findings have identified *crt* polymorphisms as markers of a genetic background on which *kelch13* mutations are particularly likely to arise and that they correlate with the contemporary geographical boundaries and population frequencies of artemisinin resistance (Miotto *et al*., 2015).

2.7.2.2 *multi drug resistance 1 gene (mdr1)*

Multidrug resistance occurs when parasites selected for resistance to one drug become resistant to a broad range of structurally unrelated drug, for instance AQ and artemisinin. Multidrug

resistance 1 (*mdr1*) gene is located on chromosome 5 and has one exon. It encodes P-glycoprotein homologue 1(Pgh-1) which localizes on digestive vacuole membrane. *Mdr1* has 1419 a.a and 162.25kDa in molecular weight. *Mdr1* consist of two domains, each with six predicted transmembrane domains and a conserved nucleotide binding domain (Valderramos and Fidock, 2006) that act as ATP binding site. Both *mdr1* amplification and mutations may occur (Duraisingh and Cowman, 2005). Resistance to mefloquine and other structurally related aryaminoalcohols in *P.falciparum* results from amplification in *Pfmdr1*.

Mutation in *P. falciparum* in these regions: N86Y, Y184F, S1034C, N1042D, and D1246Y have been reported to involve in determining drug susceptibility to CQ, MQ, Quinine, Lumefantrine, Halofantrine and artemisinin (Sidhu *et al*., 2005; Reed *et al*., 2000; Sisowath *et al*., 2005: Sά *et al*., 2009)

2.7.2.3 Kelch 13

Kelch 13 gene is located on chromosome 13 and has one exon with 726 a.a and 83.66 kDa molecular weight. The C-terminal region of *Kelch 13* protein has six kelch motifs consisting of beta sheet that fold into propeller domains and mutation in this region is predicted to disrupt the domain scaffold and alter its function (Ariey *et al*,. 2014). Recently, SNPs in the propeller region of *Kelch 13* protein has been identified as a key determinant for artemisinin resistance in *Plasmodium falciparum*. Nonsynonymous polymorphism at Y493H, R539T,I54T and C580Y protein position observed in the *Kelch* repeat region of kelch propeller domains have been associated with higher resistance to artemisinin (Ariey *et al*,. 2014: Miotto *et al*., 2015). Thus polymorphism in *Kelch 13* propeller protein is a potent molecular marker in determining the emergency and spread of artemisinin-resistant *P.falciparum* (Ariey *et al*., 2014; Miotto *et al*., 2015).

2.7.2.4 *Deubiquitinating Proteinase 1*

Deubiquitinating Proteinase 1 (*Ubp1)* is a 3.3-kb gene located on chromosome 2 that encodes for deubiquitination enzyme. This protein is associated with increased artesunate resistance in the rodent malaria parasite *P. chabaudi*. i.e. Mutations in V739F and V770F in *ubp1* of *P. chabaudi* were recently identified by linkage group analysis of an elegant genetic-cross experiment to confer resistance to artesunate in this rodent malaria parasite (Hunt *et al*,. 2007).

2.7.2.5 *Sodium Hydrogen Exchanger gene 1 (nhe1)*

This is a transmembrane protein localized in the plasma membrane of the parasite with 1920 a.a of 226kDka molecular weight and predicted to have 12 transmembrane domains. This protein is found in chromosome 13 of *P. falciparum* containing 2 exons and it encodes for *sodium hydrogen exchanger* (*Na/H*$^{+}$). It is associated with quinine resistance (Bennet *et al*. 2007). The role of *Pfnhe1* is not fully understood but hypothesized that it is involved in active efflux protons to maintain PH 7.4 within the parasite in response to acidification by anaerobic glycolysis, the primary energy source for the parasite.

2.7.2.6 *V type Pyrophosphatase 2* (*vp2*) and *Calcium Hydrogen Antiporter 1* (*cvx1*)

V type Pyrophosphatase 2 (*Vp2*) and *Calcium Hydrogen Antiporter 1* (*cvx1*) gene are H^{+} channel molecule that regulate pH balance in the parasite's food vacuole (Jiang *et al.,* 2008). Previous studies associated the putative drug transporter, *Pfcvx1* with CQ resistance by either in response to K76T mutation in *Pfcrt* or to the modulation of CQ resistance (Jiang *et al*., 2008). Recently, *Pbcvx1 and pbvp2* were associated with PQ resistance in *P. berghei* ANKA (Kiboi *et al*., 2014).

2.8 Rodent malaria parasites

There are four African rodent malaria parasites namely *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei* (Smith and Parsons, 1996). Rodent parasites often represent a practical means towards *in vivo* experimentation (Janse and Waters, 1995). Housekeeping genes and biochemical processes are conserved between rodent and human malaria parasites (Carlton *et al*., 1998a). Molecular basis of resistance in some drug resistant rodent parasites has shown similarities to resistance in human parasite. Atovaquone resistance in *P. berghei* and SP resistance in *P. chabaudi* has shown correlation to resistance in *P. falciparum* (Gervais *et al*., 1999; Carlton *et al*., 2001). However, CQ resistant *P. chabaudi* and artemisinin resistant *P. chabaudi* have no correlation to resistance in *P. falciparum* (Carlton *et al*., 1998b; Afonso *et al*., 2006). *P. berghei* has successfully been used in drug testing investigations (Ridley, 2002) and is probably the best practical model for experimental studies of human malaria drug resistance selection (Peters, 1999; Peters and Robinson, 2000; Xiao *et al*., 2004).

CHAPTER THREE: METHODOLOGY

3.1 Laboratory Animals

The animals were housed in experimental room in the animal house in a standard marrolon type 2 cages clearly labelled with experimental details. They were maintained at 22^{o}C and 60-70% relative humidity. The mice were fed on commercial rodent food and water adlibitum.

3.2 Experimental Design

Independent Measures Experimental Design was adopted in this study. The six test compounds (CQ, PQ, PMQ, LM and ATM) were defined as the dependent variable while the mice were the independent variable. For the drug pressure, random sampling method was adopted. Here, 10 mice were selected randomly from a population of about 30 mice at the beginning of each of the passage.

For the sensitive and cross resistance test, the mice were randomly divided into five mice per group (six groups) for each of the test drug for both the resistant and the parent line and put in different cages. The mice were then labelled 1-5 in each of the group. The first five groups were the test group while the sixth group served as the control group (placebo). Each of the test group received different dosage of the test drug while the control group was given water.

3.3 Parasites, host and compounds

Transgenic ANKA strain of *P. berghei* expressing fusion protein GFP-Luciferase (*P. berghei* ANKA GFP-Luciferase, reference line: 676m1cl1 (Janse *et al*., 2006) obtained from Leiden University Medical Center, Netherlands was used to select AQ. AQ drug pressure against *P. berghei* was previously initiated by Kiboi *et al* 2009. However, the resistance index obtained was

low and hence this study further subjected the parasite to drug pressure. Male swiss albino mice weighing 20±2g outbred at KEMRI Animal House, Nairobi, Kenya were used as the host. A selection of AQ, CQ, PMQ, PQ LM and ATM were prepared freshly by dissolving the required amount equivalent to (50mg/kg for AQ, 10mg/kg for CQ, PQ, PMQ, LM and 5mg/kg for ATM) in a solvent containing 3% ethanol and 7% Tween-80 (solvent for aqueous compounds).

3.4 Selection of amodiaquine resistance

The 20^{th} passage *P.berghei* was revived from -80^0C. Then 10 naïve mice were inoculated intraperitoneally with $1x10^6$ parasitized red blood cells in a 0.2ml on day 0 (D0). Once the parasitemia rose to 2-7%, the mice were orally treated once with 50mg/kg of AQ (this dose cleared the parasites to a level that could not even be detected microscopically. The parasitemia rose to >2% after 7-10 days post treatment on average and this dosage was thus selected for the drug pressure) on day 3(D3). Parasite growth was then followed until the parasitaemia rose to 2-7% when donor mouse was then selected for subsequent passage into the next naïve group of mice.

The level of resistance was evaluated at interval of four drug pressure passages by measurement of ED_{50} and ED_{99} in the standard 4 Day Test (4DT) which permits the calculation of an index of resistance I_{50} and 1_{99} (the ratio of the ED_{50} or ED_{99} of the resistant line to that of sensitive, parent line) (Fidock *et al* 2004; Xiao *et al*., 2004). To further phenotype the resistant parasite, the stability of the resistance line was assessed by freezing the parasite at -80 for four weeks and then determined the ED_{50} and ED_{99} in the 4 Day Test as detailed in section 3.5. The 1_{99} values were then grouped into four categories: 1) I_{99}= 1.0 (sensitive), 2) I_{99}= 1.01-10.0 (slightly resistance), 3) I_{99}=10.01-100, (moderate resistance), 4) $I_{99}\geq$100 (high resistance).

3.5 Determination of 50% and 99% Effective Doses (ED_{50}, ED_{99}) Of Antimalarial Drugs

The 50% (ED_{50}) and 99% (ED_{99}) effective doses, the doses that reduces parasitaemia by 50%/ and 99% respectively of each of the test compound, were measured in a quantitative standard 4 Day Test Suppressive Test (Fidock *et al*., 2004). Briefly; mice were infected intraperitoneally each with 1×10^6 parasites (*P.berghei*). Oral treatment with drug (at least four different drug concentration as shown in Table 3.1) was initiated on day 0, (4 hrs post-infection) and continued for four days, days 0–3 (24, 48 and 72 hrs post-infection). Parasite density was estimated microscopically (×100) on day 4 (96 hrs) post parasite inoculation using thin blood films made from tail blood snips. Parasite growth was then followed on D2, D3, D4, D7, D9, D11 and D15 days post infection. To calculate % chemosuppression of each dose this formula (Fidock *et al*., 2004) was used: A-B/A×100

Where A = the mean parasitemia in the negative control group and B the parasitemia in the test group. The ED_{50} and ED_{99} were analysed using version 5.5 statistica 2000.

Table 3.1: Drug dosages used in the determination of the ED_{50} and ED_{99} of AQ, CQ, PMQ, PQ, LM and ATM

Antimalarial drug	**Dosage**
Amodiaquine	40mg/kg, 20mg/kg, 10mg/kg, 5mg/kg
Lumefantrine	10mg/kg, 5mg/kg, 2.5mg/kg, 1.25mg/kg, 0.625mg/kg
Chloroquine	10mg/kg, 5mg/kg, 2.5mg/kg, 1.25mg/kg, 0.625mg/kg
Piperaquine	10mg/kg, 5mg/kg, 2.5mg/kg, 1.25mg/kg, 0.625mg/kg
Primaquine	10mg/kg, 5mg/kg, 2.5mg/kg,1.25mg/kg, 0.625mg/kg
Artemether	5mg/kg, 2.5mg/kg, 1.25mg/kg, 0.625mg/kg,0.3125mg/kg

3.6 Dilution Cloning of Amodiaquine Resistant Parasites

Genetically homogenous resistant parasites were obtained from different generations of AQr by dilution cloning based on the protocol by Janse *et al*., 2004. Briefly, mouse with parasitemia between 0.5 and 1% was selected as a donor mouse and 5µl of infected blood was collected from the tail of the mouse in 1µl of heparin and diluted in 1ml of 1× PBS. The number of infected erythrocytes per 1µl was estimated from 20µl of diluted blood. The cell suspension was then diluted further with 1×PBS to an estimated final concentration of 0.5 parasites/ 0.2ml PBS. Ten mice were then injected with the infected blood. When 30-50% of the mice become positive and showed a parasitemia of between 0.3-0.5percent at day 8 post infection, dilution was considered successful. The fastest growing clone was selected for cross resistance and molecular studies.

3.7 Drug Sensitivity and Cross Resistance Profile Test

The assessment of the resistance and cross resistance profile of individual clone generations by dilution cloning, the fastest clone in each generation was selected and evaluated for its response to AQ in the 4-Day suppressive protocol as described by Fidock *et al.*, 2004 as detailed in section 3.5 on determination of effective doses. To this purpose, four different drug dosages were selected for each of the test drug (Table 3.1) and administered orally. The 50% and 99% indices of resistance were calculated as in section 3.4.

3.8 Molecular Analyses

3.8.1 DNA extraction

Parasite DNA was extracted by first preparing a parasite pellet. To this effect, 500µl of mouse blood (5-10% parasitaemia) was collected through cardiac puncture and then diluted with 500µl of PBS. The solution was then span for 1 min at 500xg. The supernatant was discarded and the

pellet was the re-suspended in 30ml volume of cold 4^0C 1x RBC lysis buffer and incubated for 15-30min on ice. The mixture was then centrifuged at 500xg for 10 min at 4^0C. The supernatant was then discarded and the pellet washed in 30ml PBS by centrifuging at 500xg for 10 min at 4^0C to obtain the parasite pellet. Genomic DNA was then extracted using a Qiamp® Blood DNA extraction kit following manufacturer's instructions. The genomic DNA extracted was the used as a template for PCR analysis.

3.8.2 PCR Analysis of *Pbmdr1, Pbubp1, Pbkelch13* and *Pbcrt*

Target fragments from *P. falciparum* ortholog genes *Pbmdr1* (PBANKA_1237800), *Pbcrt* (PBANKA_1219500), *Pbubp1* (PBANKA_0208800) and *Pbkelch13* (PBANKA_1356700) were amplified. Briefly, 1µl of genomic DNA was used as the template in 25µl PCR reactions using DreamTaq (Thermo-Scientific™). Other reagents; $MgCl_2$, dNTPs, forward and reverse primers (Table 3.2) and cycling conditions were optimized accordingly as shown in table 3.3. PCR products were analyzed in 1% agarose gel, purified using GeneJet™ PCR purification kit (Thermo-scientific™).

Table 3.2: primer sequences for amplification and sequencing of *Pbmdr1*, *Pbcrt*, *Pbubp1* and *Pbk13* (Kiboi *et al.*, 2014).

Primer Name:	**PCR primers sequence (5' to 3'):**	**Primer annealing Position**	**Expected band size**
Pbcrt – Forward	GGA CAG CCT AAT AAC CAA TGG	69-89	
Pbcrt – Reverse	CGA CCA TAG CAT TCA ATC TTA GG	751-729	1.3kb
Pbcrt – Forward	CCT AAG ATT GAA TGC TAT GGT CGT	729-751	
Pbcrt – Reverse	GTT AAT TCT GCT TCG GAG TCA TTG	1230-1253	1.4kb
	Sequencing primers (5' to 3'):		
Pbcrt – Forward	TCA GGA AGA AGT TGT GTC A	109-127	
Pbcrt – Reverse	GAT AAG GAA AAA CTG CCA TC	383-402	
Pbcrt – Forward	GTG TTG GCA TGG TCA AAA TG	908-927	
Pbcrt – Reverse	CTT GGT TTT CTT ACA GCA TCG	1124-1104	
	PCR primers (5' to 3')		
Pbkelch13 - Forward	AGT CAA ACA GTA TCT CTA ACT	1272- 1291	
Pbkelch13 – Reverse	ACG GAA TGT CCA AAT CTT G	1879-1899	627bp
	Sequencing primers (5' to 3')		
Pbkelch13 - Forward	TCC ACT AAC CAT ACC TAT AC	1272-1291	
Pbkelch13 – Reverse	AGC TTC TAA TAA TGC ATA TGG	1899-1879	
	PCR and Sequencing primers (5' to 3')		
Pbmdr1 – Forward	GTG CAA CTA TAT CAG GAG CTT CG	176-198	
Pbmdr1 – Reverse	CAC TTT CTC CAC AAT AAC TTG CTA CA	742-717	566bp
Pbmdr1 – Forward	GGA TTT TTA TCG TCG CAT ATT AAC AG	2647-2672	
Pbmdr1 – Reverse	TAG CTT TAT CTG CAT CTC CTT TGA AG	3259-3234	612bp
Pbmdr1 – Forward	CTT CAA AGG AGA TGC AGA TAA AGC TA	3234-3259	
Pbmdr1 – Reverse	GAT TCA ATA AAT TCG TCA ATA GCA GC	3887-3862	653bp
	PCR and Sequencing primers (5' to 3')		
Pbubp1 – Forward	AGT TCC AAT GAA TAT ATT CAT GTG AA	1990-2015	
Pbubp1 – Reverse	CTA AGT TGC ATA GCT TTA TCA TTT TC	2621-2596	631bp

Table 3.3: Optimized conditions for PCR amplification of *Pbcrt*, *Pbmdr1*, *Pbubp1*, *Pbkelch13* gene.

PCR amplifying profiles	**Temperature (°C) /Time (**min**)**			
	Pbcrt ***1st and 2nd Fragment***	***Pbmdr1*** ***1st, 2nd and 3rd fragments***	***Pbubp1***	***Pbkelch13***
Initial denaturation	95°C, 5 min	95°C, 5 min	95°C, 5 min	95°C, 5 min
Denaturation	95°C, 1 min	95°C, 30 secs	95°C, 30 secs	95°C, 1 min
Annealing Temperature	50°C, 30 secs	52°C, 30 secs	50°C, 30 secs	51°C, 30 secs
Elongation	72°C, 3 min	72°C, 1 min	72°C, 1.5 min	72°C, 1.5 min
Primer (Forward & reverse)	2.5µM each	2.5µM each	2.5µM each	2.5µM each
MgCl2 (mM)	2.0	1.5	1.5	2.0
dNTPs (mM)	2.0	2.0	2.0	2.0
Cycles	35	30	30	30
Final elongation	62°C, 10 min	62°C, 10 min	62°C, 10 min	62°C, 10 min

3.8.3 Sequencing of *Pbmdr1, Pbubp1, Pbkelch13* and *Pbcrt*

The PCR products were then sequenced based on BigDyev3.1 using a 3730xlsequencer. Briefly, the DNA sample was divided into four separate sequencing reactions, containing all the four standard dNTPs, the DNA polymerase, and only one of the four ddNTPs for each reaction. After rounds of template DNA extension, the DNA fragments that were formed were denatured and separated by size using gel electrophoresis with each of the four reactions in one of the four separated lanes. The DNA bands were then visualized by UV light. The resulting contigs were assembled using Lasergene 11 Core Suite, the DNA sequences and the predicted amino acid

sequences were analyzed using CLUSTAL W available in EBI website (www.ebi.ac.uk) and PlasmoDB (PlasmoDB, 2017).

3.8.4 RNA extraction

In this experiment, all buffers, solutions and tubes for parasite preparation were first treated with 0.1% (v/v) of diethyl pyrocarbonate (DEPC). Total RNA was prepared from approximately 1x 10^6 fresh parasites pellet. In preparation of parasite pellet, parasitized red blood cells were first washed in 1xPBS and then lysed in 5 volumes of ammonium chloride solution. The parasite pellet was washed twice in 10ml of 1xPBS and then resuspended in 200µl of 1xPBS. The total RNA was then extracted based on high pure RNA extraction kit. Total RNA extracted was then purified using Quick-RNA™ MiniPrep (Zymo Research) following manufacturer's instructions.

3.8.5 cDNA synthesis

The total RNA extracted was used immediately for cDNA synthesis. The first strand cDNA synthesis was performed in a final volume of 20µl using Thermo-Scientific RevertAid First Strand cDNA Synthesis kit and oligo-DT as primers. 5ng/µl of total RNA, 1µl of oligo-DT and nuclease free water were mixed with 4µl of transcriptor reverse transcriptase buffer(5x), 0.5µl Ribolock RNase inhibitor (20U/µl), 2µl of dNTPs (10mM) and 1µl of RevertAid M-MuLV Reverse Transcriptase (200U/µl) was added and mixed gently. The RT reaction mix was incubated at 42^{o}C for 60min, then at 70^o C for 5min to terminate the reaction and finally chilled on ice. The cDNA was then used as template for qRT-PCR assays.

3.8.6 Quantitative Real Time-PCR Assays

To evaluate the mRNA transcript levels of *Pbmdr1, Pbvp2, Pbvcx1* and *Pbnhe1,* qRT-PCR was used in a final volume of 20µl using Maxima SYBR Green/Rox qPCR Master Mix (Thermo-

Scientifc^{TM}). Oligonucleotide for *Pbmdr1*, *Pbvp2*, *Pbvcx1* and *Pbnhe1* were designed to run using similar cycling conditions relative to the *Pbβ-actin*, as the house keeping gene. *ROX Dye* is used to normalize the fluorescent reporter signal in *real-time* quantitative *PCR* or *RT-PCR*. Briefly, 12µl of maxima SYBR Green, 2µl of forward and reverse primers each as shown in Table 3.4, 1µl cDNA and 3µl nuclease free water were added and mixed thoroughly. The reaction mix was run for pre-treatment at 50^0C for 2 min, initial denaturation at 95^0C for 10 min, denaturation at 95^0C for15 secs, and annealing at 60^0C for 60 secs for 45 cycles.

Table 3.4: Oligonucleotide primers used to measure the transcriptional level profiles of *Pbmdr1, Pbvp2, Pbvcx1,* and *Pbnhe1* with *Pbβ-actin* as housekeeping using Maxima SYBR Green chemistry in quantitative Real-Time PCR.

Name	**Primer sequence (5' – 3')**	**Position**	**Tm**
Pbmdr1- Forward	ACGGTAGTGGCTTCAATGGA	917-936	54.2
Pbmdr1- Reverse	CTGTCGACAGCTGGTTTTCTG	1082-1062	54.7
Pbnhe1 – Forward	TGGAGAGTTTGATTTAGGCTTACC	2022-2045	54.0
Pbnhe1 – Reverse	GCTAGGCGATGTTTTGTTAGGAG	2202-2180	55.3
Pbvp2 – Forward	TGCAGCAGGAAATACAACAGC	1449-1469	55.2
Pbvp2 – Reverse	GTCGTACTTTTGCACTACTTGCGT	1558-1535	56.5
Pbcvx1 – Forward	TCAAATTGCTCTTTTTGTTGTACCAA	1101-1126	57.9
Pbcvx1 – Reverse	ACACCTTCTAGCCAATTACTTTCACC	1265-1240	57.1
Pbβ-actin – Forward	CAGCAATGTATGTAGCAATTCAAGC	392-416	56.8
Pbβ-actin – Reverse	CATGGGGTAATGCATATCCTTCATAA	523-498	58.9

3.9 Statistical analysis

The means of expression levels of each gene from three independent experiments and from triplicate assays obtained from AQ resistant were compared to AQ sensitive using student's t-

test; p value was set at 0.05. The relative expression levels results were normalized using *Pb-β actin* as the housekeeping using the formula 2^^CT based on (Livak and Schmittgen, 2001). The means for cross resistance profiles for each drug from at least four different drug concentrations were analysed using student's t test with *p* value set at 0.05.

3.10 Ethical Consideration

Permission to carry out this study and ethical clearance was sought from KEMRI's Scientific Ethics Review Unit (SERU) (see attached). All animal work was carried out according to relevant national and international standards as approved by KEMRI-Animal Use and Care Committee (see attached).

CHAPTER FOUR: RESULTS

4.1 Amodiaquine Drug Pressure Induces Stable Resistant Phenotypes

The AQ pressure dose (50mg/kg) was only 50 times and 10 times the ED_{50} and ED_{99} respectively of the parent line (ED_{50} = 0.95mg/kg, ED_{99} = 5.05mg/kg) as shown in table 4.1. At 24^{th} passage, the parasitemia rose from 0.63 on day 4 post infection to 0.96, 1.2, and 1.76 on day 7, 9 and 11 post infection respectively. At the 28^{th} passage, the ED_{50} and the ED_{99} were 5mg/kg and 13mg/kg respectively. While at 36^{th} passage the ED_{50} and ED_{99} increased to 12.01mg/kg and 20.73mg/kg respectively. The I_{50} and I_{99} at 36^{th} passage was only 12 and 4 fold respectively (Table 4.1). The AQ resistant parasites remained stable with ED_{50} and ED_{99} of 5.86mg/kg and 18.22mg/kg respectively. These effective doses were equivalent to I_{50} and I_{99} of 6.0 and 3.6 folds respectively (Table 4.1). Although not expected, the I_{50} dropped marginally while the I_{99} remained relatively unchanged but remained within the resistance threshold. This study was thus able to successfully select stable AQ resistant *P. berghei.*

Table 4.1: The 50% and 99% Effective Dose (ED_{50} and ED_{99}) in mg/kg/day of amodiaquine resistant *Plasmodium berghei* ANKA line.

Passage No	**50% and 99% effective doses**		**Index of resistance**	
	ED_{50} (mg/kg)	**ED_{99} (mg/kg)**	**I_{50}**	**I_{99}**
Parent line	0.95	5.05	1.00	1.00
24^{th}	1.26	8.74	1.33	1.73
28^{th}	5.00	13.00	5.26	2.57
36^{th}	12.01	20.73	12.64	4.10
Stability after freezing for four weeks	5.86	18.22	6.17	3.6

4.2 Amodiaquine resistance associated with cross resistance to CQ, LM, ATM, PQ and PMQ

Artemether had the highest I_{99} of 9.8 (Table 4.2). Lumefantrine recorded a higher I_{99} of 3.9 than CQ and PQ which recorded 1_{99} of 3.22 and 1.28 respectively (Table 4.2) despite CQ and PQ.

Table 4.2: Cross resistance profiles of the amodiaquine resistant line Plasmodium berghei ANKA line and the sensitive parent line

Antimalarial drug	**Sensitive parent line**	**AQ resistant line**	**Index of resistance**
	ED_{99} units	**ED_{99} units**	**1_{99}**
Artemether	3.93	38.5	9.8
Chloroquine	9.36	30.12	3.22
Lumefantrine	3.93(Kiboi *et al*,. 2009)	15.35	3.9
Piperaquine	7.72	9.86	1.28
Primaquine	1.33(Langat *et al*,. 2012)	8.34	6.27

4.3 Evaluation of point mutations in *Pbmdr1, Pbubp 1, Pbkelch13* and *Pbcrt*

4.3.1 PCR Analysis

The specific regions of each of the genes were amplified and the expected bands sizes were achieved indicating that the desired regions were successfully amplified (Plate 4: 1, 2, 3, 4). The expected band sizes of mdr1 in regions 1, 2 and 3 were 566bp, 612bp and 653bp respectively (Plate 4.1). Thus the targeted regions of *mdr1* were successfully amplified. The expected band size of *ubp1* was 631bp (Plate 4.2). Thus the targeted region was successfully amplified. The expected band sizes of *crt* were1.3kband 1.4bk (Plate 4.3). Thus the targeted region was successfully amplified. The expected band size of *kelch13* was 627bp (Plate 4.4). Thus the targeted region was successfully amplified.

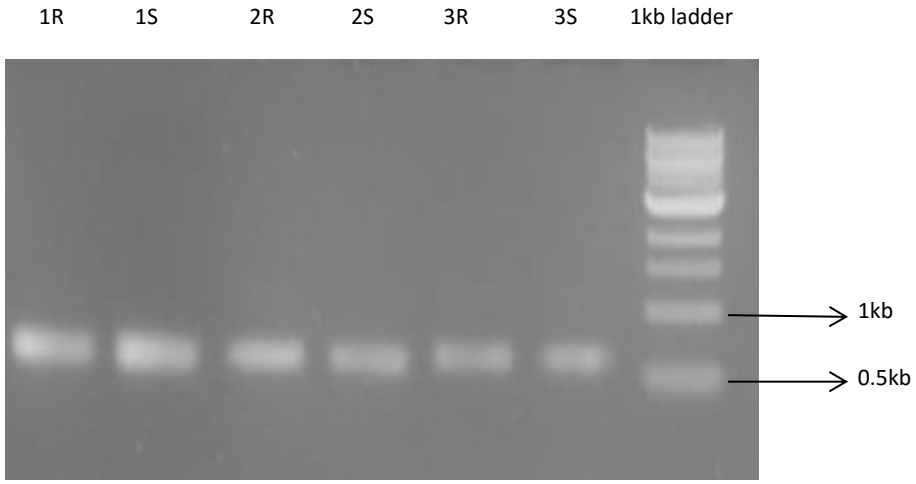


Plate 4.1: Gel photo showing analysis of PCR products of: *mdr-1*gene with the three amplified regions: 1, 2, 3 of both the AQ resistant line (R) and the sensitive line (S). The 1R and 1S represent region 86 and 184 of the *mdr1* of both the sensitive and the resistant line. 2R and 2S represent region 1034 and 1042 of the *mdr1* of both the sensitive and the resistant line while 3R and 3S represent region 1246 of the *mdr1* of the sensitive and the resistant line. The PCR

products were analysed on a 1% agarose gel. The expected band sizes of regions were: 566bp (1), 612bp (2) and 653bp (3).

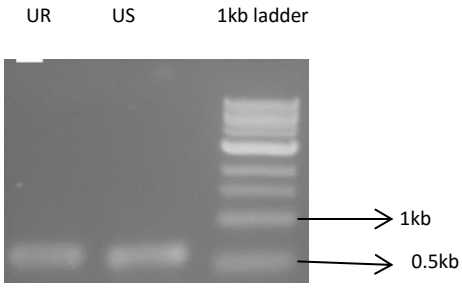


Plate 4.2: Gel photo showing analysis of PCR products of: *ubp1*gene with the amplified region of both the AQ resistant line (R) and the sensitive line (S). The PCR products were analysed on a 1% agarose gel. The expected band size of amplified region was 631bp.

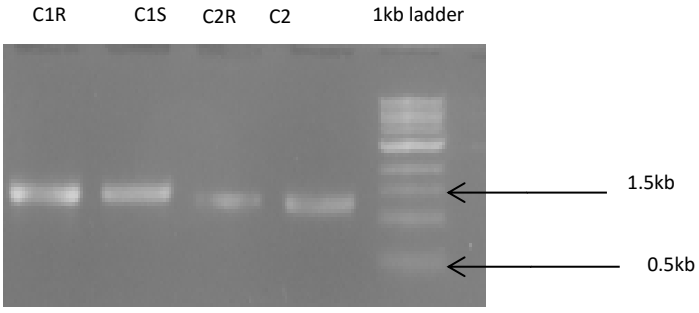


Plate 4.3: Gel photo showing analysis of PCR products of: *crt* gene with the two amplified regions: 1, 2 of both the AQ resistant line (R) and the sensitive line (S). The C1R and C1S represent region 76 of the *crt* of both the sensitive and the resistant line. C2R and C2S represent region 326 and 356 of the *crt* of both the sensitive and the resistant line. The PCR products were analysed on a 1% agarose gel. The expected band sizes of regions were: 1.4bp (C1) and 1.3bp (C2).

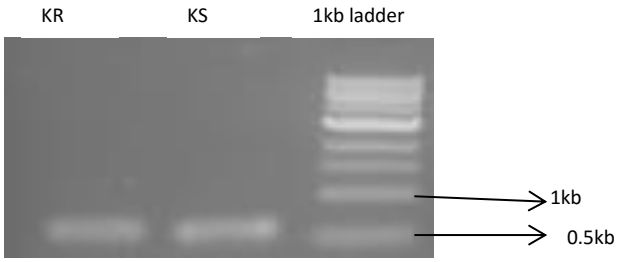


Plate 4.4: Gel photo showing analysis of PCR products of: *kelch13* gene with the targeted amplified region of both the AQ resistant line (R) and the sensitive line (S). The KR and KS represent amplified region of both the sensitive and the resistant line. The PCR products were analysed on a 1% agarose gel. The expected band size of region was 627bp**.**

4.3.2 Sequencing analysis

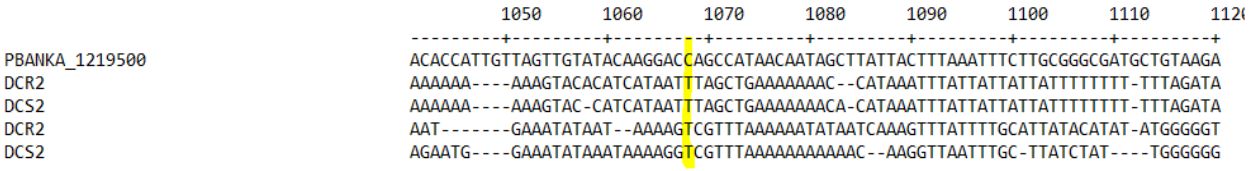
Nucleotide codons corresponding to protein position 76, 326 and 356 of *Pbcrt* protein were found not to harbor any mutation (Fig 4.1: a, b, c). Similarly, *Pbmdr1* at 86, 184, 1034, 1042 and 1246 positions of the protein had no mutation (Fig 4.2: a, b, c, d, and e). The study further assessed any point mutation in *kelch 13* and *ubp1*. No nucleotide sequence variation was mapped in *Pbubp1* and *Pbkelch13* genes between the AQ resistant and its sensitive progenitor (Fig 4.4: a, b, Fig 4.3: a, b).

```
                    170       180       190       200       210       220       230       240
                    ----------+---------+---------+---------+---------+---------+---------+
PBANKA_1219500      AAAATAATGTTTATGTTTATTTACTAAGTATATTATATTTATGTGTTTGTGTAATGAATAAAGTTTTTGCAAAAAGAACC
DCR1                AAAATA-TGTT-ATGTTTATTTACTA-GTATATTATATTTATGTGTTTGTGTAATGAATAAAGTTTTTGCAAAA-GAACC
DCS1                AGAATAATGTTTATGTTTATTTACTAAGTATATTATATTTATGTGTTTGTGTAATGAATAAAGTTTTTGCAAAAAGAACC
DCR1                ------------------------------TTTGACCTTAACCAATGGAAGTAATAATCAAATAAAATAAAAAAAGATGA
DCS1                --------------------------------TGGCT---AACAATGGAAGTA-TAATCAAATAAAATAAAAAAAGATGA
```

(a)

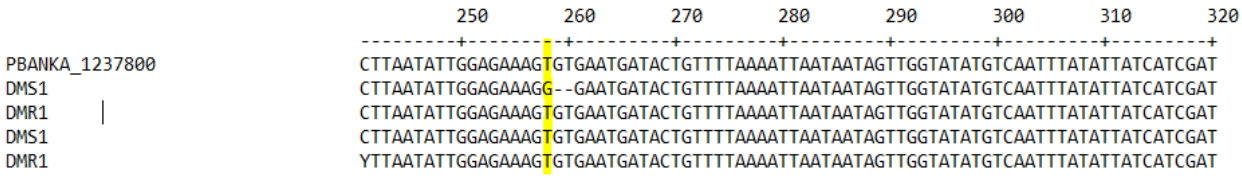
```
                    970       980       990       1000      1010      1020      1030      1040
                    ----------+---------+---------+---------+---------+---------+---------+
PBANKA_1219500      TTTATAACATTTTCCTTTTTCAACATATGTGATAATTTACTTGCTTGCTATATAATTGATAAATTTTCAACA-ATGACAT
DCR2                ATTTTTTTTTTTTTTTTTCAGAAAACCTTTA-TAACATTTTCCTTTTTCACATA-TGGATAATTTTGCTGTA-GTAAGGA
DCS2                ATTTTTTTTTTTTTTTTTCAGAAAACCTT-A-TAAC--TTTTCTTTTTCA-ATA-TGGATAATTCTTCT-TA-GTAAGGA
DCR2                CTCCTAAATTTTTTTCTCCTAAATTTTTTTCCTCCCTAAATTTTTTCTCCTACG-TTGGTACTTATTGGACA-ATATTAC
DCS2                CTCCTAAATTTTTTTCTCCTAAATTTTTTTC-TCCCTAAATTTTTCTCCTAACG-TTGGTACTTATTGGGAACAAATTAC
```

(b)

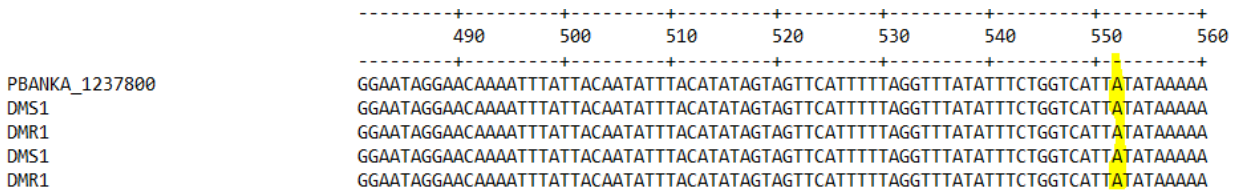


(c)

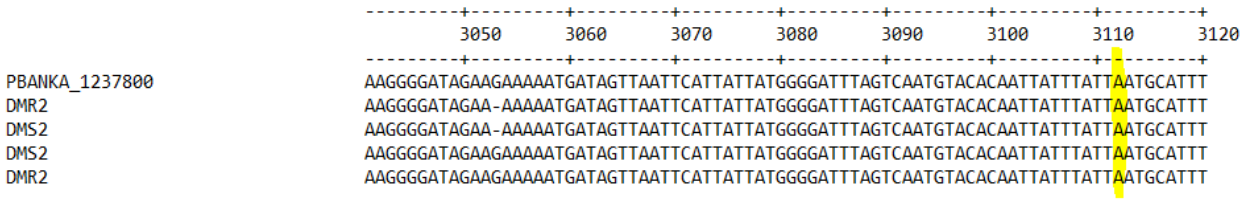
Fig 4.1: The nucleotide sequences at protein position: (a) 76, (b) 326 and (c) 356 of chloroquine resistance transporter (crt) gene of both the amodiaquine resistance (DCR) and sensitive (DCS) *P.berghei* ANKA after sequencing based on BigDyev3.1 using a 3730xlsequencer and Contigs assembled using Lasergene 11 Core Suite, the DNA sequences and the predicted amino acid sequences were analyzed using CLUSTAL W available in EBI website (www.ebi.ac.uk) and PlasmoDB (PlasmoDB, 2017).



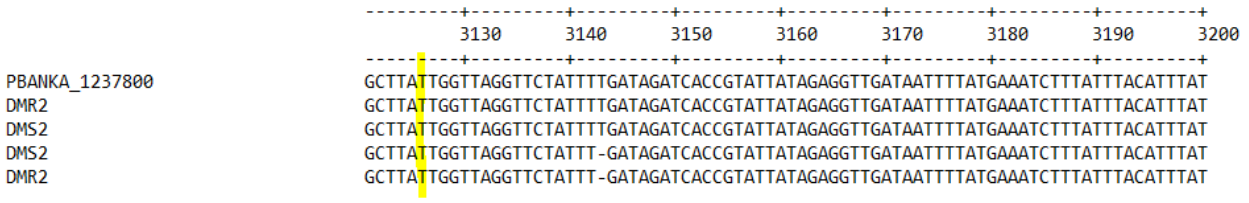
(a)



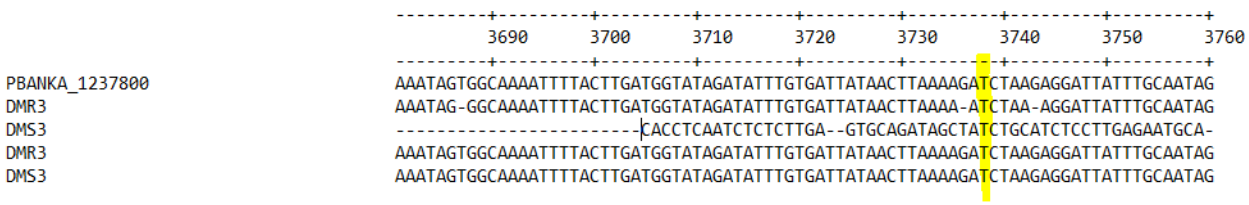
(b)



(c)



(d)



(e)

Fig 4.2: The nucleotide sequences at protein position: (a) 86, (b) 184, (c) 1034, (d) 1042 and (e) 1246 of multi drug resistance 1 (*mdr1*) gene of both the amodiaquine resistance (DMR) and sensitive (DMS) *P.berghei* ANKA after sequencing based on BigDyev3.1 using a 3730xlsequencer and Contigs assembled using Lasergene 11 Core Suite, the DNA sequences and the predicted amino acid sequences were analyzed using CLUSTAL W available in EBI website (www.ebi.ac.uk) and PlasmoDB (PlasmoDB, 2017).

(a)

(b)

Fig 4.3: The nucleotide sequences at protein position: (a) 476, 493, (b) 539, 543 and 580 of *kelch 13* (*k13*) gene of both the amodiaquine resistance (DKR) and sensitive (DKS) *P.berghei* ANKA after sequencing based on BigDyev3.1 using a 3730xlsequencer and Contigs assembled using Lasergene 11 Core Suite, the DNA sequences and the predicted amino acid sequences were analyzed using CLUSTAL W available in EBI website (www.ebi.ac.uk) and PlasmoDB (PlasmoDB, 2017).

Fig 4.4: The nucleotide sequences at protein position:) a) 739 and 770 of *ubp1* gene of both the amodiaquine resistance and sensitive *P.berghei* ANKA after sequencing based on BigDyev3.1 using a 3730xlsequencer and Contigs assembled using Lasergene 11 Core Suite, the DNA sequences and the predicted amino acid sequences were analyzed using CLUSTAL W available in EBI website (www.ebi.ac.uk) and PlasmoDB (PlasmoDB, 2017).

4.4 Assessment of expression profile of ortholog genes.

The mRNA transcript was measured using the quantitative RT-PCR. The expression means of each of the compensatory and modulatory genes in the AQ resistant line were compared to that of the parent line using student t-test. The *Pbmdr1* and *Pbvp2* mRNA transcript were elevated 2.0 fold ($p<0.001$) and 1.4 fold ($p<0.02$) respectively (Fig 4.5). mRNA transcript of *Pbnhe1* and

Pbcvx1 were evaluated indicating a 3.4 fold (p<0.0001) and 2.9 fold (p<0.0001) respectively.

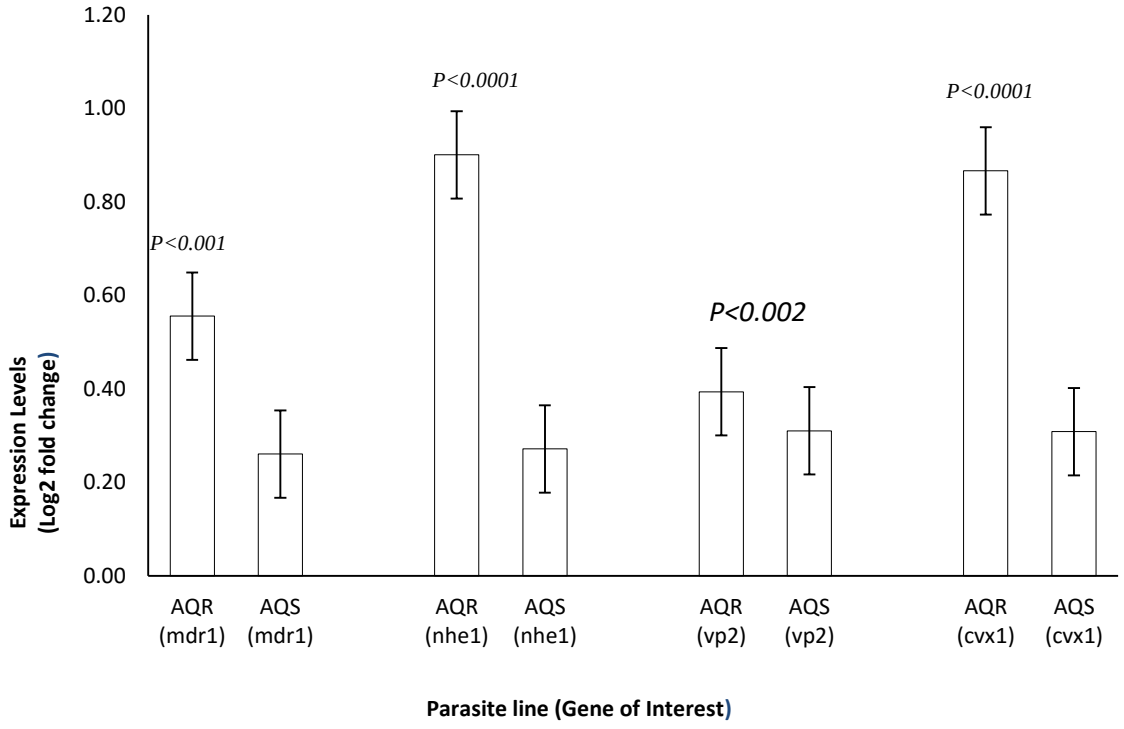


Fig 4.5: Expression profiles in multidrug resistance gene 1 (*mdr1*), V-type H+ pumping Pyrophosphatase (*VP2*), Ca2+/H+ antiporter (*vcx1*)and *nhe1* as measured from cDNA amount derived from 5 µg/ul of total RNA isolated from amodiaquine resistant clones relative to their wild type drug sensitive parental clones amodiaquine sensitive (AQS).The differential expression from a mean of three independent experiments were significantly different for *mdr1(p<0.001)*, *nhe1(p<0.0001)*, *vp2(p<001)* and *cvx1(p<0.0001)* after Student's t-test analysis with *p* value set at 0.05.

CHAPTER FIVE: DISCUSSION

Recently, AQ has been reintroduced as an ACT by the WHO (Gil, 2008). Its reintroduction as an ACT has caused great need of understanding its mechanism of resistance. However, artesunate is partnered with a drug against which resistance can arise fast. The mechanism of resistance of AQ is controversial. This study focused in understanding the genetic mechanism by which the *P. berghei* evades the drug action as well as the testing for any cross resistance that exist between AQ and other chemically and mechanistically related and unrelated drugs.

5.1 Amodiaquine drug pressure induces stable resistant phenotypes

From the results obtained in this study, there was slow development of resistance with an I_{99} of only 4.1 after 36 passages. This is consistent with other results from selection of AQ resistant *P. berghei* N line in 2% RT method where slow emergence of resistance was also recorded (Peters and Robinson, 1992). It would appear that emergence of AQ resistance is slow regardless of the method or the dose used to induce the resistance. The results conform to other studies that administration of a constant dose at every passage results to slow emergence of resistance as compared with stepwise increase of the dosage at every passage (Xiao *et al*., 2004). Evidence has also been presented that resistance to single compounds may emerge more rapidly when a high dose is employed in the 2% RT than a lower dose (Peters and Robinson, 2000). The confirmation of increased AQ selection pressure was clearly shown by the drastic increase in the I_{50} to 12.64 at 36th passage however the I_{99} seems not to increase by a big margin, with only 4.10 recorded at the same passage (Table 4.1). This increase confirms the ease of maintaining and increasing AQ resistance once the initial probable physiological adaptations or genetic changes occurs. The observed results emphasize importance of monitoring AQ resistance in areas where the drug is in use.

This study demonstrates that stable AQ resistant *P. berghei ANKA* can be achieved by submitting sensitive parasite to thirty-six continuous drug pressure passages. The parasite retained resistance level after cryopreservation for one month with I_{50} and I_{99} of 6.27 and 3.6, meaning the mechanisms are encoded in the genome.

5.2 Amodiaquine resistance associated with cross resistance to CQ, LM, ATM, PQ and PMQ

Cross resistance patterns of dilution cloned AQ resistant parasites against chemically and mechanistically related and unrelated drugs was then evaluated. From results obtained, all the test compounds were cross resistant to AQ resistant parasite line. This cross resistance profiles is attributed to strong specific mechanisms (linked to mode of action) and nonspecific mechanisms (independent of mode of action) (Raynes, 1999). CQ, PMQ, PQ and AQ are all aminoquinolines derivatives and are therefore likely to share the same mechanism of action as well as mechanism of resistance (Ginsburg *et al*., 1998; Carlton *et al*., 2001: Robert *et al*., 2001).

Resistance to AQ and CQ in *P. falciparum* is reported to be inversely correlated to resistance to arylamino alcohol such as lumefantrine (Durasing and cowman, 2005). This study however, indicated a slight cross resistance between LM and AQ with a 4 fold decrease in LM activity against AQ resistant strains. LM is chemically unrelated to AQ but predicted to have the same mode of action and thus may also share a similar mechanism of resistance (Carlton *et al*., 2001: Robert *et al*., 2001). From the results, a slight cross resistance in AQ and LM in *P. berghei* exists. This indicates that AQ and LM may have similar mechanism of resistance.

Chloroquine is a structural analog of AQ (chemically related) and thus may have similar mode of action as well as similar mechanism of resistance (Ginsburg *et al*., 1998). The study data

indicated a 3 fold decrease in CQ activity against AQ resistant parasites. This was expected and confirms previous in vitro studies that have correlated CQ resistance with AQ resistance (Ochong *et al*., 2003). Several other in vitro studies and clinical reports have shown cross resistance between CQ and AQ (Carlton *et al*., 2001; Platel *et al*., 1998). This study therefore confirms the existence of cross resistance between AQ and CQ.

Primaquine as earlier mentioned is an aminoquinolines derivative (8- aminoquinoline) and as such is predicted to share the same mechanism of action as well as mechanism of resistance with AQ (O' Neill *et al.,* 2006). Together with tefanoquine which is its analog, have gametocidal activity against all human malaria parasite species (Vangapandu *et al*., 2006). Primaquine is mainly used against hypnozoites responsible for the relapsing forms of *P. vivax* and *P. ovale* and interferes with the mitochondrial function of plasmodium (Baird, 2005). Primaquine is currently used for hepatic malarial chemoprophylaxis to eliminate *P. falciparum* at the early stage of infection (Chiang *et al*., 2006). The study data indicate the existence of slight cross resistance between AQ and PMQ with a 6 fold decrease in PMQ activity against AQ resistant strain. Thus PMQ and AQ may thus share the same mechanism of resistance.

Piperaquine is a potent bisquinoline antimalarial available as piperaquine base (PQ) or as its water-soluble tetra-phosphate salt, Piperaquine phosphate (PQP) (Davis *et al*., 2005; Raynes, 1999). PQ is chemically and mechanistically related to AQ and thus may share the same mode of action and possibly the same mechanism of resistance. Surprisingly, PQ recorded the lowest index of resistance with 1.3 fold decrease in PQ activity against AQ resistant strain. This may suggest a different mechanism of resistance may exist between PQ and AQ.

Artemether is an artemisinin derivative. These derivatives are currently the most effective antimalarial drugs against uncomplicated malaria. They have thus been recommended in combination with other antimalarials as first and second line treatment of uncomplicated malaria in sub Saharan Africa by the WHO (WHO 2006). This study noted a significant 10 fold decrease in artemether activity against AQ resistant strains. This was unexpected since artemether is chemically and mechanistically unrelated to AQ (Robert *et al.,* 2001, Tilley *et al.,* 2016). This indicates the existence of moderate cross- resistance between AQ and artemether in *P.berghei*. The artemisinin are predicted to have a different mode of action and potentially different mechanism of resistance from AQ (Mbengue *et al.,* 2015, Tilley *et al.,* 2016). Since artemisinin has many targets in the rings stage and also metabolically active trophozoite (Eckstein-Ludwig *et al.,* 2003), this study envisage a complex mechanisms controlling loss of ATM efficacy in the AQ resistant phenotype could exist. These complex networks may be revealed by examining the whole genome and transcriptome profile. This worsens the already bad state of artemisinin resistant isolates that have been confirmed in the South East Asia (Miotto *et al*., 2015). This decrease in artemether activity against AQ resistant strains prompted the evaluation SNPs in genes that are associated with artemisinin resistance such as *Kelch13* and *ubp1 (*Hunt *et al*., 2010; Miotto *et al*., 2015*)*. This study thus confirms the existence of cross resistance between AQ and artemether in *P. berghei*.

It is known that the mechanism of resistance in *P. falciparum* may be different from that in murine plasmodium malaria species. For instance, the mechanism of resistance to CQ is different in *P. falciparum* and in murine malaria parasites *P.chabaudi* and there is still a debate whether those of artemisinin derivatives will be similar (Afonso *et al*., 2006; Carlton *et al* 2001; Hunt *et*

al., 2007, 2004a, b). However, for drug such as mefloquine, antifolates and Atovaquone, similar mechanism of resistance have been reported (Carlton *et al*., 2001).

5.3 Evaluation of sequence variation of *Pbcrt, Pbk13, Pbubp1* and *Pbmdr1* genes

After amplifying and sequencing specific coding regions of *Pbcrt, Pbmdr1*, *Pbubp1* and *Pbk13*, there was no change in the nucleotide and their translated protein sequences in *Pbmdr1*, *Pbcrt*, *Pbubp1* and *Pbk13*.

Multi drug resistance gene 1 (*Pbmdr1*) encodes P-glycoprotein homologue 1(Pgh-1) which localizes on digestive vacuole membrane. *Mdr1* may contribute to drug resistance through amplification (increase in copy no) and mutations (Duraisingh and Cowman, 2005). Copy number variation is a key denominator between multidrug resistant phenotypes and 4 amino quinolones (Holmgren *et al.,* 2006b, Duraisingh and Cowman, 2005, Borges *et al.,* 2011). Changes in amino acid; 86, 184, 1034, 1042, and 1246 in *Pfmdr1* have been demonstrated to mediate and/or modulate CQ, LM and mefloquine resistance (Price *et al.,* 2004, Sisowath *et al.,* 2005, Ecker *et al.,* 2012). In *Pbmdr1*, there were no SNPs that were detected. This was expected since recent studies using LM and PQ resistant *P. berghei* parasite found no polymorphism in *mdr1* gene (Kiboi *et al*., 2014). These findings not only give clues of possible differences in AQ resistance mechanisms between *P. falciparum* and rodent malaria *P.berghei* parasite since, *mdr1* is associated with AQ resistance in *P.falciparum* while in *P.berghei* it is not, but also provide basis for understanding new drug evasion mechanisms.

Chloroquine resistance transporter gene (*Pbcrt*) encodes a putative transporter CRT protein. Lys76Thr mutation in the *Pfcrt* gene is the most common marker for CQ resistance and has also been strongly associated with 4-aminoquinoline resistance such as AQ in *P. falciparum* (Fidock

et al., 2000; Ochong *et al.,* 2003; Ecker *et al.,* 2012). Recent studies have also identified potential *crt* background mutations; Ile356Thr and Asn326Ser that associate with artemisinin resistance (Miotto *et al.,* 2015). From the data obtained, there was no point mutation in *Pbcrt* gene. Previous studies however associated *crt* with CQ resistance. The absence of SNPs in this gene was in agreement with previous studies involving LM and PQ in *P. berghei* parasites in which no SNPs in *crt* gene was mapped. This study thus gives insight of a possible different mechanism of resistance between AQ and CQ. Moreover, AQ and CQ mode of action may be different since AQ is still effective against some CQ resistant strains of *P. falciparum.*

Due to the high cross resistance between AQ and ATM, genes such as *kelch 13* and *ubp1* (genes associated with artemisinin resistance) (Hunt *et al*., 2010; Miotto *et al*., 2015) were analyzed for polymorphisms. Artemisinin resistance in *P. falciparum* was previously associated with multiple SNPs in a gene on chromosome 13 (*Kelch 13*) mapping to the b-propeller domain of the encoded kelch-like protein, PF3D7-43700 (Ariey *et al*., 2009). From recent studies, M476I, Y493H, R539T, 543T and C580Y variant showed strong association with artemisinin resistance (Miotto *et al*., 2015; Straimer *et al.,* 2015). Thus in this study, the same region was targeted. From the results, *Pbkelch13* no SNPs were mapped in all the regions analysed. The fact that index of resistance to ATM was double the index of AQ provide solid clues that AQ and ATM share some resistance mechanisms independent of *crt* and *Kelch13*. Importantly, the AQ resistant line fills a valuable niche of identifying new resistance markers and targets, not only for AQ and other quinoline drugs but for artemisinin as well. This study thus does not associate *Pbk13* polymorphism with AQ resistance.

From the data, *Pbup1* is not linked to AQ resistance in *P. berghei* ANKA since there were no SNPs that were detected. However, previous studies in *P. chabaudi* associated polymorphism at

protein position V739F and V770F with mediating CQ resistance (Hunt *et al*., 2007; 2010). The acquisition of resistance to AQ in *P. chabaudi* and *P. berghei* may be thus different. Although *ubp1* as a genetic marker involved in artesunate and chloroquine resistance in *P. falciparum* remains unconfirmed, several have focused on it as possible artesunate resistance marker (Hunt *et al*., 2007; Rodrigues *et al*., 2010). This study does not therefore associate *ubp1* with AQ resistance in *P. berghei* ANKA.

5.4 Expression profile of *Pbvp2, Pbmdr1, Pbnhe1* and *Pbcvx1*

Resistance to drug may arise as a result of amplification of a gene or genes i.e. increase in the copy number. This study reveals that increase in transcript levels of *mdr1* is associated with AQ resistance. Overexpression of *mdr1* is a common marker for multidrug resistant *P. falciparum* (Borges *et al.*, 2011, Gonzales *et al.*, 2008). In addition, the *mdr1* is a master transcriptional regulator for other genes associated with resistance (Gonzales *et al.*, 2008, Jiang *et al.*, 2008). This is in agreement with earlier studies where increase in *mdr1* transcript controls resistance to multiple drugs in *P. chabaudi, P.yoeii* and *P. falciparum* (Chavchich *et al*., 2010; Ferrer-Rodriguez *et al* 2004; Rodriguez *et al*., 2010). The high expression of *mdr1* gene may have three possible implications; first, *mdr1* directly mediate AQ resistance and cross resistance levels; second, *it* acts a master regulator of unknown resistance causal gene and thirdly, *mdr1* transcript changes play duo functions of regulating unknown causal gene and directly mediate AQ resistance or its cross resistance profiles.

The study further revealed significant increase in *Pbcvx1* and *Pbvp2* transcript levels ($p<0.0001$ and $p<0.002$ respectively). *Vp2* and *cvx1* gene are H^+ channel molecule that regulate pH balance in the parasite's food vacuole (Jiang *et al.*, 2008). The differential expression of *Pbcvx1* and *Pbvp2* is thus associated with AQ resistance. This further confirms earlier findings in which, the

putative drug transporter, *Pfcvx1* was associated with CQ resistance by either in response to K76T mutation in *Pfcrt* or to the modulation of CQ resistance (Jiang *et al*., 2008). Recently, *Pbcvx1 and pbvp2* were associated with PQ resistance in *P. berghei* ANKA (Kiboi *et al*., 2014). The elevation of *vp2* and *cvx1* transcript in AQ resistance could play two roles; pH balance and compensate for deleterious mutations. However, the AQ resistant line harbours no mutation in *PfCRT* protein, thus elevation of *vp2* and *cvx1* does not compensate for any mutation in the transporter. Nonetheless, the compensatory role may be directed at unknown variants within the site of action (s). The predicted modes of action for CQ, AQ and PQ is the inhibition of heme polymerization within the food vacuole (O'Neill *et al.*, 2011). This study thus suggests that high *vp2* and *cvx1* expression may play role in regulating pH balance in AQ resistance.

This study also revealed that *Pbnhe1* was significantly differentially expressed. The elevated *nhe1* mRNA transcript is associated with AQ resistance in *P. berghei* ANKA. This is in agreement with previous studies in which alteration of polymorphism in *nhe1* results in variation of Na^+/H^+ regulation in quinine and quinoline based drug resistance (Bennett *et al.,* 2007). The Na^+/H^+ exchanger protein is involved in maintaining transmembrane pH, digestive vacuole pH as well as cytosolic pH (Bennett *et al.*, 2007). This study thus suggests that the AQ resistant parasite evades drug action by elevating *nhe1* transcripts to alter the pH at the site of action thus reducing the drug-target binding affinities.

CHAPTER 6: CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The study has established that:

i) Stable multi drug resistant *P. berghei* are developed by continuous submission of the parasites to AQ drug pressure for 36 passages.

ii) Amodiaquine is associated with cross resistance in lumefantrine, artemether, piperaquine, chloroquine and primaquine. These are chemically and mechanistically related and unrelated to amodiaquine.

iii) Increased transcript level of *Mdr1*, *cvx1*, *vp2* and *nhe1* level is associated with amodiaquine resistance in *P.berghei*. The genes thus play modulatory and compensatory roles in the acquisition of amodiaquine resistance. These findings are consistent with multi drug resistant phenotypes in *P.falciparum* (Gonzales *et al*. 2008; Jiang *et al*., 2008; Mwai *et al* 2012) suggesting that some mechanism in *P.falciparum* and *P.berghei* are similar.

iv) *mdr1*, *crt*, *ubp1* and *kelch13* are not associated with amodiaquine resistance and its cross resistance profile. This suggests that AQ resistance is controlled by unknown causal gene.

6.2 Recommendation

This study thus recommends the following:

i) Due to the limitation of time, the study did not acquire highly stable amodiaquine resistant *P. berghei*; hence, drug pressure selection should be continued.

ii) More studies on cross resistance profile of AQ with other antimalarials should be carried out. This will increase the understanding of different mechanism of resistance among different drugs.

iii) Whole genome sequencing and transcriptome sequencing of the amodiaquine resistant *P.berghei* parasite should be done to reveal other novel mutation that could be present and study their involvement in amodiaquine resistance as well as reveal AQ resistance causal gene.

iv) Validation of the suspected genes using PlasmoGEM resources in *P. berghei* as well as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) in *P. falciparum.* These are gene editing tools that can be used to confirm if the suspected genes are indeed associated with AQ resistance in *P.berghei* and *P. falciparum*.

REFERENCES

Adeoye GO, Nga IC (2007). Comparison of Quantitative Buffy Coat technique (QBC) with Giemsa-stained Thick Film (GTF) for diagnosis of malaria. *Parasitology International*, 56:308–312.

Afonso, A., Hunt, P., Cheesman, S., Alves, A. C., Cunha, C. V., do Rosario, V. and Cravo, P.(2006). Malaria Parasites Can Develop Stable Resistance to Artemisinin but Lack Mutations in Candidate Genes *atp6* (Encoding the Sarcoplasmic and Endoplasmic Reticulum Ca2ATPase), *tctp*, *mdr1*, and *cg10*. *Antimicrobial Agents and Chemotherapy*, 50: 480–489.

Alonso, P. L., Sacarlal, J., Aponte, J. J., Leach, A., Macete, E., Milman, J., Mandomando, I., Spiessens, B., Guinovart, C., Espasa, M., Bassat, Q., Aide, P., Ofori- Anyinam, O., Navia, M. M., Corachan, S., Ceuppens, M., Dubois, M., Demoitié, M., Dubovsky, F., Menéndez, C., Tornieporth, N., Ballou, W. R., Thompson, R and Cohen, J. (2004). Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomized controlled trial. *Lancet,* 364:1411-1420.

Aonuma H, Suzuki M, Iseki H, Perera N, Nelson B, Igarashi I, Yagi T, Kanuka H, Fukumoto S (2008). Rapid identification of *Plasmodium*-carrying mosquitoes using loop-mediated isothermal amplification. *Biochem Biophysiology Research Communication,* 376:671–676.

Ariey F, *et al* (2014). A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature,* 505:50–55. [PubMed: 24352242]

Baird, J.K. (2005). Effectiveness of Antimalarial Drugs. *The New England Journal of Medicine*, 352:1565-1577.

Barman D, Mirdha BR, Samantray JC, Kironde F, Kabra SK, Guleria R (2003). Evaluation of quantitative buffy coat (QBC) assay and polymerase chain reaction (PCR) for diagnosis of malaria. *Journal Communication Discovery,* 35:170–181.

Basco, L. K. and Ringwald, P. (2003). In vitro activities of piperaquine and other 4-Aminoquinolines against clinical isolates of *Plasmodium falciparum* in Cameroon. *Antimicrobial Agents and Chemotherapy*, 47: 1391-1394

Beck HP (1999). How does molecular epidemiology help to understand malaria? *Tropical Medicine and International Health* 4:1–3.

Bell D, Wongsrichanalai C, Barnwell JW (2006). Ensuring quality and access for malaria diagnosis: how can it be achieved? *Nature Review Microbiology*, 4:S7–S20.

Bennett TN, Patel J, Ferdig MT, Roepe PD. (2007). *Plasmodium falciparum* Na+/H+ exchanger activity and quinine resistance. *Molecular Biochemical Parasitology*, 153:48–58

Bloland P., Ettling M. (1999). Making malaria-treatment policy in the face of drug resistance. *Ann Tropical Medicine Parasitology*, 93:5-23

Biagini G., O'Neill P., Nzila A., Ward, S., Bray P., (2005). Antimalarial chemotherapy: young guns or back to the future. *Trends in Parasitology,* 19, 479–487.

Borges, S., Cravo, P., Creasey, A., Fawcett, R., Modrzynska, K., Rodrigues, L., Martinelli, A. & Hunt, P. (2011). Genome wide Scan Reveals Amplification of *mdr1* as a Common Denominator of Resistance to Mefloquine, Lumefantrine, and Artemisinin in *Plasmodium chabaudi* Malaria Parasites. *Antimicrobial Agents and Chemotherapy,* 55**,** 4858–4865.

Breman, J. G., Mills, A., Snow, R. W., Mulligan, J., Lengeler, C., Mendis, K., Sharp, B., Morel, C., Marchesini, P., White, N. J., Steketee, R. W. and Doumbo, O. K. (2006). "Conquering Malaria." Disease Control Priorities in Developing Countries (2nd Edition), ed. 413-432.

Carlton, J., Vinkenoog, R., Waters, A. P., Walliker, D. (1998a) Gene synteny in species of Plasmodium. *Molecular and Biochemical Parasitology*, 93: 285–294.

Carlton, J. M-R., Hayton, K., Cravo, P. V. L. and Walliker, D. (2001) of mice and Malaria mutants: unraveling the genetics of drug resistance using rodent malaria models. *Trends in Parasitology*, 17: 236- 241.

Carlton, J., Mackinnon, M. and Walliker, D. (1998b). A chloroquine resistance locus in the rodent malaria parasite *Plasmodium chabaudi*. *Molecular and Biochemical Parasitology*, 93: 57-72.

CDC, (2016). Laboratory Identification of Parasites of Public Health Concern. Parasites and

Health, Malaria [http://www.dpd.cdc.gov/DPDx/HTML/Malaria.htm]

Chiang, P. K., Bujnicki, J. M., Xinzhuan, S. and Lanar, D. E. (2006). Malaria: Therapy, Genes and Vaccines. *Current Molecular Medicine*, 6: 309-326.

Clendennen TE, 3rd, Long GW, Baird KJ (1995). QBC and Giemsa stained thick blood films: diagnostic performance of laboratory technologists. Trans R Soc *Tropical Medicine Hygiene,* 89:183–184.

Cooper R., Hartwig C., Ferdig M., (2005). *Pfcrt* is more than the *Plasmodium falciparum* chloroquine resistance gene: a functional and evolutionary perspective. *Acta Tropical,* 94, 170–180.

D'Alessandro, U. and Buttiens, H (2001). History and importance of antimalarial drug resistance. *Tropical Medicine and International Health*. 6: 845-848.

Davis, T. M. E., Hung, T., Sim, I., Karenajeewa, H. A. and Ilett, K. F. (2005). Piperaquine: A Resurgent Antimalarial Drug. *Drugs*, 65: 75-87.

de Koning-Ward, T. F., Janse, C. J and Waters, A. P. (2000). The Development of Genetic Tools for Dissecting the Biology of Malaria Parasites. *Annual Review of Microbiology*. 54: 157-185

Duraisingh M.T., Cowman A.F., (2005). Contribution of the *pfmdr1* gene to antimalarial drug-resistance. *Acta Tropica*, 94: 181–190.

Echeverry DF, Holmgren G, Murillo C, Higuita JC, Björkman A, Gil JP, et al (2007). Short report: Polymorphisms in the *pfcrt* and *pfmdr1* genes of *Plasmodium falciparum* and *in vitro* susceptibility to amodiaquine and desethylamodiaquine. *Am Journal Tropical Medicine Hygiene*. 77:1034–8.

Ecker, A., Lehane, A. M., Clain, J. & Fidock, D. A. (2012*). PfCRT* and its role in antimalarial drug resistance. *Trends in Parasitology*, 28**,** 504-514.

Eckstein-Ludwig, U., Webb, R. J., Van Goethem, I. D. A., East, J. M., Lee, A. G., Kimura, M., O'neill, P. M., Bray, P. G., Ward, S. A. & Krishna, S. (2003). Artemisinin target the SERCA of *Plasmodium falciparum. Nature*, 424**,** 957 - 961.

Endeshaw T, Gebre T, Ngondi J, Graves PM, Shargie EB, Ejigsemahu Y, Ayele B, Yohannes G, Teferi T, Messele A, Zerihun M, Genet A, Mosher AW, Emerson PM, Richards FO (2008). Evaluation of light microscopy and rapid diagnostic test for the detection of malaria under operational field conditions: a household survey in Ethiopia. *Malaria Journal*, 7:118.

Eriksen J, Nsimba S., Minzi O., Sanga A, Petzold M, Gustafsson L, Warsame M, Tomson G (2005). Adoption of the new antimalarial drug policy in Tanzania–a cross-sectional study in the community. *Tropical Medicine International Health,* 10:1038-1046.

Eastman R.T. and D. A. Fidock, (2009). "Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria," *Nature Reviews Microbiology*, vol. 7, no. 12, pp. 864–874.

Franke-Fayard, B., Waters, A. P. and Janse, C. J. (2006) Real-time in vivo imaging of transgenic bioluminescent blood stages of rodent malaria parasites in mice. *Nature Protocols*, 1: 476-485.

Ferrer-Rodrıguez I., Perez-Rosado J., Gervais G., Peters. Robinson, B., Serrano, A., (2004). *Plasmodium yoelii*: identification and partial characterization of an *mdr-1* gene in artemisinin-resistant line. *Journal Parasitology*, 90, 152–160.

Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su XZ, *et al*. (2004). Dissecting the loci of low-level quinine resistance in malaria parasites. *Molecular Microbiology*, 52:985–97.

Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, et al (2000). Mutations in the *Plasmodium. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Molecular Cellology*, 6:861–7.

Fidock D., Rosenthal P., Croft S.L., Brun R., Nwaka S. (2004). Antimalarial drug discovery: efficacy model for compound screening. *Nature Review Drug Discovery*, 3, 509–520.

Florens, L., Washburn, M. P., Raine, J. D., Anthony, R. M., Grainger, M., Haynes, J. D., Moch, J. K., Muster, N., Sacci, J. B., Tabb, D. L., Witney, A. A., Wolters, D., Wu, Y., Gardner, M. J., Holder, A. A., Sinden, R. E., Yates, J. R. and. Carucci, D. J. (2002). A proteomic view of the *Plasmodium falciparum* life cycle. *Nature*, 419: 520-526.

Gamble C., Ekwaru, P. J., Garner, P. and ter Kuile, F. O. (2007). Insecticide-Treated Nets for the Prevention of Malaria in Pregnancy: A Systematic Review of Randomized Controlled Trials. *PLoS Medicine*, 4: 0506-051**.**

Gabrielle. A.J and Manuel .L (2015) Sexual development in *Plasmodium* parasites: knowing when it's time to commit. *Nature Review Microbiology*, 13: 573-587.

Gervais G., Trujillo K., Robinson B., Peters W., Serrano A., (1999). *Plasmodium berghei*: identification of an mdr-like gene associated with drug resistance. *Experimental Parasitology*, 91, 86–92.

Ginsburg H., Ward S.A., Bray P., (1999). An integrated model of chloroquine action. *Parasitology Today.* 15: 357–360.

Gonzales, J. M., Patel, J. J., Ponmee, N., Jiang, L., TAN, A., Maher, S. P., Wuchty, S., Rathod, P. K. & Ferdig, M. T. (2008). Regulatory Hotspots in the Malaria Parasite Genome Dictate Transcriptional Variation. *PLoS Biology,* 6**,** e238.

Gorissen, E., Ashruf, G., Lamboo, M., Bennebroek, J., Gikunda, S., Mbaruku, G. and Kager, P. A. (2000). *In vivo* efficacy study of amodiaquine and sulfadoxine/pyrimethamine in Kibwezi, Kenya and Kigoma, Tanzania. *Tropical Medicine and International Health*. 5: 459-463.

Greenwood, B. (2005). Malaria vaccines Evaluation and implementation. *Acta Tropica*, 95: 298–304

Han ET, Watanabe R, Sattabongkot J, Khuntirat B, Sirichaisinthop J, Iriko H, Jin L, Takeo S, Tsuboi T (2007). Detection of four *Plasmodium* species by genus- and species-specific loop-

mediated isothermal amplification for clinical diagnosis. *Journal Clinical Microbiology*. 45:2521–2528.

Holland CA, Kiechle FL (2005). Point-of-care molecular diagnostic systems-past, present and future. *Current Opinion Microbiology*. 8:504–509.

Holmgren, G., Gil, J. P., Ferreira, P. M., Veiga, M. I., Obonyo, C. O. & Bjorkman, A. (2006b). Amodiaquine resistant *Plasmodium falciparum* malaria *in vivo* is associated with selection of *pfcrt* 76T and *pfmdr1* 86Y. *Infection, Genetics and Evolution,* 6**,** 309-314.

Hung, T. Y., Davis, T. M. E., Ilett, K. F., Karenajeewa, H., Hewitt, S., Denis, M. B., Lim, C. and Socheat, D (2004). Population pharmacokinetics of piperaquine in adults and children with uncomplicated falciparum or vivax malaria. *British Journal of Clinical Pharmacology*, 57: 253-262.

Hunt P., Martinelli A., Fawcett R., Carlton J., Carter R., Walliker D. (2004a). Genesynteny and chloroquine resistance in *Plasmodium chabaudi*. *Molecular Biochemistry Parasitology*. 136, 157–164.

Hunt P., Cravo P., Donleavy P., Carlton J.-R., Walliker D., (2004b). Chloroquine resistance in *Plasmodium chabaudi*: are chloroquine-resistance transporter (*crt*) and multi-drug resistance (*mdr1*) orthologues involved? *Molecular Biochemistry Parasitology*. 133, 27–35.

Hunt P., Afonso A., Creasey A., Culleton R., Sidhu A., Logan J., (2007). Gene encoding a deubiquitinating enzyme is mutated in artesunate and chloroquine resistant rodent malaria parasites. *Molecular Microbiology.* 65:27–40.

Hunt P., Martinelli A., Modrzynska K., Borges S., Creasey A., Rodrigues L. (2010). Experimental evolution, genetic analysis and genome re-sequencing reveal the mutation conferring artemisinin resistance in isogenic lineage of malaria parasites. *BMC Genomics,* 11, 499.

Janse, C. and Waters, A. (1995). *Plasmodium berghei*: The application of cultivation and purification techniques for molecular studies of malaria parasites. *Parasitology Today,* 11: 138-143.

Janse C., Ramesar J., Waters A., (2004). *Plasmodium Berghei*: General Parasitological Methods. Leaiden Malaria Research Group, Leiden. *Leiden University Medical Center, Parasitology*.

Janse, C. J., Franke-Fayard, B., Mair, G. R., Ramesar, J., Thiel, C., Engelmann, S., Matuschweski, K.,Jan van Gemert, G., Sauerwein, R. W and Waters, A. P. (2006) High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. *Molecular and Biochemical Parasitology,* 145: 60-70.

Jiang, H., Patel, J. J., YI, M., Mu, J., Ding, J., Stephens, R., Cooper, R. A., Ferdig, M. T. & Su, X. 2008. Genome Wide Compensatory Changes Accompany Drug-Selected Mutations in the *Plasmodium falciparum crt* Gene. *PLos ONE,* 3**,** e2484.

Kamya M, Bakyaita N, Talisuna A, Were W, Staedke S. (2002). Increasing antimalarial drug resistance in Uganda and revision of the national drug policy. *Tropical Medicine International Health,* 7:1031-1041.

Kiboi, D. M., Irungu, B. N., Langat, B., Wittlin. S., Brun. R., Chollet, J., Obiodun, O., Ng'ang'a, J. K., Nyambati, V. C. S., Rukunga, G. M., Bell, A. and Nzila A. (2009). .*Plasmodium berghei*

ANKA: selection of resistance to piperaquine and lumefantrine in a mouse model. *Experimental. Parasitology*, 122, 196–202.

Kiboi, D., Irungu, B., Orwa, J., Kamau, L., Ochola-Oyier, L. I., Ngángá A, J. & Nzila, A. (2014). Piperaquine and Lumefantrine resistance in *Plasmodium berghei* ANKA associated with increased expression of Ca2+/H+ antiporter and glutathione associated enzymes. *Experimental Parasitology*, 147**,** 23–32.

Kublin J., Cortese J., Njunju E., Mukadam R., Wirima J., Kazembe P., Djimde A., Kouriba B., Taylor T., Plowe C., Contreras C., Caraballo A (2003). Re-emergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *Journal Infectious Diseases*, 187:1870-1875.

Langat B., Kiboi D., Irungu B., Kimoloi S.,Nyambati V., Rukunga G., (2010). Lumefantrine-resistant and piperaquine resistant *Plasmodium berghei* show cross resistance to primaquine but not to atovaquone. *Africa Journal Pharmacology Thermometer.* 1, 35–40.

Lengeler, C. (2004). Insecticide-treated bed nets and curtains for preventing malaria. Cochrane *Database of Systematic Reviews*.

Li, X., Bjorkman, A., Andersson, T. B., Ridderstrom, M and Masimirembwa, C. M. (2002). Amodiaquine Clearance and Its Metabolism to N-Desethylamodiaquine Is Mediated by CYP2C8: A New High Affinity and Turnover Enzyme-Specific Probe Substrate. *The Journal of Pharmacology and Experimental Therapeutics*, 300: 399-407.

Livak K., Schmittgen, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 25, 402–408.

Mariga, S. T., Gil, J. P., Sisowath, C., Wernsdorfer, W. H. and Bjorkman, A. (2004). Synergism between Amodiaquine and Its Major Metabolite, Desethylamodiaquine, against *Plasmodium falciparum In Vitro*. *Antimicrobial Agents and Chemotherapy*, 48: 4089–4096

Marianne E Sinka, Michael J Bangs, Sylvie Manguin, Maureen Coetzee, Charles M Mbogo, Janet Hemingway, Anand P Patil, Will H Temperley, Peter W Gething, Caroline W Kabaria, Robi M Okara, Thomas Van Boeckel, H Charles J Godfray, Ralph E Harbach and Simon I Hay (2010) The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. *Biomedical central*, 43:21-56

Martinelli A., Henriques G., Cravo P., Hunt P., (2011). Whole genome re-sequencing identifies a mutation in an ABC transporter (*mdr2*) in a *Plasmodium chabaudi* clone with altered susceptibility to antifolate drugs. *International Journal Parasitology*, 41, 165–171.

Mbengue, A., Bhattacharjee, S., Pandharkar, T., Liu, H., Estiu, G., Stahelin, R. V., Rizk, S. S., Njimoh, D. L., Ryan, Y., Chotivanich, K., Nguon, C., Ghorbal, M., Lopez-Rubio, J. J., Pfrender, M., Emrich, S., Mohandas, N., Dondorp, A. M., Wiest, O. & Haldar, K. (2015). A molecular mechanism of artemisinin resistance in *Plasmodium falciparum* malaria. *Nature,* 520**,** 683-687.

Menard D, Yapou F, Manirakiza A, Djalle D, Matsika-Claquin MD, Talarmin A. (2006). Polymorphisms in *pfcrt, pfmdr1, dhfr* genes and *in vitro* responses to antimalarials in *Plasmodium falciparum* isolates from Bangui, Central African Republic. *Am Journal Tropical Medicine Hygiene.* 75:381–7.

Merkli, B. and Richle, R. W. (1980). Studies on the resistance to single and combined antimalarials in the *Plasmodium berghei* mouse model. *Acta Tropica*, 37, 228–231.

Miotto, O., Amato, R., Ashley, E. A., Macinnis, B., Almagro-Garcia, J., Amaratunga, C., Lim, P., Mead, D., Oyola, S. O., Dhorda, M., Imwong, M., Woodrow, C., Manske, M., Stalker, J., Drury, E., Campino, S., Amenga-Etego, L., Thanh, T. N. N., Tran, H. T., Ringwald, P., Bethell, D., Nosten, F., Pyae Phyo, A., Pukrittayakamee, S., Chotivanich, K., Chuor, C. M., Nguon, C., Suon, S., Sreng, S., Newton, P. N., Mayxay, M., Khanthavong, M., Hongvanthong, B., Htut, Y., Han, K. T., Kyaw, M. P., Faiz, A., Fanello, C. I., Onyamboko, M., Mokuolu, O. A., Jacob, C. G., Takala-Harrison, S., Plowe, C. V., Day, C. P., Dondorp, A. M., Spencer, C. C. A., Mcvean, G., Fairhurst, R. M., White, N. J. & Kwiatkowski, D. P. (2015). Genetic architecture of artemisinin-resistant *Plasmodium falciparum*. *Nature Genetics,* 47**,** 226–234.

Moorthy, V. S., Good, M. F. and Hill, A. V. S. (2004). Malaria vaccine developments. *Lancet,* 363: 150–156

Muangnoicharoen S, Johnson D.J, Looareesuwan S, Krudsood S, Ward SA (2009). Role of known molecular markers of resistance in the antimalarial potency of piperaquine and dihydroartemisinin *in vitro*. *Antimicrobiology Agents Chemotherapy*, 53:1362–6.

Mungai M, Tegtmeier G, Chamberland M, Parise M (2001). Transfusion-transmitted malaria in the United States from 1963 through 1999. *N England Journal Medicine*, 344:1973–1978.

Mwai L., Kiara S., Abdirahaman A., Pole L., Rippert A., Diriye, A., (2009a). .*In vitro* activities of piperaquine, lumefantrine and dihydroartemisinin in Kenyan *Plasmodium falciparum* isolates and polymorphisms in *Pfcrt* and *pfmdr-1*. .*Antimicrobiology* Agents *Chemotherapy*, 5069–5073.

Mwai L., Diriye A., Masseno V., Muriithi S., Feltwell T., Musyoki J., (2012). Genome wide adaptations of *Plasmodium falciparum* in response to lumefantrine selective drug pressure. PLoS ONE 7, e31623.

Nzila A., Mwai L., (2010). *In vitro* selection of *Pasmodium falciparum* drug resistant parasite lines. *Journal of Antimicrobiology Chemotherapy*. 65, 390–398.

Nzila, A. (2006). The past, present and future of antifolates in the treatment of *Plasmodium falciparum* infection. *Journal of Antimicrobial Chemotherapy*, 57:1043–1054

O'neill, P. M., Barton, V. E., Ward, S. A. & Chadwick, J. (2011). 4-aminoquinolines: chloroquine, amodiaquine and next-generation analogues, Basel, *springer.*

Ochong, E. O., Van Den Broek, I., Keus, K. & Nzila, A. (2003). Association between chloroquine and amodiaquine resistance and allelic variation in the *Plasmodium falciparum* multiple drug resistance 1 gene and the chloroquine resistance transporter gene in isolates from the upper Nile in southern Sudan. *American journal of tropical medicine and hygiene,* 69**,** 184–187.

Palacios G, Quan PL, Jabado OJ, Conlan S, Hirschberg DL, Liu Y, Zhai J, Renwick N, Hui J, Hegyi H, Grolla A, Strong JE, Towner JS, Geisbert TW, Jahrling PB, Büchen-Osmond C, Ellerbrok H, Sanchez-Seco MP, Lussier Y, Formenty P, Nichol MS, Feldmann H, Briese T, Lipkin WI (2007). Panmicrobial oligonucleotide array for diagnosis of infectious diseases. *Emerging Infectious Diseases*. 13:73–81.

Peters W (1971). Malaria. Chemoprophylaxis and chemotherapy. *BMC,* 2:95-98.

Peters, W. and Robinson, B. L. (1999). The chemotherapy of rodent malaria. LVI. Studies on the development of resistance to natural and synthetic endoperoxides. *Annals of Tropical Medicine and Parasitology*, 93: 325-339**.**

Peters, W. and Robinson, B. L. (2000). The chemotherapy of rodent malaria. LVIII. Drug combinations to impede the selection of drug resistance, part 2: the new generation—artemisinin

or artesunate with long-acting blood schizontocides. *Annals of Tropical Medicine and Parasitology*, 94: 23-35**.**

Philippe J Guerin, Piero Olliaro, Francois Nosten, Pierre Druilhe, Ramanan Laxminarayan, Fred Binka, Wen L Kilama, Nathan Ford, Nicholas J White (2002). Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development. *The lancet infectious diseases*, 9:564-573.

Poon LL, Wong BW, Ma EH, Chan KH, Chow LM, Abeyewickreme W, Tangpukdee N, Yuen KY, Guan Y, Looareesuwan S, Peiris JS (2006). Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. *Clinical Chemotherapy*, 52:303–306.

Pornsilapatip J, Namsiripongpun V, Wilde H, Hanvanich M, Chutivongse S (1990). Detection of Plasmodia in acridine orange stained capillary tubes (the QBC system) Southeast Asian. *Journal Tropical Medical Public Health*, 21:534–540.

Price, R. U., A., Brockman, A., Mcgready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S., White, N. J., Nosten, F. & Krishna, S. (2004). Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet*, 364**,** 438–447.

Randall M (2014).The origin of Antimalarial-Drug Resistance. 371:397-399.

Ratsimbasoa A, Fanazava L, Radrianjafy R, Ramilijaona J, Rafanomezantsoa H, Ménard D (2008). Evaluation of two new immunochromatographic assays for diagnosis of malaria. *Am Journal Tropical Medicine Hygiene*. 79:670–672.

Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF (2000). *Pgh1* modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature,* 403:906–9.

Reesing HW (2005). Europea4n strategies against the parasite transfusion risk. *Transfus Clinical Biology,* 12:1–4.

Ridley, R.G. (2002). Medical need, scientific opportunity and the drive for antimalarial drugs. *Nature*, 415: 686-693**.**

Robert, A., Benoit-Vical, F., Dechy-Cabaret, O. and Meunier, B. (2001) From classical antimalarial drugs to new compounds based on the mechanism of action artemisinin. *Pure and Applied Chemistry*, 73: 1173-1188**.**

Rodrigues L., Henriques G., Borges S., Hunt P., Sanchez C., Martinelli A. (2010). Experimental evolution of resistance to artemisinin combination therapy results in amplification of the *mdr1* gene in a rodent malaria parasite. *PLoS ONE*5, e11593.

Sá JM, Twu O, Hayton K, Reyes S, Fay MP, Ringwald P, *et al* (2009). Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine. *Procedure Nature Academy Science U S A*, 106:18883–9.

Salako LA, Akinyanju O, Afolabi BM (1999). Comparison of the standard Giemsa-stained thick blood smear with the Quantitative Buffy Coat Technique in malaria diagnosis in Nigeria. Niger Q *Journal Hospital Medicine*, 9:256–269.

Schlitter M., (2008). Antimalarial drugs – what is in use and what is in the pipeline. *Archnometer. Pharmacology*, 341, 149–163.

She RC, Rawlins ML, Mohl R, Perkins SL, Hill HR, Litwin CM (2007). Comparison of immunofluorescence antibody testing and two enzyme immunoassays in the serologic diagnosis of malaria. *Journal Travel Medicine,* 14:105–111.

Shretta R, Omumbo J, Rapuoda B, Snow RW (2000). Using evidence to change antimalarial drug policy in Kenya. *Tropical Medicine International Health,* 5:755-764.

Sibley CH (2014). Understanding drug resistance in malaria parasites: Basic science for public health. *Molecular Biochemistry Parasitology*. 195:107–14.

Sidhu AB, Valderramos SG, Fidock DA (2005). *Pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. *Molecular Microbiology*, 57:913–26.

Sisowath C., Stromberg J., Martensson, A., Msellem, M., Obondo, C., Bjorkman, A., (2005). *In vivo* selection of *Plasmodium falciparum pfmdr1* 86N coding alleles by artemether-lumefantrine (Coartem). *Journal Infectious Disease*. 191, 1014–1017.

Sisowath C., Ferreira P., Bustamante L., Dahlstrom S., Martensson A., Bjorkman A.(2007). The role of *pfmdr1* in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa. *Tropical Medicine International Health* 12, 736–742.

Sisowath C., Petersen I., Veiga M., Martensson A., Premji Z., Bjorkman A.(2009). *In vivo* selection of *Plasmodium falciparum* parasites carrying the chloroquine susceptible *Pfcrt*K76allele after treatment with artemether lumefantrine in Africa. *Journal Infectious Disease.* 5, 750–757.

Smith, D. F. and Parsons, M. (1996). Molecular Biology of Parasitic Protozoa. Oxford University *Press New York USA*, p 81.

Straimer, J., Gnädig, N. F., Witkowski, B., Amaratunga, C., Duru, V., Ramadani, A. P.,Dacheux, M., Mhim, N., Zhang, L., Lam, S., Gregory, P. D., Urnov, F. D., Mercereau-Puijalon, O., Benoit-Vical, F., fairhurst, r. m., ménard, d. & Fidock, D. A. (2015). K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. *Science*, 347**,** 428-431.

Sulzer AJ, Wilson M, Hall EC (1969). Indirect fluorescent-antibody tests for parasitic diseases. An evaluation of a thick-smear antigen in the IFA test for malaria antibodies. *Am Journal Tropical Medicine Hygiene*, 18:199–205.

Tarning, J., Lindegardh, N., Annerberg, A., Singtoroj, T., Day, N. P. J., Ashton, M and White, N. J. (2005). Pitfalls in Estimating Piperaquine Elimination. *Antimicrobial Agents and Chemotherapy*, 49: 5127–5128.

Tilley, l., Straimer, J., Gnädig, N. F., Ralph, S. A. & Fidock, D. A. (2016). Artemisinin action and resistancein *Plasmodium falciparum*. T*rends in parasitology*, 32**,** 682 - 696.

Valderramos, S. G. and Fidock D. A. (2006). Transporters involved in resistance to antimalarial drugs. *Trends in Pharmacological Sciences*, 27:594-601

Vangapandu, S., Jain, M., Kaur, K., Patil, P., Patel, S. R. and Jain, R. (2006) Recent Advances in Antimalarial Drug Development. *Medicinal Research Review*, 1: 65-107.

Warhurst DC, Williams JE (1996). Laboratory diagnosis of malaria. *Journal Clinical Pathology*, 49:533–538.

Warhurst, D. C., Steele, J. C. P., Adagu, I. S., Craig, J. C. and Cullander, C. (2003). Hydroxychloroquine is much less active than chloroquine against chloroquine-resistant

Plasmodium falciparum, in agreement with its physicochemical properties. *Journal of Antimicrobial Chemotherapy*, 52:188–193.

Webster, D and Hill, A.V.S. (2003) Progress with new malaria vaccines. *Bulletin of the World Health Organization*, 81: 902–909**.**

Winstanley, P., Ward, S., Snow, R. and Breckenridge, A. (2004) Therapy of Falciparum Malaria Sub-Saharan Africa: from Molecule to Policy. *Clinical Microbiology Reviews*, 17: 612-637.

White, N. J. (2004) Antimalarial drug resistance. *The Journal of Clinical Investigation*, 113:1084–1092.

WHO 2001: Drug resistance in Malaria: Malaria Epidemiology Branch Centers for Disease Control and Prevention Chamblee, GA, United States of America. WHO/CDS/CSR/DRS/2001.4.

WHO 2006. Guidelines for the Treatment of Malaria, second ed. World Health Organization, Geneva.

WHO 2010. Guidelines for the treatment of malaria. Geneva: World Health Organization.

WHO 2016. World Malaria Report. Geneva: World Health Organization.

Xiao, S.H., Yao, J.M., Utzinger, Y.C., Chollet, J. and Marcel, T. (2004). Selection and Reversal of *Plasmodium berghei* resistance in the mouse model following repeated high doses of artemether. *Parasitology Research*, 92: 215-21

APPENDICES

APPENDIX 1: *IN VIVO* ACTIVITY PROFILE OF AQ

Table 5: *In vivo* activity of AQ against *P. berghei* ANKA at 24th passage

No of cages	Drug	Dosage(mg/kg)	Average parasitaemia D3	Average parasitaemia D4	Average parasitaemia D7	Average parasitaemia D9	Average parasitaemia D11
1	AQ	10	0.13	0.63	0.96	1.2	1.76
2	AQ	5	0.3	2.64	3.36	4.06	4.42
3	AQ	2.5	0.54	4.99	5.56	9.62	13.42
4	AQ	1.25	0.69	8.58	10.56	13.34	15.22
5(control)			0.99	15.86	23.46	28	32.99

Fig 5: *In vivo* activity profile of AQ against *P. berghei* at 24th passage

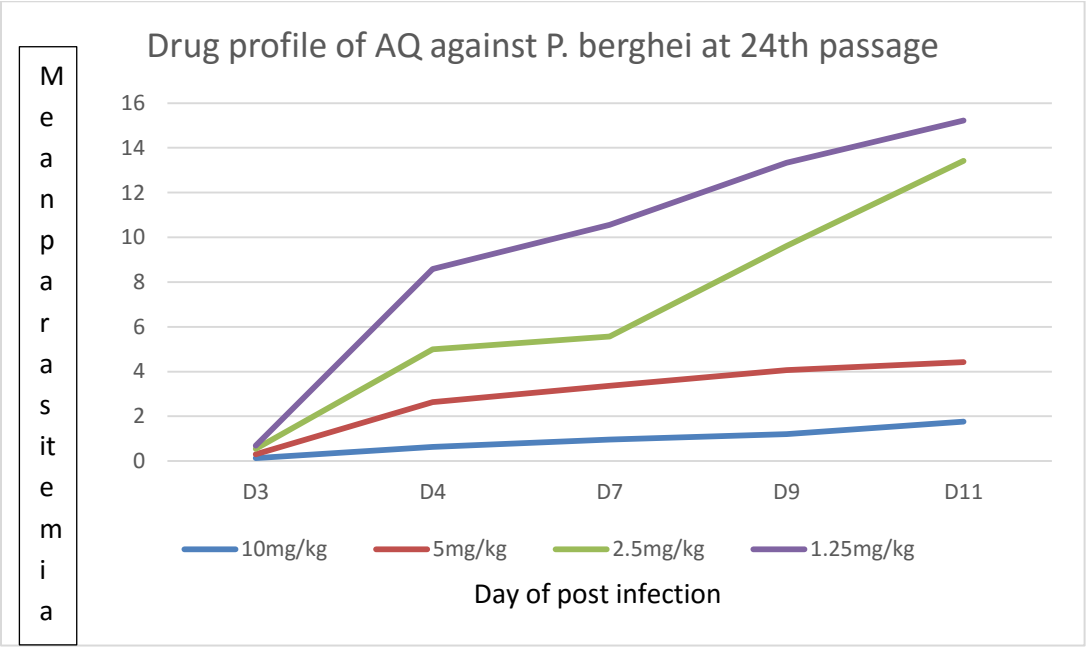
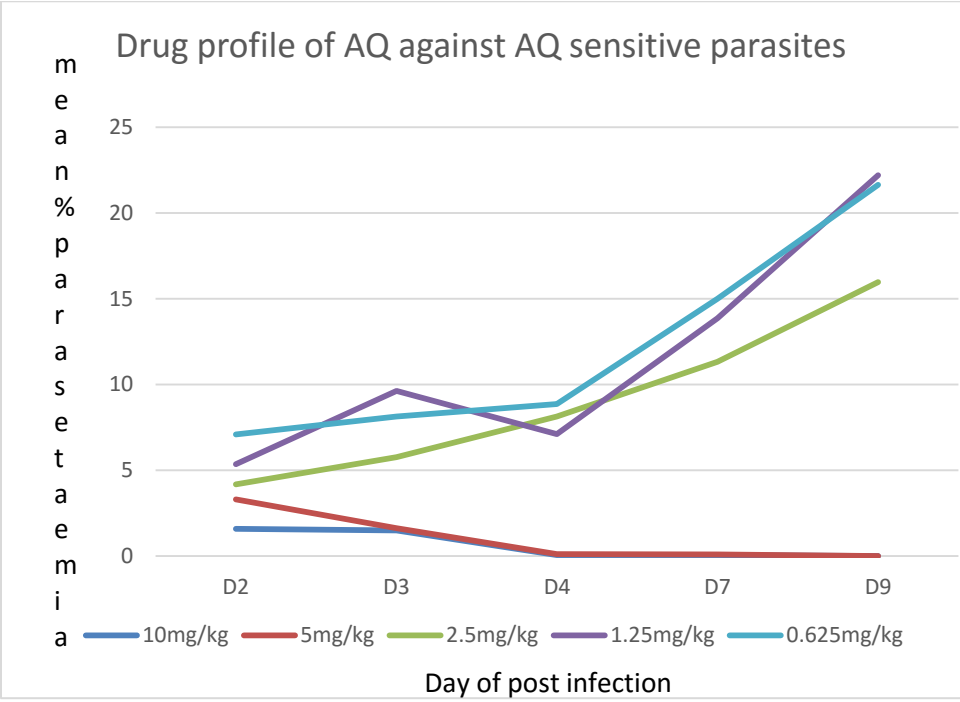


Table 6: *In vivo* activity of AQ against AQ sensitive parasite *P. berghei* ANKA

No of cages	Drug	Dosage(mg/kg)	Average parasitaemia D2	Average parasitaemia D3	Average parasitaemia D4	Average parasitaemia D7	Average parasitaemia D9
1	AQ	10	1.57	1.48	0.05	0.04	0
2	AQ	5	3.3	1.61	0.11	0.08	0
3	AQ	2.5	4.18	5.76	7.1	11.32	15.98
4	AQ	1.25	5.27	7.63	8.7	13.86	22
5	AQ	0.625	7.08	8.0	8.86	15	21.65
6(control)			9.02	18.52	23.30	28.01	32.17

Fig 6: *In vivo* activity of AQ against AQ sensitive parasite *P. berghei* ANKA



APPENDIX 2: *IN VIVO* ACTIVITY PROFILE OF PIPERAQUINE

Table 7: *In vivo* activity of Piperaquine against Amodiaquine resistant parasite *P. berghei* ANKA

No of cages	Drug	Dosage(mg/kg)	Average parasitaemia D3	Average parasitaemia D4	Average parasitaemia D7	Average parasitaemia D9
1	PQ	10	0.34	1.22	2.62	3.59
2	PQ	5	1.25	2.3	3.63	4.61
3	PQ	2.5	1.36	2.68	5.3	6.08
4	PQ	1.25	1.54	4.21	5.94	7.38
5	PQ	0.625	1.69	6.24	7.29	9.24
6(control)			2.59	10.53	14.66	22.86

Fig 7: *In vivo* activity of Piperaquine against Amodiaquine resistant parasite *P. berghei* ANKA

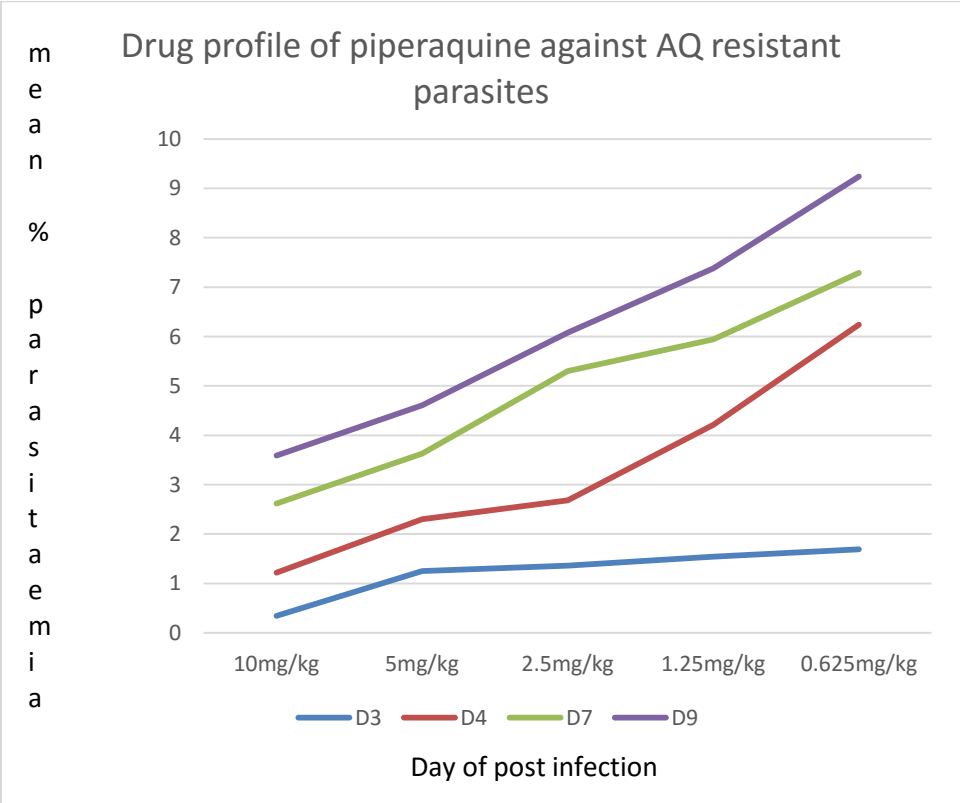
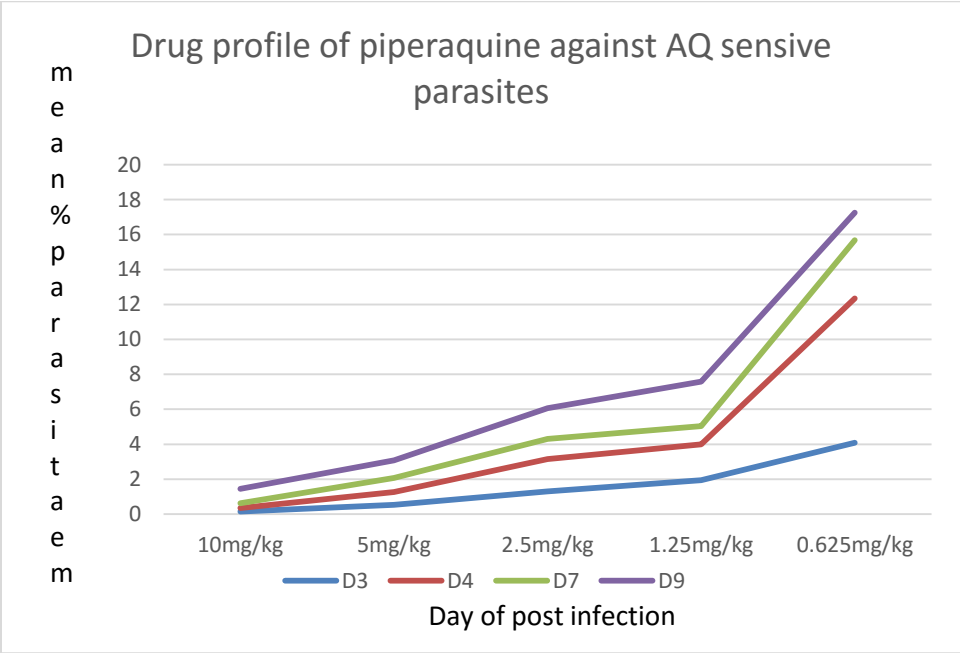


Table 8: *In vivo* activity of Piperaquine against Amodiaquine sensitive parasite *P. berghei* ANKA

No of cages	Drug	Dosage(mg/kg)	Average parasitaemia D3	Average parasitemia D4	Average parasitaemia D7	Average parasitaemia D9
1	PQ	10	0.14	0.34	0.63	1.44
2	PQ	5	0.53	1.27	2.06	3.08
3	PQ	2.5	1.3	3.14	4.30	6.06
4	PQ	1.25	1.94	4	5.04	7.58
5	PQ	0.625	4.09	12.35	15.68	17.25
6(control)			9.26	20.3	23.7	27.75

Fig 8: *In vivo* activity of Piperaquine against Amodiaquine sensitive parasite *P. berghei* ANKA



APPENDIX 3: *IN VIVO* ACTIVITY PROFILE OF ARTEMETHER

Table 9: *In vivo* activity of Artemether against Amodiaquine resistant parasite *P. berghei* ANKA

No of cages	Drug	Dosage(mg/kg)	Average parasitaemia D3	Average parasitemia D4	Average parasitaemia D7
1	ATM	5	2.55	6.06	9.06
2	ATM	2.5	3.76	8.23	11.21
3	ATM	1.25	4.99	10.3	13.37
4	ATM	0.625	6.13	11.58	13.91
5	ATM	0.3125	9.61	13.06	15.85
6(control)			10.3	13.10	20.7

Fig 9: *In vivo* activity of Artemether against Amodiaquine resistant parasite *P. berghei* ANKA

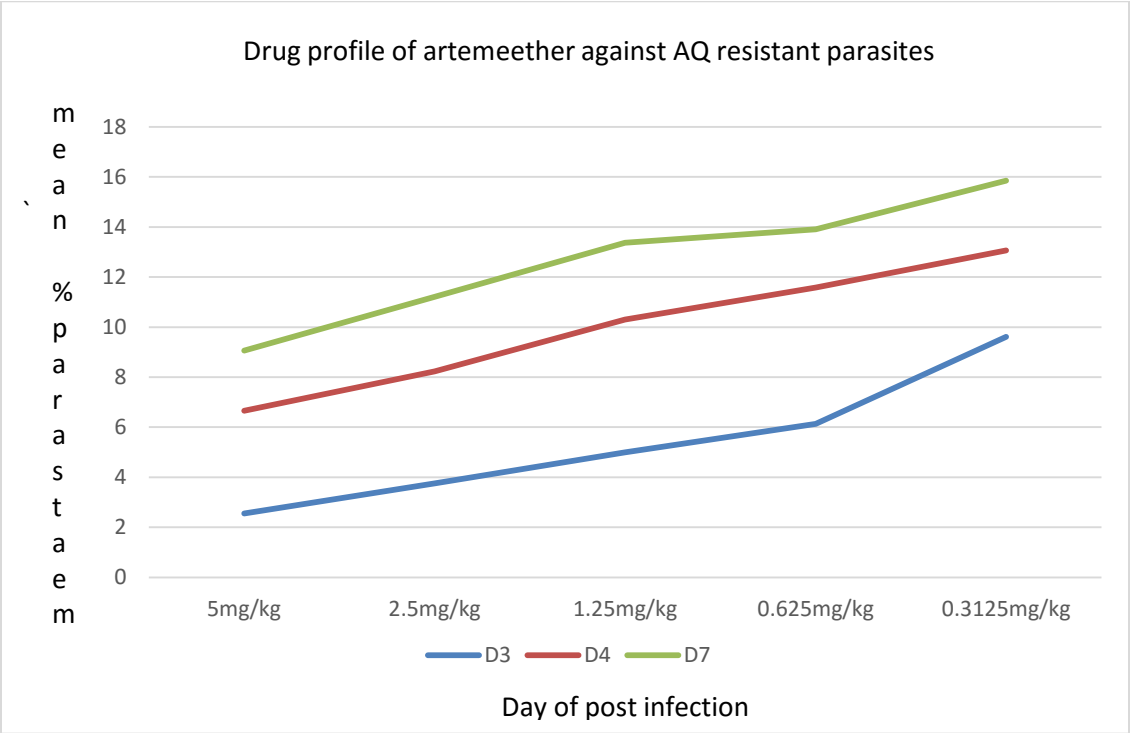
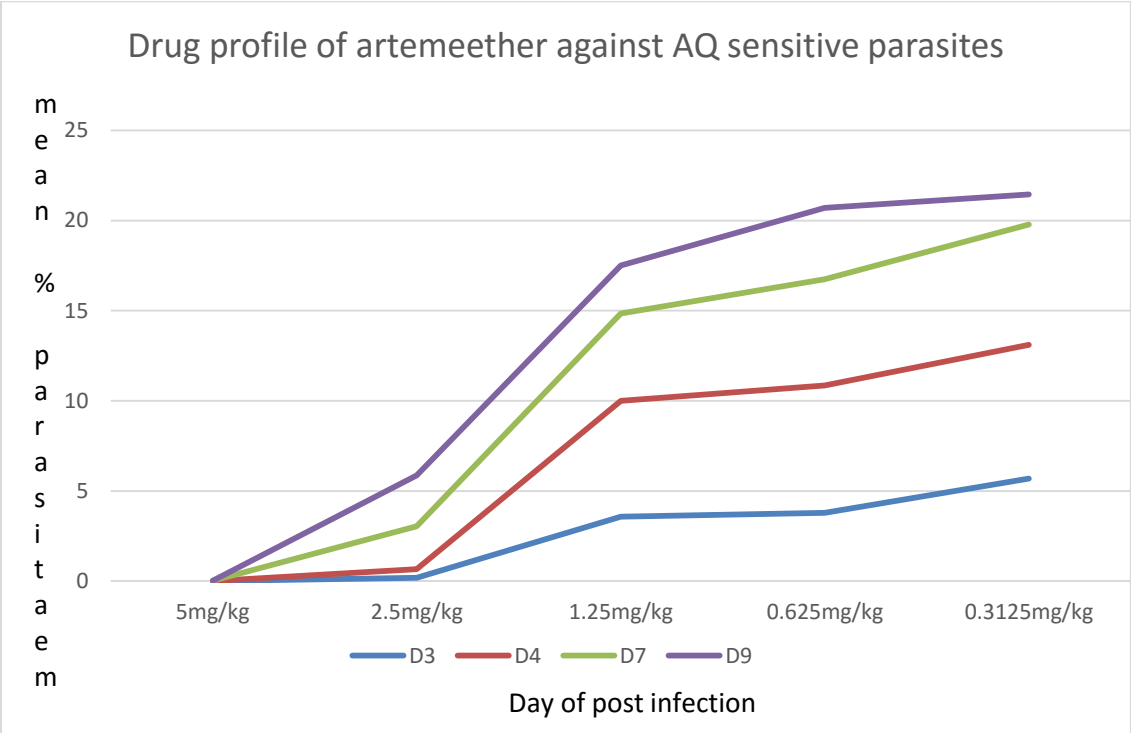


Table 10: *In vivo* activity of Artemether against Amodiaquine sensitive parasite *P. berghei* ANKA

No of cages	Drug	Dosage(mg/kg)	Average parasitaemia D3	Average parasitemia D4	Average parasitaemia D7	Average parasitaemia D9
1	ATM	5	0.00	0	0.02	0.03
2	ATM	2.5	0.18	0.66	3.05	5.86
3	ATM	1.25	3.58	9	14.84	17.51
4	ATM	0.625	3.79	10.86	16.74	20.7
5	ATM	0.3125	5.69	13.1	19.78	21.45
6(control)			9.26	20.3	23.7	27.75

Fig 10: *In vivo* activity of Artemether against Amodiaquine sensitive parasite *P. berghei* ANKA



APPENDIX 4: *IN VIVO* ACTIVITY PROFILE OF CHLOROQUINE

Table 11: *In vivo* activity of Chloroquine against Amodiaquine resistant parasite *P. berghei* ANKA

No of cages	Drug	Dosages	Average parasitaemia D2	Average parasitaemia D3	Average parasitemia D4
1	CQ	10	9.64	13.2	15.8
2	CQ	5	11.6	15.14	17.2
3	CQ	2.5	15.6	17.21	19.15
4	CQ	1.25	16.64	18.9	20.04
5	CQ	0.625	17.2	20	22.4
6(control)			19.3	22.2	25.2

Fig 11: *In vivo* activity of Artemether against Amodiaquine resistant parasite *P. berghei* ANKA

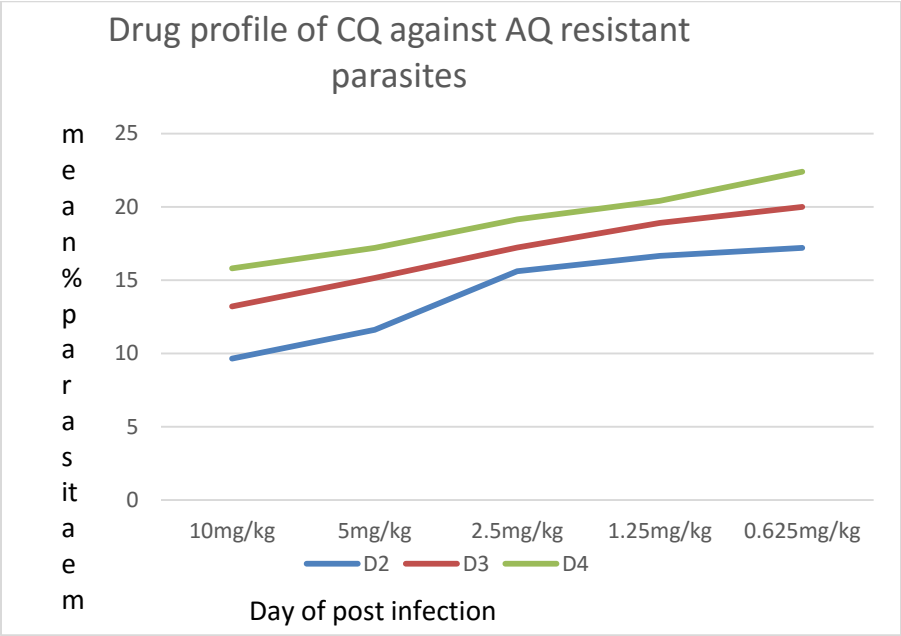


Table 12: *In vivo* activity of Chloroquine against Amodiaquine sensitive parasite *P. berghei* ANKA

No of cages	Drug	Dosage(mg/kg)	Average parasitemia D2	Average parasitemia D3	Average parasitemia D4	Average parasitemia D7	Average parasitemia D9
1	CQ	10	0	0	0	0	0
2	CQ	5	0.2	0.13	0.3	0.47	15.33
3	CQ	2.5	0.45	0.3	3.8	14.28	31.23
4	CQ	1.25	1	1.85	7.9	27.38	33.7
5	CQ	0.625	1.35	2.8	11.7	39.83	47.33
6(control)			7.5	9.34	25.2	42.9	49.8

Fig 12: *In vivo* activity of Chloroquine against Amodiaquine sensitive parasite *P. berghei* ANKA