GENETIC BASIS OF RESISTANCE IN *Plasmodium falciparum* PARASITES EXPOSED TO PURE ARTEMISININ AND *Artemisia annua* EXTRACTS

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Genetic basis of resistance in *Plasmodium falciparum* parasites exposed to pure artemisinin and *Artemisia annua* extracts

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DECLARATION

This thesis is my original work and has not been presented for the award of degree in any other university

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

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DEDICATION

This thesis is dedicated to my entire family of Stephen Moffat Maranga and Esther Maranga.

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ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin based combination therapy
ART	Artemisinin
ARP-10	Apicoplast ribosomal protein-10
ATP	Adenosine triphosphate
BAM	Binary alignment map file
BQSR	Base quality score recalibration
BWA	Burrow-wheeler aligner
CDC	Center for disease control
CDS	Coding sequences
CNV	Copy number variation
CQ	Chloroquine
DDT	Dichlorodiphenyltrichloroethane
DHA-PQ	Dihydroartemisinin-piperaquine
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
DV	Digestive vacuole
FD	Ferrodoxin
HIV	Human immunodeficiency virus
HRP-II	Histidine rich protein II
GATK	Genome analysis tool kit
IC_{50}	Inhibition concentration
IRS	Insecticide residual spraying
K13	Kelch 13 gene
LLIN	Long lasting insecticide treated nets
MDR2	Multi-drug resistant 2 gene

MFQ	Mefloquine
MSP	Merozoite surface protein
NGS	Next generation sequencing
PI3K	Phosphatidyl inositol-3-kinase
PCR	Polymerase chain reaction
PFCRT	*Plasmodium falciparum* chloroquine resistant transporter
PFMDR1	*Plasmodium falciparum* multi-drug resistant 1 gene
PLDH	Plasmodium lactate dehydrogenase
PRD	Pyronaridine
QBC	Quantitative buffy coat
QC	Quality control
RBC	Red blood cells
RDS	Rapid diagnostic test
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RSA	Ring-stage survival assays
SAM	Sequence alignment map format
SEA	Southeast Asia
SERCA	Sarco endoplasmic reticulum calcium independent ATPase
SNP	Single nucleotide polymorphism
SP	Sulfadoxine-Pyrimethamine
TNF	Tumor necrosis factor
UBP-1	Ubiquitin specific protease 1
WHO	World Health Organization
WGS	Whole genome sequencing
VCF	Variant call file

ABSTRACT

Artemisinin-based combination therapies are the current front-line antimalarials in majority of the countries where the disease is endemic. Their safety and tolerability profile is excellent. However, the emergence of artemisinin resistance in Southeast Asia is alarming. Resistance is characterized by prolonged parasite clearance rates (CR) following treatment and reduced ring-stage susceptibility to artemisinin. Therefore, understanding the genetic basis of resistance would be critical to the success treatment and intervention strategies. This study aimed at identifying single nucleotide polymorphisms associated with artemisinin and *Artemisia annua* resistance. Genetic analysis was done on *Plasmodium falciparum* lines W2 (CQ-resistant strain from Indochina) and D6 (CQ-sensitive strain from Sierra Leone), previously selected under pure artemisinin and *Artemisia annua* extract for 3 years. Genomic DNA was extracted using QIAamp blood mini kit and libraries were sequenced on Illumina Miseq platform using 151bp paired-end chemistry. D6 and W2 parasites cultured without drug exposure were used as controls. Paired-end short reads were mapped against *P.falciparum* reference genome sequence version 3.1. One non-synonymous mutation K189T (70%) was identified in the *Plasmodium*/Apicomplexa-specific domain of K13 gene. The *Pfmdr1* mutation N86Y was detected in W2 parasites exposed to artemisinin drug at IC_{50} equivalents and notably, all the parasites showed a single *Pfmdr1* copy number. The *Pfcrt* K76T mutation was detected in two samples, W2 parental line and W2 parasites exposed to ART drug. Also, one background mutation was identified in *Pfcrt* gene (I356T) in W2 parasites exposed to pure artemisinin at $1C_{50}$ equivalents. This study showed a very limited variability in K13 gene sequence and the K189T mutation described here has not been linked to reduced parasite clearance or *in vitro* artemisinin tolerance. The *Pfmdr1* gene may putatively play a role in artemisinin resistance although additional studies are required to conclusively confirm this. Additionally, parasites exposed to *Artemisia annua* harbored wildtype alleles in all the gens analyzed here. Therefore, no mutation was associated with *Artemisia annua* resistance.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria, a parasitic infectious disease, is widespread in the tropic and sub-tropic regions of the world. It is a major health problem, taking each year 438,000 lives and causing 214 million infections (World Health Organization, 2015). Although malaria is disseminated throughout more than 100 countries, sub-Saharan Africa shares 90% of the global malaria burden (WHO, 2017). The severity of the disease is observed in children under 5 years of age and pregnant women. In Kenya 5.1% mortality rate has been reported among patients admitted with severe malaria (Sultan *et al*., 2017). Malaria in humans is caused by five main species namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. knowlesi* and *P. malariae*. *Plasmodium falciparum* is the most prevalent species accounting for majority of morbidity and mortality in Africa (WHO, 2006a).

Antimalarial drug resistance has rendered most drugs useless and facilitated the transmission of drug resistant strains in many endemic areas of the world (Laxminarayan, 2004). Over the past decades, chloroquine was the mainstay of malaria therapy. However, resistance rendered it clinically ineffective in most areas of the world. This led to introduction of sulfadoxine-pyrimethamine (SP) as the first line drug for uncomplicated malaria (Hyde, 2005). The rapid spread of SP resistance required attention for an alternative treatment strategies to replace the failing drugs. In 2006, the World Health Organization recommended the use of artemisinin combination therapies (ACT) as first-

line treatment of both uncomplicated and severe *falciparum* malaria (WHO, 2010b). ACTs have then remained the most powerful chemotherapeutic tool as shown by their rapid activity against certain stages of gametocytes. The rationale behind ACTs is that combining two drugs with differing mechanism of action reduces the chance of parasites simultaneously developing resistance as a result of genetic mutation (Nosten and white, 2007). Since artemisinins have a short half-life of ~1-2 hours, they are co-administered with longer half-life partner drugs (WHO, 2006a). The combination provides good protection against the emergence of resistance to the partner drug, but once resistance has developed, the residual concentration of unprotected partner drug provides a selective filter enhancing the spread of resistance to the partner compound (White, 2004). The widespread adoption of ACTs has been a key factor behind the reduction of malaria transmission in many regions of the world (Peak *et al*., 2015). However, the emergence of artemisinin resistance in western Cambodia threatens to reverse these impressive gains.

Clinical artemisinin resistance is characterized by reduced parasite clearance following treatment with artemisinin derivatives (Dondorp *et al*., 2009, Phyo *et al*., 2012), and it was correlated with *in vitro* artemisinin resistance (Witkowski *et al*., 2013). This reduced sensitivity to artemisinins may spread to other regions or arise independently elsewhere, including Africa, where the malaria burden is highest. Thus, creating a major drawback in malaria control especially in high transmission African settings.

Although, parasite clearance rates have been widely used to assess the *in vivo* drug responses to artemisinin derivatives (Stepniewska *et al*., 2010), its precise measurement

requires frequent sampling and this is often logistically difficult to implement in resource-constrained settings. Consequently, there is a need to develop *in vitro* assays to mimic clinical drug exposure and also to identify molecular markers of artemisinin resistance. Genetic markers of resistance constitute a complex pattern of single nucleotide polymorphisms (SNPs) in the pertinent genes. Regions on chromosome 10, 13, 14 that are likely to show strong associations with the slow-clearance phenotype have been identified (Cheeseman *et al*., 2012). A molecular marker for artemisinin resistance was discovered following whole genome sequencing of artemisinin-resistant parasite line from Africa and clinical parasite isolates from Cambodia (Ariey *et al*., 2014). The study showed that reduced ring stage survival rates ($RSA_{0\text{-}3}$hrs) and prolonged parasite clearance rates were associated with single nucleotide polymorphisms in K13 propeller domain (Pf3D7_1343700). Mutation with M476I allele was identified in Tanzanian *P.falciparum* strain F32-ART that had undergone *in vitro* artemisinin selection for five years. Further research on parasite isolates from Cambodia revealed mutations in C580Y, Y493H and R539T. Other studies confirmed that reduced parasite clearance half-life > 5hrs were strongly associated with K13 propeller mutations across Southeast Asia, where mutation at codon 580 (C580Y) was the most predominant (Ashley *et al*., 2014). Non-synonymous mutations in the BTB-POZ domains (KPBD) have also shown to result in reduced sensitivity of *P.falciparum* to artemisinin (Miotto *et al*., 2015). Parasites with KBPD mutations tend to grow more slowly in the early part of the erythrocytic cycle, and they can withstand against oxidative damage caused by artemisinin (Dogovski *et al*., 2015; Mok *et al*., 2015). The K13 variants that confer resistance in *P.falciparum* parasites from

Southeast Asia were however not detected in isolates of Africa origin (Ashley *et al*., 2014; Kamau *et al*., 2014, Taylor *et al*., 2015). This is expected since the SNPs conferring resistance to artemisinin Southeast Asia might be different from the ones causing resistance in Africa.

Artemisia annua is rich in terpenes (mainly mono and sesqui), artemisinin compound, flavonoids, and polyphenolic acids (Weathers *et al*., 2015). Traditionally, it was used by Chinese herbalists for treatment of intermittent fevers associated with malaria infections. *Artemisia annua* extract was recently shown to overcome existing resistance to pure artemisinin in rodent malaria parasites *Plasmodium yoelli* (Elfawal *et al*., 2015). This is an interesting finding and therefore, there is need to understand the basis of *Artemisia annua* extract resistance, yet, to date very few studies have been done in Kenya.

Understanding the molecular basis associated with resistance is critical since the information could be used in designing better therapeutic strategies and identification of new drug targets. This study focused on the identification of point mutations associated with artemisinin and *artemisia annua* resistance as well as copy number variation in *Pfmdr1* gene. Two parasite lines of different levels of resistance (W2-CQ resistance and D6-CQ sensitive) were sequenced and the differences between them mapped.

1.2 Statement of the problem

Artemisinin, a rapidly acting antimalarial agent, has been the foundation of *P. falciparum* malaria treatment globally. However, clinical resistance to artemisinin and its derivatives have been reported in Southeast Asia (Ariey *et al*., 2014; Ashley *et al*., 2014). This is

devastating as there are presently no further well established treatment options alternative to ACTs. If artemisinin resistance were to spread to sub-Saharan Africa it could have a tremendous impact on malaria-related morbidity and mortality. Therefore, there is need to understand the genetic basis of *Plasmodium falciparum* resistance to key short half-life artemisinin and its parent plant *Artemisia annua* extract. While there have been considerable efforts geared towards identification of the genetic markers of artemisinin resistance, a lot need to be done. Few studies have been done in Kenya to assess the genetic changes associated with antimalarial drug resistance in laboratory selected lines. These selected parasites provide insight about genotypic changes associated with resistance.

1.3 Justification

Molecular techniques have been used to identify markers coding for artemisinin resistance. However, most studies analyze only a few SNPs as primary detectors of resistance. This creates a practical obstacle in monitoring the current epidemic of artemisinin resistance. Today, many pathogen genomes have been sequenced and it's hoped that genomic studies will open new avenues that can be exploited to control ART resistance. Various regions that appear to play a role in regulation of artemisinin resistance have already been identified. For example, genetic association studies strongly linked locus on chromosome 10, 13 and 14 to artemisinin resistance (Cheeseman *et al*., 2012). However, the genetic basis of *P. falciparum* drug resistance to ART have not been completely elucidated. The present study exploited whole genome sequencing of drug-pressured *Plasmodium falciparum* clones D6 and W2 to identify single nucleotide polymorphisms associated

with resistance to artemisinin and *Artemisia annua*. This study will improve on the available knowledge on the genetics of ART resistance and in effect potentiate development of strategies to contain it. In addition, the study could contribute to clarifying if resistance to *Artemisia annua* extract builds up as a result of gene mutation, and eventually guide us on whether to make use of the crude extract as an additional tool for malaria control.

1.4 Hypothesis

1.4.1 Null hypothesis

1) Artemisinin and *Artemisia annua* pressure does not select mutations in *Plasmodium falciparum* parasites.

1.5 Objectives

1.5.1 General objective

To determine the genetic basis of resistance in *Plasmodium falciparum* parasites exposed to pure artemisinin and *Artemisia annua* extracts

1.5.2 Specific objectives

1) To identify point mutations in genes associated with artemisinin resistance.
2) To identify point mutations in selected genes associated with *Artemisia annua* resistance.
3) To determine the *Pfmdr1* gene copy number variation in *Plasmodium falciparum* parasites exposed to artemisinin and *Artemisia annua* extracts.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria burden

Despite considerable scientific advances, intensive research and development of modern medicine, malaria still remains one of the most deadly human diseases, causing ~216 million infections annually (WHO, 2017). Currently, a half of the world's populations live in potential malaria transmission areas. Malaria is widespread throughout the tropic and sub-tropic regions of the world (Figure 2.1). However, sub-Saharan Africa share 90% of the global malaria burden. In high endemic areas, the disease burden is greatest in young children below 5 years and pregnant women (Carneiro *et al*., 2010).

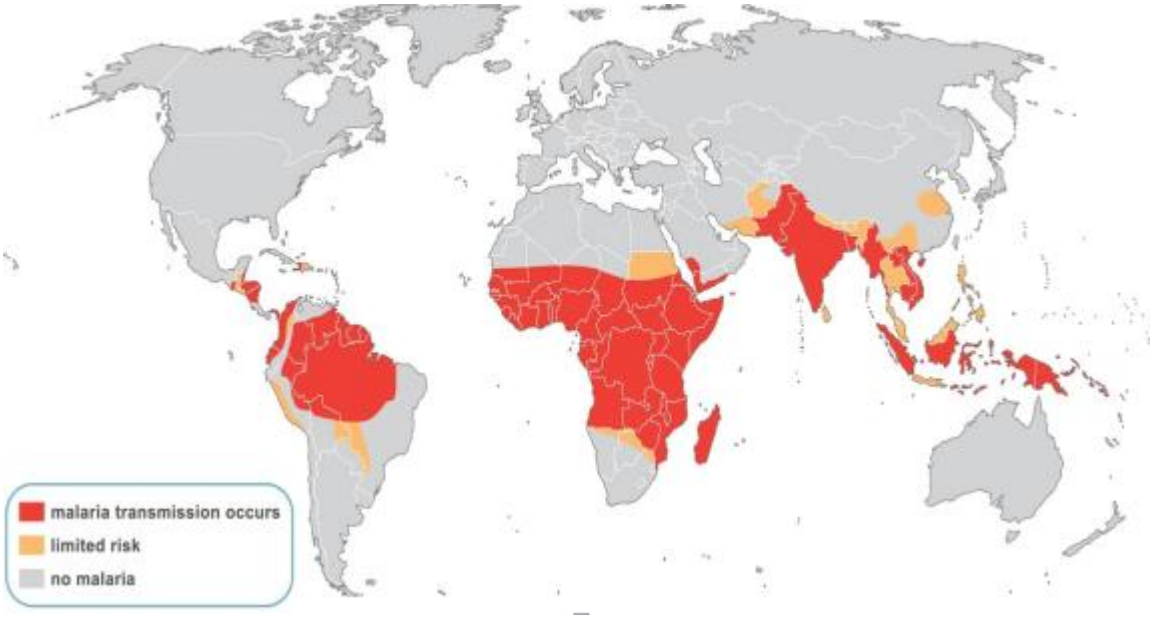


Figure 2.1: Global distribution of *Plasmodium falciparum* malaria (World Health Organization, 2017).

Malaria burden extends far beyond the direct measures of morbidity and mortality. It is responsible for approximately 40% of public-health expenditure (WHO, 2012). Moreover, acute febrile illness, anemia, school and employment absences, and complications in the course of pregnancy contribute to a heavy individual household and health service burden, which has a negative impact on social and development of endemic countries (Barnes and White, 2005).

2.2 *Plasmodium* species

Malaria is caused by protozoan parasites of the genus *Plasmodium,* which are transmitted to humans by the bites of infected female *Anopheles* mosquitoes. Today, five *Plasmodium* species that cause malaria are known, *P.falciparum, P. vivax, P malariae, P. knowlesi* and *P.ovale*. However, it has been argued that *P.knowlesi*, a simian parasite that causes human malaria should be added on this list (Cox-singh *et al*., 2008). *P. knowlesi* infections have been found in Thailand (Jongwutiwes *et al*., 2004) and China. Also, it has been reported to cause severe malaria in Malaysia (Cox-singh *et al*., 2008).

Plasmodium falciparum is the most prevalent species worldwide, causing the most severe form of malaria and being responsible for over 90% of the deaths in sub-Saharan Africa (WHO, 2006). *P. vivax* and *P. ovale* cause benign tertian malaria (Baird *et al*., 2016). *P.vivax* is prevalent in Southeast Asia accounting for up to 50% of the malaria cases with prevalent rates between 1-6% of the population (Tjitra *et al*., 2008). Different *Plasmodium* species vary in geographical distribution, clinical presentation, microscopic morphology, and susceptibility to anti-malarial drugs (WHO, 2000).

2.3 Life cycle of *Plasmodium falciparum*

All pathogenic manifestations and clinical symptoms associated with mammalian malaria infection are caused by asexual erythrocytic phase of *Plasmodium* life cycle. The parasites life cycle is complicated and requires a vertebrate host for the asexual cycle and a female *Anopheles* mosquito for completion of the sexual cycle (Figure 2.2). The gametocyte bridges the two hosts, beginning in the human host and ending in the mosquito midgut.

Natural infection of humans by *Plasmodium falciparum* is initiated when a parasite-infected *Anopheles* mosquito injects sporozoites into the bloodstream during a blood meal. The infectious sporozoites in the mosquitos' saliva enter the host bloodstream and invade its hepatocytes where asexual reproduction occurs. Exoerythrocytic schizogony occurs in the hepatocytes leading to production of several thousands of merozoites. The infected liver cells rupture and liberate thousands of merozoites, which invades the red blood cells in the general circulation. Erythrocytic schizogony follows producing 2 to 36 merozoites per infected red blood cell (Soulard *et al*., 2015) and erythrocyte eventually raptures releasing more merozoites into the blood stream.

The merozoites arrest their life cycle and develop into male (micro) gametocytes or female (macro) gametocytes, which a mosquito ingests during a blood meal (Barry, 2005). Once inside the mid-gut of the mosquito, the male gametocytes fertilize the female gametocyte to form ookinete. The ookinete form oocyst just outside the mosquito's stomach, which then grow, divide and rupture to give rise to sporozoites which migrate to salivary glands. The sporozoites are then injected into the humans during a blood meal.

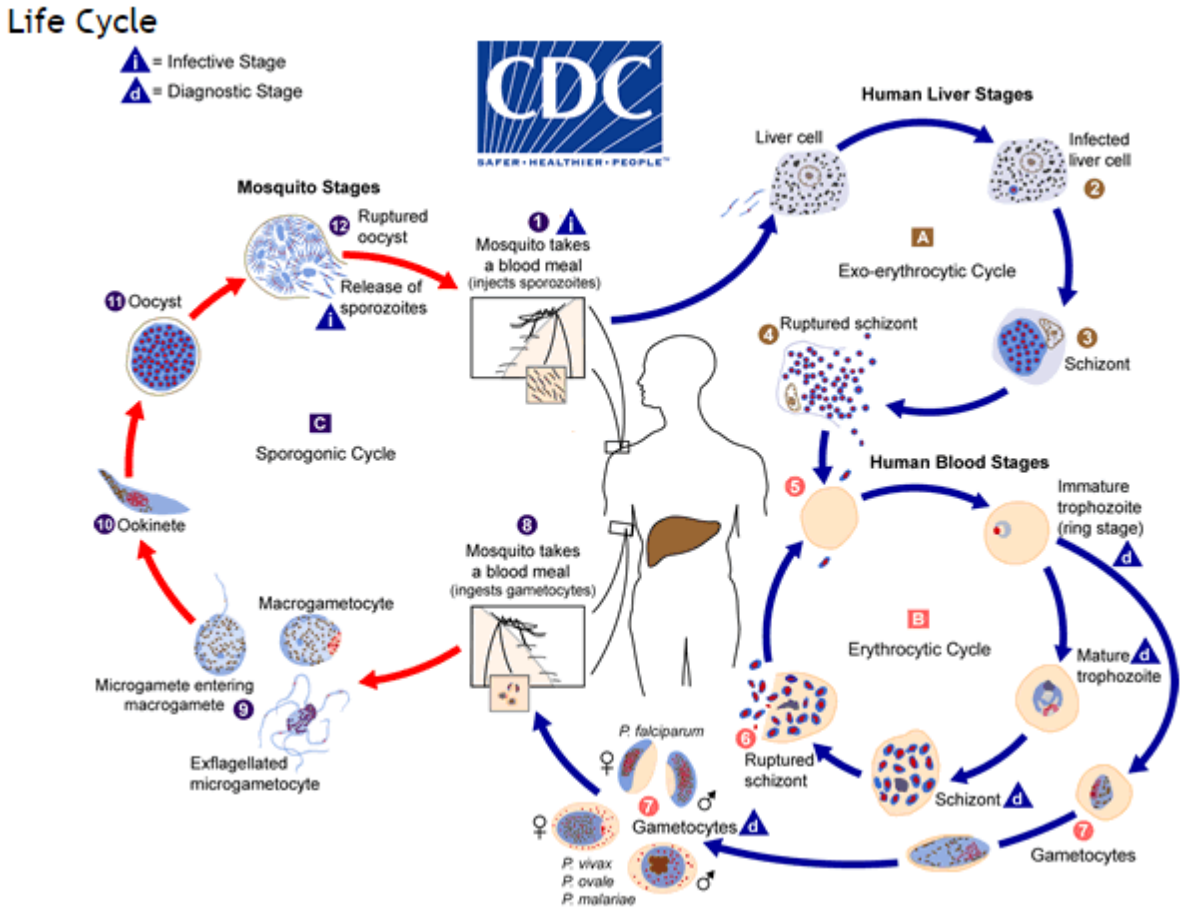


Figure 2.2: The life cycle of *Plasmodium falciparum* (*https://www.cdc.gov/dpdx/* malaria)

The malaria parasite life cycle involves two host. During a blood meal, a malaria-infected female *Anopheles* mosquito injects sporozoites into the human host

2.4 Malaria diagnosis

Malaria management have relied heavily on accurate diagnosis of the patients. Currently, there are two diagnostic approaches used most often, clinical diagnosis and laboratory

diagnosis (Tangpukdee *et al*., 2009). Clinical diagnosis, the most practiced approach, is unreliable because the earliest symptoms of malaria are very non-specific and variable. Malaria symptoms include fever, headache, weakness, chills, dizziness, abdominal pain, diarrhea, nausea, vomiting and pruritus. These symptoms however overlap with other tropical diseases such as common viral or bacterial infections thus impairing specificity of clinical diagnosis.

Laboratory diagnosis of malaria use different techniques, for example, conventional microscopic diagnosis by staining thin and thick peripheral blood smears (Ngasala *et al*., 2008) , quantitative buffy coat (QBC) method (Bhandari *et al*., 2008), rapid diagnostic tests and molecular diagnostic methods, such as polymerase chain reaction (PCR) (Holland and Kiechle *et al*., 2005). Microscopic detection of malaria remains the gold standard for laboratory diagnosis. The technique is performed by staining thick and thin blood films on a glass slide, to visualize malaria parasites. (Mcmorrow *et al*., 2011). Despite microscopy being effective in detecting *Plasmodium* parasites, the technique is laborious and requires high-skilled personnel. This led to introduction of rapid diagnostic tests (RDTs) to serve as a complimentary tool. RDTs use immunochromatographic methods to detect *Plasmodium* specific antigens such as histidine rich protein 11 (HRP-11) or *Plasmodium* lactate dehydrogenase (pLDH) in a patient's blood sample (Chotivanich *et al*., 2006)

Polymerase chain reaction (PCR) based technique have proven to be one of the most specific and sensitive diagnostic methods for malaria. The technique allow the specific

amplification of a targeted region of the *Plasmodium* genome as well as diagnosis of mixed infection. Furthermore, PCR have been used to detect drug resistant parasites (Imwong *et al*., 2001).

2.5 Pathogenesis of severe malaria

The immunity to malaria is provided by innate mechanism. However, at a molecular level the immune system of *Plasmodium falciparum* is rather complex, and it is essentially stage specific (Stevenson *et al*., 2004). Immune system cells play a major role in controlling the early progression of the disease through phagocytosis and production of inflammatory mediators (Autino *et al*., 2012). Pathogenesis of malaria fever and nausea are due to rapture of the late stage infected erythrocytes which stimulate the production of pro or anti-inflammatory cytokines, such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin 1 beta (IL1β), or interleukin-10 (IL-10) by the macrophage cells (Taramelli *et al*., 2000). Studies have correlated elevated levels of TNF-α in the serum of severe patients with cerebral malaria (Stevenson *et al*., 2004).

Cytoadherance of mature parasites to the vascular endothelium cells through electron-dense knobs on the packed RBC surface is another pathogenetic characteristic of severe malaria (Beeson *et al*., 2000). This phenomena, called parasite sequestration occurs during the second half of the intra-erythrocytic asexual growth phase of the parasite. Sequestration is thought to be associated with a number of features of severe malarial pathology such as cerebral malaria and pregnancy-associated malaria.

Sequestration of parasitized erythrocytes occurs approximately 20 hours after the RBC invasion (Autino *et al*., 2012). Mature parasites produce new proteins that are exported to the infected red blood cells. These parasites make malaria diagnosis more difficult since they are not seen in the peripheral blood. Importantly, sequestration enhances cytoadherance of late stage infected red blood cells to non-parasitized red blood cells or platelets (Rowe *et al*., 2009), a process known as rosetting.

2.6 Control of malaria

During the last decade, malaria control and intervention have been scaled up and intensified to reduce and interrupt disease transmission in many countries (Komatsu *et al*., 2007). This has resulted in major reductions in the burden of malaria caused by *P. falciparum*. Globally, the estimated incidence of malaria has been reduced by 29% and the mortality rates among children have declined by 45% (WHO, 2013). These interventions include, insecticide-treated nets (ITNs), indoor residual spraying (IRS), rapid diagnostic tests (RDTs), intermittent preventive treatment during pregnancy, and the introduction of artemisinin combination therapy (ACTs).

Three living entities are involved in malarial infection; parasite, vector, and host. Therefore, to reduce the burden of malaria, interventions can be designed to target several stages of *Plasmodium* lifecycle. For example, parasite can be targeted in the host either directly with drugs or indirectly with vaccines and strategies that reduce vector contact with the host can be employed to reduce malaria transmission (Greenwood, 2008).

2.6.1 Vector control

Malaria parasite is absolutely dependent on its vector, therefore, the interventions that reduce vectorial capacity are a necessary first step towards malaria eradication. Effective vector control strategies are based on knowledge and understanding of vector biology and ecology, public education and implementation of effective control measures. Primary vector control strategies used to prevent malaria in Africa include indoor residual spraying of insecticides (IRS) and long-lasting insecticidal nets (LLINs) (WHO, 2014b). LLINs are treated with pyrethroid only once, at factory level and they do not require any re-treatment during their expected lifespan (4-5 years). The wide-spread use of insecticide treated nets reduces the feeding frequency and survival of mosquitoes at the population level by killing mosquitoes with insecticide or blocking their contact with humans (WHO, 2006b)

Indoor residual spraying is the practice of spraying long-acting chemical insecticides on the interior walls and roofs of the houses and domestic animal shelter in a given area to kill the adult vector mosquitos that land on these surfaces. Since many malaria vectors are endophilic, resting on a nearby surface after taking a blood meal, they are particularly susceptible to be controlled through IRS. Indoor residual spraying kills the mosquitos after they have fed, thereby reducing malaria transmission. This method have led to control of major vector *Anopheles gambiae S. S.*, which rests and bites mostly indoors (WHO, 2006b). However, another vector, *An. Arabiensis*, which does not rest indoors, is less affected by IRS even at high coverage levels, and is responsible for low level transmission and seasonal increase and outbreaks (Mabaso *et al*., 2004).

In 2006, WHO recommended use of 12 different insecticides for IRS, belonging to four chemical groups including DDT and a series of alterative insecticides (such as the pyrethroid spermethrin and deltamethrin) (WHO, 2006c). Insecticides may however put evolutionary pressure on mosquitoes to develop resistance against the insecticides used. DDT resistance in major malaria vectors has been found throughout west, central Africa as well as several parts of South Africa. Insecticide resistance have led to development of new methods of vector control aimed at manipulating the *Anopheles* population (so that it is less likely to transmit malaria) without eliminating mosquitoes from the environment (Marshall & Taylor, 2009). An alternative strategy is to use fungal biopesticides and genetically engineered Wolbachia bacteria to control mosquitoes (Bian *et al*., 2013). These interventions maybe complemented by other methods such as larval control or environmental management, which aims to reduce mosquito breeding sites.

2.6.2 Malaria vaccines

Despite huge investment and intensive research, no fully effective vaccine for malaria has been licensed so far (Girard *et al*., 2007), however circumsporozoite protein repeat T-cell epitopes hepatitis B surface antigen (RTS,S) vaccine have shown to be the most advanced malaria vaccine candidate and the first to undergo large-scale phase III evaluation in Africa (Agnandji *et al*., 2012). The vaccine targets the pre-erythrocytic stage of *P. falciparum* parasites. It induces humoral and cellular immune response to the circumsporozoite protein present on the surface of sporozoites and liver stage schizonts. RTS,S vaccine has consistently shown protection against clinical episodes of malaria in

the range of 30-60% (Aponte *et al*., 2007; Abdulla *et al* 2008). The efficacy against malaria was 47% among young children and 37% against infants (Agnandji *et al* 2012). The available data from clinical trials suggests that this vaccine is likely to be implemented as an addition to, and not a replacement for, existing preventive, diagnostic and treatment measures.

2.6.3 Malaria chemotherapy

Chemotherapy remains the key tool for malaria control, reducing morbidity and mortality as well as decreasing malaria transmission. Currently used antimalarials fall into several chemical groups 4-Aminoquinolines, Aryl-alcohols, 8-Aminoquinolines, Antifolates, quinolone alcohols, antibiotics and artemisinins (Robert *et al*., 2001). Antimalarial drugs can also be classified according to their action on particular stages of the parasite's lifecycle (Daily, 2006). Blood schizontcidal drugs act on the asexual erythrocytic stages of malaria parasites. Tissue schizontcidal kill the hepatic schizonts and prevent invasion of erythrocytes, thereby preventing the establishment of clinical malaria. Gametocytocides destroy the intraerythrocytic sexual forms (gametes) of the parasite in the blood, and thus prevent human to human transmission (Schlitzer, 2008). Gametocytes are often resistant to standard antimalarials used to treat the asexual-stage parasites (Daily, 2006). Their unique biology shuts down a subset of metabolic pathways rendering the antimalarials ineffective. However artemisinin compounds and primaquine have reported gametocytocidal activity (Schlitzer, 2008). Sporontocides prevent development of oocyst in the mosquito and thus ablate the transmission.

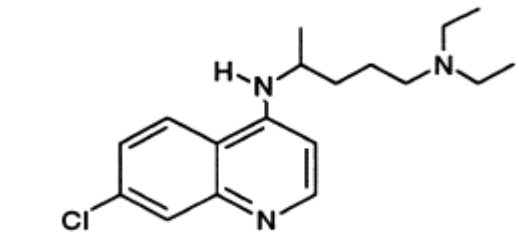
2.6.3.1 Aminoquinolines

Aminoquinoline drugs can be categorized into two main subgroups; 4-aminoquinolines which include chloroquine (CQ), amodiaquine (AQ) and piperaquine (PQ) and 8-Aminoquinolines include three members, primaquine and tafenoquine (Daily, 2006).

4-aminoquinoline drugs are active against the intra-erythrocytic stage of the parasite. The main antimalarial drugs in this group are chloroquine and amodiaquine. They are able to accumulate to high concentrations within the food vacuole of *Plasmodium* to kill the parasite (Robert *et al*., 2001). Chloroquine **(**Figure 2.3a**)** was introduced in 1944-1945 and became the cornerstone of malaria therapy and prevention. Chloroquine binds to heme, preventing the detoxification of heme by crystallization into hemazoin (Winstanley *et al*., 2004). Chloroquine-heme complex accumulates to a level capable of killing the parasite. Recent studies suggest that chloroquine inhibits hematin formation by complexing with ferriprotoporphorin IX (FPIX), thereby preventing its polymerization into hemazoin (Deshpande & Kuppast, 2016).

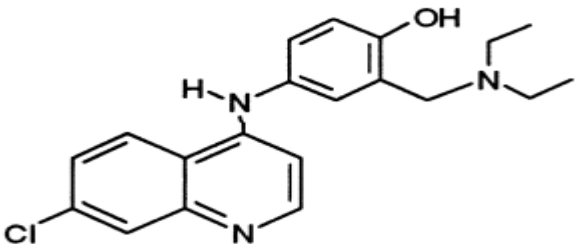
Amodiaquine **(**Figure 2.3b**)** is an analogue of chloroquine and is thought to have the same mode of action (Li *et al*., 2002). This drug is effective against low level chloroquine resistant parasites. It has been hypothesized to act by inhibiting heme detoxification, and have also been shown to accumulate within digestive vacuole (Hayeshi *et al*., 2008). The global use of amodiaquine has declined owing to its association with hepatoxicity, myelotoxicity and drug resistance (Biagini *et al*., 2005). The immunological basis of amodiaquine toxicity is formation of a stable, inactive metabolite desethylamodiaquine

(O'Neill *et al*., 2004). It also forms an unstable reactive quinoneimine in liver and white cells, which acts as a hapten, resulting in possible immune-based toxicity.



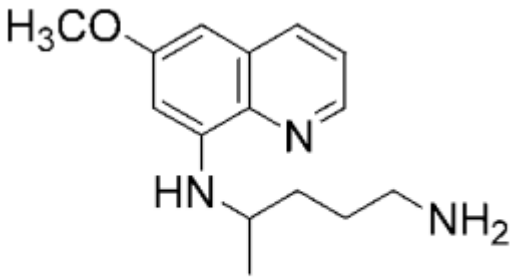
Chloroquine

A



Amodiaquine

B



Primaquine

C

Figure 2.3: 4-amonoquinolines; (A) chloroquine and (B) amodiaquine and 8-amimoquinolines: (C) primaquine (Robert *et al*., 2001)

Tafenoquine and primaquine are the main 8-aminoquinolines drugs. They have gametocytocidal activity against all human malaria parasite species (Robert *et al*., 2001). Primaquine (Figure 2.3c) has been widely used to eradicate the hyponozoites responsible for the relapsing forms of *P. vivax* and *P. ovale* malaria (Baird, 2005). This drug has also

been shown to cause adverse reactions, serious toxicity can be a major problem in patients with glucose-6-phosphate dehydrogenase deficiency.

Tafenoquine is a primaquine analog with a longer half-life of 14 days compared to 4 hours of primaquine (Chiang *et al*., 2006). Tefanoquine has a larger therapeutic index than primaquine hence it is useful for prevention of relapses of vivax malaria and chemoprophylaxis of *P. falciparum* (Robert *et al*., 2001).

2.6.3.2 Quinoline alcohols

Quinoline alcohols include Quinine (QN) (Figure 2.3.1a) and Mefloquine (MFQ) (Figure 2.3.1b). Quinine is the most abundant alkaloid in the bark of the cinchona tree and was introduced into Europe from South America in the 17th century (Meshnick and Dobson, 2001). Quinine has a rapid schizontcidal activity against intra-erythrocytic malaria parasites, and its peak plasma concentrations occur 1 to 3 hours after oral administration of the bisulfate. This drug has been used as a mainstay therapy for severe *falciparum* malaria because preparations for intravenous application are available (Schlitzer, 2008). Quinine has been shown to inhibit the detoxification of heme, an essential process within the parasite (Fitch, 2004).

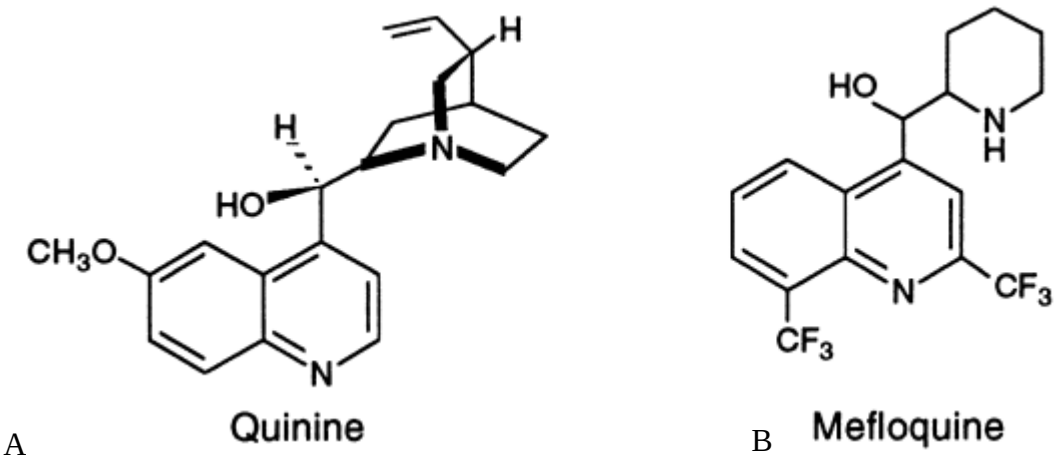


Figure 2.3.1: Structure of quinoline alcohols: (A) Quinine and (B) mefloquine (Robert *et al.*, 2001)

Mefloquine (Figure 2.3.1b) has a long half-life of 14-21 days (Robert *et al.*, 2001). The drug acts against the asexual stages of all human malaria parasite species. Mefloquine emerged as a successor to CQ in the 1980s after chloroquine resistance was reported at the Thai-Cambodia border (Duraisingh and Cowman, 2005). Although the exact mechanism of action remains unclear, *in vitro* studies have shown that MFQ can bind to heme and exert antimalarial activity by inhibiting heme detoxification (Eastman and Fidock, 2009). It also binds with high affinity to membranes, causing morphological changes in the food vacuole of *Plasmodium*.

2.6.3.3 Folate antagonists

Folate antagonists inhibit the synthesis of tetrahydrofolate co-factors essential in the synthesis of pyrimidine deoxythymidylate for parasite DNA (Gregson and Plowe, 2005). There are two groups of Antifolates: the dihydropteroate synthase (DHPS), like

sulfadoxine and dihydrofolate reductase (DHFR), like pyrimethamine and proguanil (Bloland, 2001). Due to marked synergistic effect, the DHFR and DHPS are used in combination for treatment of malaria (Robert *et al*., 2001).

Sulfadoxine-pyrimethamine (SP) combination (Fansidar) was introduced in response to emergence of CQ resistance (Gregson and Plowe, 2005). The drug is cheap, safe in many parts of Africa and is primarily used as intermittent preventive malaria treatment during pregnancy. Unfortunately, SP resistance is widespread in high-transmission areas of Africa due to long half-life of 4 days (Winstanley *et al*., 2004). Resistance is caused by point mutations in the genes of DHFR and DHPS (Uhlemann and Krishna, 2005).

Proguanil, a prodrug has also been widely used for malaria prophylaxis. However, due to widespread drug selection pressure on the parasite, its clinical response deteriorated (Gregson and Plowe, 2005). Chlorproguanil-dapsone (Lapdap) combination was recommended for prophylaxis since it has a short half-life (<50hrs) hence lower selection pressure for resistance than SP (Nzila, 2006).

In addition, methotrexate and aminopterin are also potent antifolates although they inhibit both human and parasite DHFR enzyme (Bertino *et al*., 1996). Methotrexate is a slight modification of the dihydrofolate, the normal substrate of DHFR, thus competes with the substrate in the DHFR active site (Nduati *et al*., 2005). These drugs are however not common for treatment of malaria due to their narrow therapeutic indices and life-threatening toxicity to the human host (Nzila, 2006).

2.6.3.4 Aryl-alcohols

Common aryl-alcohol antimalarial drugs are lumefantrine, halofantrine and, pyronaridine (Ezzet *et al*., 2000). Lumefantrine (also known as benflumetol) is a synthetic fluorene derivative also chemically related to mefloquine. This drug was synthesized in the 1970s by the Academy of Military Medical Sciences in Beijing, China and had undergone preliminary clinical studies in China (Basco *et al*., 1998). Lumefantrine has an elimination half-life of 4-5 days with absorption being enhanced by co-administration with a fatty meal (Toover and Jamieson, 2004).

A fixed dose of artemether-lumefantrine combination (each tablet contains 20 mg of artemether and 120 mg of lumefantrine) has been introduced as the first or second line treatment in several Africa countries (Sisowath *et al*., 2007). The fixed dose regime ensures that malaria parasites always encounter artemether and metabolites in the presence of lumefantrine and protects against development of resistance to both drugs. Single nucleotide polymorphism in the *Plasmodium falciparum multi-drug resistant gene (Pfmdr1)* particularly the variant N86, and amplification of the encoding gene *Pfmdr1* have been associated with reduced susceptibility to lumefantrine in Africa and Asia (Price *et al*., 2006; Sisowath *et al*., 2007).

Halofantrine (Figure 2.3.2a) is effective against chloroquine-resistant *P.falciparum* strains but cardio-toxicity has limited its use as a therapeutic agent (Robert *et al*., 2001). It is also an expensive drug without parenteral formulation.

Pyronaridine (Figure 2.3.2b) an acridine derivative, is a synthetic drug widely used in China (Robert *et al*., 2001). The drug is structurally related to chloroquine and has utility for multidrug resistant *falciparum* malaria. The Chinese oral formulation is reported to be effective and well tolerated, but its oral bioavailability is low contributing to an unacceptably high cost of treatment (WHO, 2006). Pyronaridine inhibits Beta-hematin formation *in vitro* and interferes with glutathione-dependent heme degradation (Auparakkitanon *et al*., 2006).

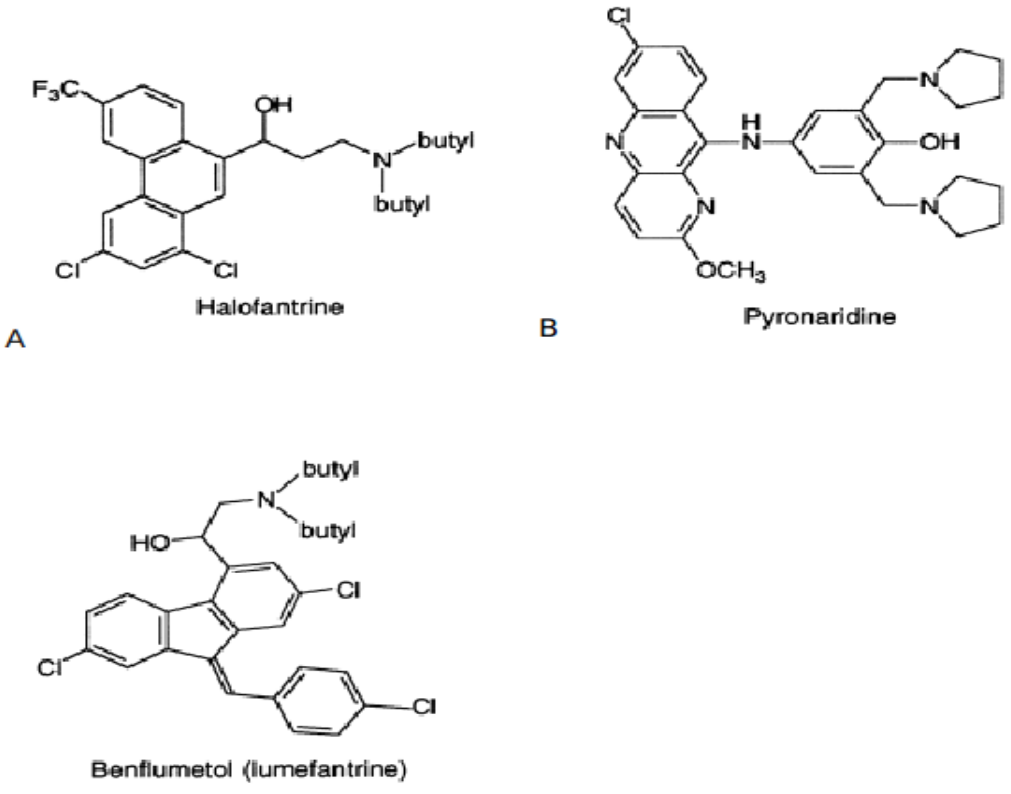


Figure 2.3.2: Structure of aryl-alcohols: (A) halofantrine, (B) pyronaridine and (C) lumefantrine (Robert *et al*., 2001)

2.6.3.5 Artemisinin derivatives

Artemisinin **(**Figure 2.3.3**)** also known as Qinghaosu, was originally extracted from *Artemisia annua* (sweet wormwood), in China. Artemisinin has been the mainstay treatment for *Plasmodium falciparum* malaria due to low prevalence of resistance and its gametocidal effects (Daily, 2006). Artemisinin derivatives include artesunate (water soluble hemisuccinate) and artemether (oil-soluble ethers). They are all sesquiterpene lactone compounds and are rapidly eliminated in the body (O'Neill and Posner, 2004).

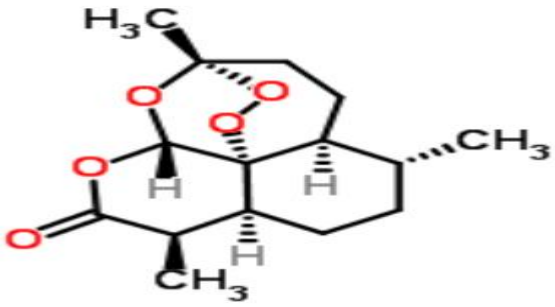


Figure 2.3.3: Structure of artemisinin antimalarial drug (Deshpande and Kuppast, 2016)

Artemisinin and its derivatives are readily metabolized to biologically active metabolite, dihydroartemisinin (Robert *et al*., 2001), which acts specifically on blood stage parasites (Ridley, 2002; Mutabingwa, 2005). This drug is also active at nanomolar concentrations *in vitro* on both CQ sensitive or resistant *P.falciparum* strains. The key structural feature of artemisinins is the endoperoxide linkage within the 1, 2, 4 trioxane system, which is believed to be cleaved by intraparasitic iron-II sources to yield carbon-centered chains (Schlitzer, 2008). Although the exact mode and site of action of ART compounds is still

unclear, *in vivo* and *in vitro* studies have suggested that ART may specifically target *Plasmodium falciparum ATP6*, a *P.falciparum* SERCA type Ca^{2+} dependent ATPase localized in the endoplasmic reticulum (Eckstein-Ludwig *et al*., 2003). Artemisinin and thapsigargin are both sesquiterpene lactone derivatives and it was therefore reasoned that they would behave in a similar manner towards SERCA-type protein. Artemisinin are also thought to interact with heme preventing detoxification of heme by polymerization into hemazoin (O'Neill and Posner, 2004).

The introduction of artemisinin derivatives was associated with a significant reduction of *falciparum* malaria in many endemic countries. Artemisinin has a short elimination half-life of 3-5 hours (Stepniewska and White, 2008). When used as a monotherapy, a treatment of 5 days is required for complete elimination of parasites. Artemisinins are preferentially used in combination with other antimalarial agents such as sulfadoxine-pyrimethamine, mefloquine and piperaquine (WHO, 2010), to increase cure rates and to shorten the duration of therapy in order to minimize the emergence of resistant parasites.

2.6.3.6 Combination therapy

Decades ago, CQ was the mainstay of antimalarial treatment. However, in many parts of the world eradication efforts were effectively thwarted by development and spread of CQ resistance (Eastman & Fidock, 2009). This led to introduction of sulphadoxine-pyrimethamine which was soon met with rapid development of resistance, rendering it ineffective in many endemic countries. Artemisinin based combination therapy was recommended by WHO in 2006 as the first-line treatment for uncomplicated *P.falciparum*

malaria (WHO, 2010a). The underlying principal behind the impact of combination therapy on drug resistance is based on the assumption that drug resistance essentially depends on rare DNA mutation. Provided that the constituent drugs administered in the combination have independent modes of action, the probability of a parasite developing resistance to both drugs simultaneously is significantly reduced compared to developing resistance to one drug (White, 1999).

ACTs comprise semi-synthetic artemisinin derivatives paired with distinct chemical classes of longer acting drugs. Artemisinin and its derivatives have a short half-life, combining a member of this class with a longer-lasting partner drug assures sustained antimalarial pressure after the plasma concentrations of the ART derivatives have fallen below therapeutic levels (Eastman & Fidock, 2009). This increases the antimalarial treatment efficacy and reduces the selective pressure for resistance. Ideally, antimalarial combination drug partners would have similar pharmacokinetics properties so that no drug is left unprotected by the other. ACTs benefit substantially from the ability of the ART derivative to rapidly reduce parasite biomass, resulting in few parasites to be cleared by the partner drug and reducing the pool of parasites from which resistance can emerge (Eastman & Fidock, 2009). ACT together with other malaria control interventions is reported to have contributed to the recent declines in malaria mortality and morbidity in many endemic countries (WHO, 2009).

Several different forms of ACTs have been evaluated, including; artesunate-amodiaquine (Brasseur *et al*., 2007), artemether-lumefantrine (Yeka *et al*., 2008), dihydroartemisinin-

piperaquine (Bassat *et al*., 2009). Two new candidate ACTs has been prequalified by WHO, artesunate-pyronaridine (ASN-PRD) and artemisinin-naphthoquine (ART-NQ) (Benjamin *et al*., 2012).

2.7 *Artemisia annua*

The genus *Artemisia* is a member of family Asteracae and comprise of more than three hundred species of annual, biennial and perennial herbs (Bertea *et al*., 2005). Species included here are *Artemisia absinthiun* (bitter wormwood), *A. dracunculus* (tarragon), *Artemisia annua* (Sweet annie), and *A. aponticum* (the roman worm wood) *(*Miller et al., 2011*)*.

Artemisia annua (Figure 2.4) also known as annual wormwood or sweet wormwood is a highly aromatic annual herb of Asian and eastern European origin and it is widely dispersed throughout the temperate region (Simon, 1990). In China, *Artemisia annua* has been used for many centuries in the treatment of fever and malaria (Brown *et al*., 2003). The plant species is a source of both essential oils (1.4-4.0%) and other phytochemicals such as lactones, flavonoids, sesquiterpene (including artemisinin), polyalykynes and coumarins (Botsaris, 2007). The composition of each category of these secondary metabolites is variable, both qualitatively and quantitatively. The extraction of the essential oils of *A. annua* is done by steam distillation. The essential oils of *A.annua* are found to contain many constituents including alpha-pipene (0.032%), campene (0.047%), B-pipene (0.882%), myrcene (3.8%), artemisia ketone (66.7%), linalool (3.4%), camphor (0.6%), and beta-caryophyllene (1.2%) (Simon *et al*., 1990).

Figure 2.4: *Artemisia annua* plant

Artemisia annua extract has a marked activity against chloroquine resistant and CQ sensitive *P.falciparum* strains, and thus useful in treatment of cerebral malaria (Liu *et al*., 2003). *Artemisia annua* plant extract has been found to be more effective than comparable dose of pure artemisinin in rodent malaria model (Elfawal *et al*., 2012). However, the WHO has cautioned against use of nonpharmaceutical sources of artemisinin because of the risk of delivering sub-therapeutic doses that could exacerbate the resistance problem (WHO, 2012b).

Recent studies have proved that whole plant *Artemisia annua* therapy which is based on oral consumption of the dried leaves of the whole plant, may be more effective than monotherapeutic artemisinin (Elfawal *et al*., 2015). The extract may constitute a naturally occurring combination therapy that augments artemisinin delivering system and synergizes the drug activity.

2.8 Drug resistance in malaria

Since the wide-scale deployment of antimalarial drugs in the 20th century, human malaria parasites have been under tremendous selection pressure to evolve mechanism of resistance (White & Pongtavornpinyo, 2003). Antimalarial drug resistance has been implicated in the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated. The emergence and spread of chloroquine resistance was considered as a leading factor in the dramatic increase in child mortality and morbidity in Africa in the 1980's (Trape, 2000) and today, resistance to nearly all established antimalarial compounds has been reported (Figure 2.5).

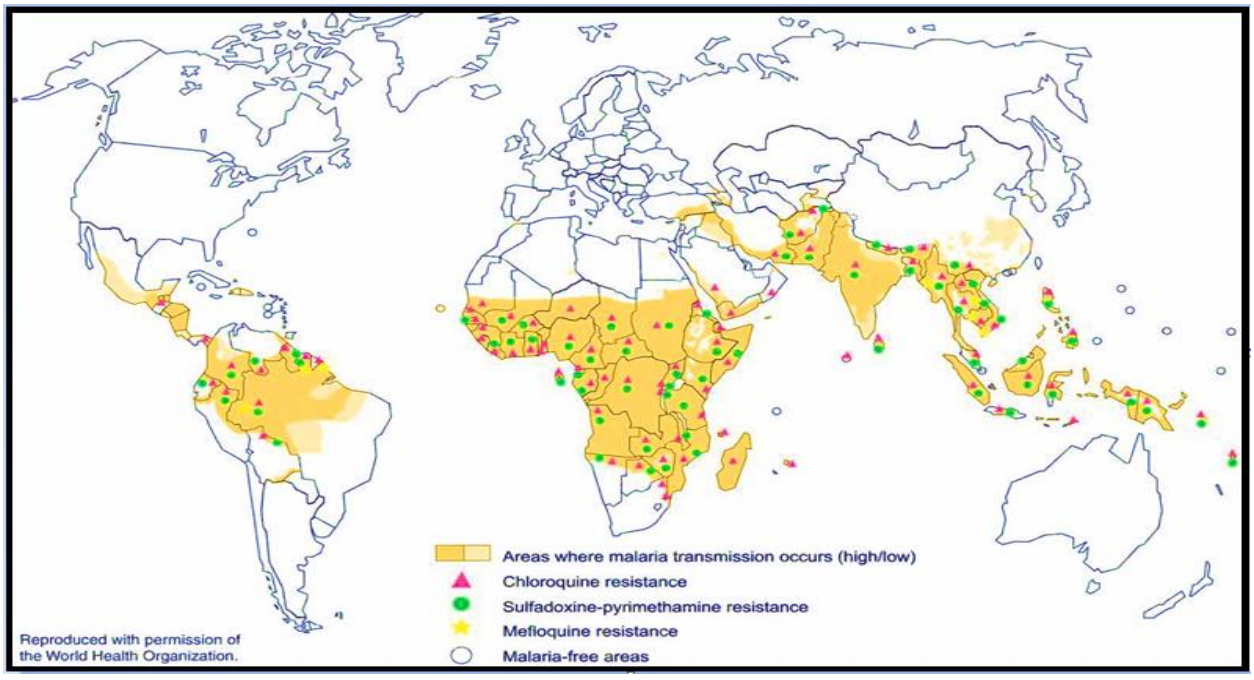


Figure 2.5: Distribution of *P. falciparum* drug resistant malaria (WHO, 2005). The map shows reported antimalarial drug resistance in Africa, Southeast Asia, and parts of South America.

Chloroquine resistance first emerged in Southeast Asia and spread to sub-Saharan Africa. As a result, many countries switched their first-line antimalarial drug to SP, but resistance to SP soon emerged and spread widely (Rooper *et al*., 2003). In the last decade, Artemisinin based combination therapy (ACTs) has played a major role in reducing malaria burden (Dondorp *et al*., 2011). Currently, no alternative antimalarial treatment offers the same level of efficacy and tolerability as ACTs (WHO, 2013b). The emergence and spread of ART resistance would therefore pose a serious threat to the malaria eradication efforts especially in sub-Saharan Africa where disease burden is high.

2.8.1 Emergence of drug resistance

From evolutionary point of view, the development of genetically determined resistance in a pathogen under drug pressure is mediated by two processes: 1) the rate that *de novo* mutations conferring resistance appear and selected through drug use within an individual, 2) subsequent spread of those resistant alleles through the pathogen population. Resistance arises through spontaneous point mutations or gene duplication, which are thought to be independent of the drug selection pressure (Barnes & White, 2005). These mutants can survive in presence of the drug and multiply in numbers to generate gametocytes densities sufficient for subsequent spread (White and Pongtavornpinyo, 2003; Barnes and White, 2005). The probability of *de novo* selection depends on several external factors, including the number of parasites exposed to a drug, concentration of the drug to which the parasite is exposed to, and the degree of resistance that results from the genetic changes (White & Pongtavornpinyo, 2003).

Drug resistance is defined by a rightward shift in the dose-response relationship, thus requiring higher drug concentration to achieve the same parasite clearance (White & Pongtavornpinyo, 2003). Drug pressure provides a strong selective advantage to the *Plasmodium* parasites and linked neutral loci increase in frequency with advantageous alleles (Takala-Harrison and Laufer, 2015). The resistant malaria parasites are transmitted in the human population by the mosquitoes. Resistance arises mainly during asexual reproduction, and may require only a single genetic event or multiple events (Barnes and White, 2005).

2.8.2 Definition of antimalarial drug resistance

Antimalarial drug resistance can be defined as the "ability of the parasite species to survive and/or multiply despite administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limit of tolerance of the subject (WHO, 2010a). This definition was later modified to specify that the drug in question must "be able to gain access to the parasite or infected red blood cells for the duration of the time necessary for the drug's normal action" (WHO, 2010a).

Treatment failure on the other hand is the incapability to clear malaria parasitemia or resolve clinical symptoms despite the administration of the drug (WHO, 2010a). Drug resistance can cause treatment failure but not all treatment failure is due to drug resistance. Incorrect dosage, poor drug quality, drug interactions, poor erratic absorption and misdiagnosis can lead to apparent treatment failure. These factors may also lead to the

development and intensification of true drug resistance through increasing the likelihood of exposure of parasite to suboptimal drug levels.

2.8.3 Fitness cost of drug resistance

In 1985, Charles Darwin in "The origin of species" suggested that the development of one trait in a population may come at the expense of another, as cited by Laufer *et al*., 2006. The mutations that allow the organisms to tolerate high level of curative agents are frequently associated with reduced replicative fitness. Fitness cost phenomenon has been demonstrated in many different systems including, bacteria (Anderson and Hughes, 2010), HIV virus (Armstrong *et al*., 2011) and fungal system (Vincent *et al*., 2013). Now, evidence suggest that resistance to all classes of antimalarial drugs, including artemisinin is associated with significant fitness cost to *P.falciparum* in absence of drug selection (Rosenthal, 2013).

Although there is lack of specific measure of fitness in malaria parasites, the most spectacular demonstrations of fitness cost of antimalarial drug resistance have come from clinical studies in Malawi (Laufer *et al*., 2006). After replacing their first-line antimalarial drug CQ with sulfadoxine-pyrimethamine, there was dramatic shift in the parasites population. The prevalence of *Pfcrt* 76T resistant alleles decreased from 85% in 1992 to 13% in 2000 in samples collected from children with malaria. This revealed that drug pressure discontinuation allowed re-emergence of minority populations of CQ-sensitive parasites.

Recent studies have shown that epistasis could be involved in fitness compensation in drug-resistant *P.falciparum* parasites (Kumpornsin *et al*., 2014). Dihydrofolate reductase (*Pfdhfr*) mutations which confer resistance to pyrimethamine, reduce parasite fitness and can be compensated for by amplification of the first gene in folate synthesis pathway GTP cyclohydrolase 1(Pfgch1), which confers low-level resistance (Babiker *et al*., 2009).

2.8.4 Spread of Antimalarial drug resistance

The factors influencing the rate of spread of drug resistance, once it has emerged in a given area, are still not defined. However, a number of plausible causes have been acknowledged. The spread of drug resistance between populations occurs in two ways. Mosquito vectors carrying drug-resistant parasites can spread resistance on a small scale, but their flight ranges are restricted to a few kilometers from breeding sites (Barnes and White, 2005). Additionally, the movement of individuals from an area of high drug resistance to one of low or no resistance is also a major factor in the spread of drug resistance between different endemic regions (Saeed *et al*., 2005). Drug resistant parasites emerging in a high transmission area would be likely present in polyclonal infection, consequently if mutations conferring drug resistance are associated with a significant fitness cost, they are more likely to be outcompeted by sensitive parasites and not transmitted efficiently (Hastings, 2006; Petersen *et al*., 2011).

Gametocytes are the transmissible stages of the parasites, and it has been argued that gametocytocidal drugs such as artemisinin can slow the spread of resistance (Barnes and White, 2005). The rate at which drug resistance spreads between populations can be

defined as the frequency by which resistance is introduced into the new population combined with the probability of the resistant parasite becoming established (Klein, 2013).

2.8.5 Artemisinin resistance

Clinical artemisinin resistance is characterized as delay in parasite clearance rate, as evidenced by >10% of cases with parasite detectable in day 3 after treatment with ACTs (Stepniewska *et al*., 2010). Slow parasite clearance rates however represents a partial resistance expressed only in ring stage parasites (Witkowski *et al*., 2013). In Pailin, western Cambodia, prolonged parasite clearance times were reported after treatment with artesunate (Dondorp *et al*., 2009). Subjects with uncomplicated *Plasmodium falciparum* malaria from Pailin, Cambodia and Wang Pha, Thailand were enrolled and randomized in blocks of 7 to 10 days artesunate at 2mg/kg of body weight per day or artesunate-mefloquine therapy administered at 4 mg/kg artesunate per day for 3 days, plus mefloquine at 15mg/kg on day 3 and 10mg/kg on day 4 with 63 day follow up. Both treatment groups in Pailin had a reduced parasite clearance of 84 hours, as compared with 48 hours in both Wang Pha groups, Thailand. Slow parasite clearance has also been documented elsewhere in Southeast Asia Myanmar (Kyaw *et al*., 2013) and Vietnam (Thriemer *et al*., 2014).

Although this clinical phenotype does not signify complete resistance, these altered parasitological responses are regarded as an early warning that this may occur in the future. Studies have shown that delay in parasite clearance times is a heritable trait

(Andersons *et al*., 2010) and several candidate genomic regions have been associated with ART resistance in Cambodia, including a region on chromosome 13 (Cheeseman *et al*., 2012), and point mutations on chromosome 10, 13, and 14 (Takala-Harrison *et al*., 2013). The role of artemisinin resistance in these candidate genes need further evaluation. Hence, the study presented here was devoted to investigate association between point mutations and artemisinin resistance using laboratory-adapted clones.

2.9 Genetic basis of drug resistance

Genetic basis of antimalarial drug resistance is spontaneous, rare mutations in genes encoding drug's parasite target of influx/efflux pumps that affect intraparasitic concentrations of the drug (Eckland and Fidock, 2007). These events confer reduced sensitivity to a particular drug and are thought to be independent of the drug used (White, 2004).

The *Plasmodium falciparum* genome encodes multiple predicted transporters. Studies have identified the *P. falciparum* chloroquine resistant transporter (PfCRT) and the multidrug resistant-1 (PfMDR1) transporter as key determinants of decreased *in vitro* susceptibility to several principal antimalarials (Valderramos & Fidock, 2006). Although the *PfATPase6* has been identified as the main target for artemisinin, preliminary studies have not associated polymorphisms in the gene encoding this enzyme with reduced susceptibility to ART (White, 2004). Rather, a recent study by Ariey *et al.*, 2014 identified a molecular marker of artemisinin resistance from whole genome sequencing of laboratory-adapted clone, the F32 Tanzanian line. The study showed that $RSA_{0\text{-}3hrs}$

survival rate and slow parasite clearance were associated with single nucleotide polymorphisms in the kelch propeller domain on chromosome 13 (Pf3D7_1343700) (Ariey *et al*., 2014).

2.9.1 Multidrug resistant 1 gene (*mdr1*)

Plasmodium falciparum multiple drug resistant-1 (Pf3D7_0523000) gene figure 2.6) is a *P.falciparum* ortholog of mammalian P-glycoproteins that mediate verapamil-reversible multi-drug resistance in mammalian cancer cells (Qi *et al*., 2004). The gene is located on chromosome 5 and has a 4.2 kb coding region. *Pfmdr1* encodes a 160kDa protein, known as P-glycoprotein homolog 1 (pgh1) that localizes to the digestive vacuole membrane, and has homology to other *MDR* genes in the ATP Binding Cassettes (ABC) transporter family (Duraisingh and Cowman, 2005). Although the function of *Pfmdr1* remains unknown, its location on the membrane of the food vacuole suggest that it is a drug transporter (Cowman, 1991).

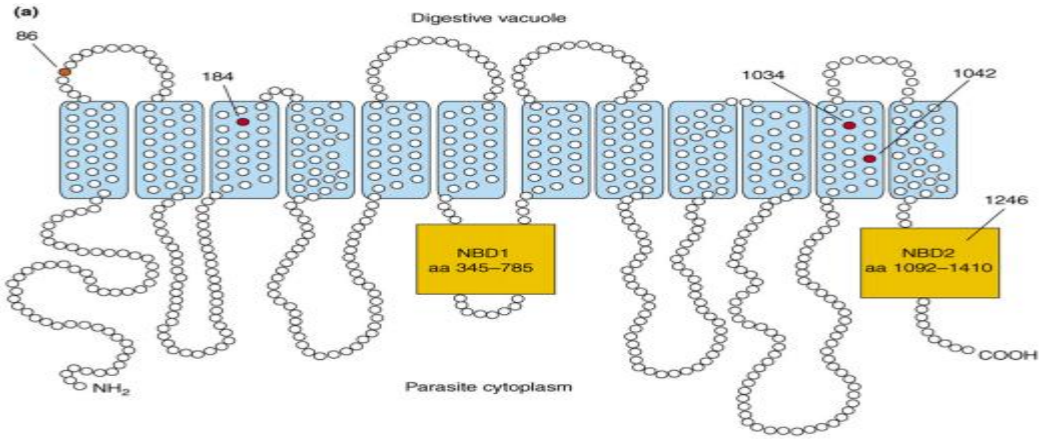


Figure 2.6: Predicted structure and polymorphisms in *Plasmodium falciparum* multidrug resistant 1 (*Pfmdr1*) transporter (Valderramos and Fidock 2006)

Pfmdr1 have two homologous halves, each consisting of six predicted trans-membrane domains and a conserved nucleotide binding domains (NBD1 and NBD2) with NH2 and COOH terminal located on the cytoplasmic side of the digestive vacuole membrane. Red circles indicates the *Pfmdr1* mutations identified in laboratory-adapted lines K1 allele (N86Y) and 7G8 allele (Y184F, S1034C, and N1042D).

The role of *Pfmdr1* in drug resistance have been elucidated in laboratory experiments. The studies reported a partial association between the N86Y mutation with CQ resistance (Babiker *et al*., 2001; Mu *et al*., 2003). Allelic exchange experiments revealed that introduction of triple mutation (S1034C, N1042D and D1246Y) into *Pfmdr1* allele of the sensitive strain does not confer CQ resistance (Sidhu *et al*., 2005). Instead it increases the level of resistance in parasites carrying *Pfcrt* K76T mutation, demonstrating that *Pfmdr1* indeed has an epistatic role in modulating CQ resistance. This effect was however strain-dependent. *Pfmdr1* polymorphisms have also been suggested to play a compensatory role in CQ resistant parasites (Duraisingh and Refour, 2005). *Pfmdr1* over-expression have been linked to drug resistance in *P.falciparum*, similar to multi-drug resistance mechanism observed in mammalian tumor cells. *Plasmodium falciparum* multidrug resistant 1 transcript levels were observed after treatment with CQ, mefloquine and quinine, but not after treatment with pyrimethamine. In summary, evidence suggested that induction of *Pfmdr1* might be a drug-specific mechanism of resistance (Myrick *et al*., 2003).

The *Pfmdr1* point mutations and duplications modulate resistance to other antimalarials including, mefloquine, halofantrine, quinine and artemisinin (Duraisingh and Cowman, 2005). Mutations 1034C, 1042D and 1246Y have been reported to alter parasite

susceptibility to quinine (QN) mefloquine (MQ) and artemisinin (Sidhu *et al*., 2005; Humphreys *et al*., 2007). Furthermore, *Pfmdr1* NFD (asparagine, phenylalanine, aspartic acid) haplotype at codon 86, 184 and 1246 have been linked to artemether-lumefantrine (AL) treatment failure. Studies have also shown that the amplification of *Pfmdr1* gene is associated with decreased ART sensitivity *in vitro* (Sidhu *et al*., 2005), and with the treatment failure of artesunate-mefloquine combination *in vivo* (Alker *et al*., 2007). *Pfmdr1* seem to influence artemisinin resistance to some extent, however its action is likely to be highly dependent on other genetic factors.

2.9.2 Chloroquine resistant transporter gene

Plasmodium falciparum chloroquine resistance transporter gene (Pf3D7_0709000) is a member of drug/metabolite transporter superfamily (Martin and Kirk, 2004). *Pfcrt* is located on chromosome 7 and encodes a 45-kDa protein with ten predicted transmembrane domains, localized in the digestive vacuole (DV) membrane of the parasitized erythrocyte (Fidock *et al*., 2000). *Pfcrt* is involved in drug flux and PH regulation (Valderramos and Fidock, 2006).

At molecular level, chloroquine resistance results primarily from point mutations in the DV transmembrane protein *Pfcrt* (Fidock *et al*., 2000). These polymorphisms include the K76T, which is ubiquitous among CQ-resistant strains, as well as 3-8 additional *Pfcrt* polymorphisms that produce region-specific haplotypes (Ecker *et al*., 2012). So far, at least 34 different *Pfcrt* haplotypes have been reported, reflecting a handful of origins of mutant *Pfcrt* that disseminate under drug pressure in selective sweeps across the world

(Laufer *et al*., 2010). The *Pfcrt* mutant forms are thought to efflux CQ out of DV, thereby preventing this drug from inhibiting the detoxification of iron-bound heme following hemoglobin proteolysis (Sanchez *et al*., 2005).

In Africa, the most prevalent mutant form of *Pfcrt* haplotype is CVIET (cysteine, valine, isoleucine, glutamic acid, and threonine) at position 72-76 (Figure 2.6.1). This haplotype however disappeared in Malawi within several years of CQ withdrawal, presumably due to a fitness cost that rendered this variant less competitive than *Pfcr*t wild-type parasites in the absence of drug pressure (Mita *et al*., 2003; Laufer *et al*., 2010).The SVMNT (serine, valine, methionine, asparagine, and threonine) haplotype, prevalent in South America, may be less severe than that of parasites harboring the CVIET signature (Sa & Twu, 2010).

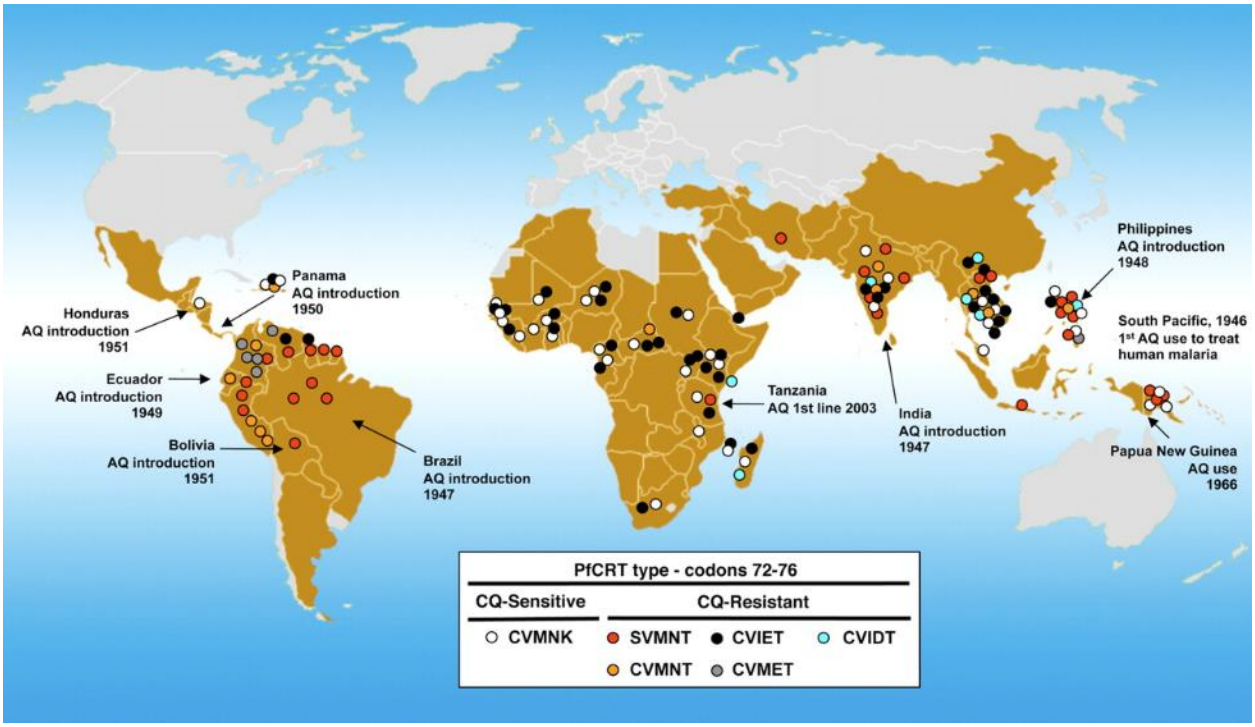


Figure 2.6.1: World distribution of *Pfcrt* haplotypes at codons 72-76 (Sa *et al*., 2009).

The CVMNK wild type allele and CVIET haplotypes are prevalent in sub-Saharan Africa. SVMNT haplotype is prevalent in South America and Southeast Asia while CVMET is prevalent in South America. The less common haplotype CVIDT is only found in parts of Southeast Asia.

A series of allelic exchange experiments have shown that CQ-resistant alleles confer *in vitro* resistance to chloroquine sensitive parasites. The introduction of a resistant *Pfcrt* allele into CQ-sensitive parasite resulted in the significant increase of IC_{50} for chloroquine (Sidhu *et al*., 2002). These studies were consistent with *in vivo* findings that documented a strong association between the *Pfcrt* K76T mutation and chloroquine treatment failure, making it a useful marker of CQ resistance (Hyde, 2005). The mutant *Pfcrt* was also shown to protect immature gametocytes from CQ action (Ecker *et al*., 2012). Interestingly,

the *Pfcrt* transporter protein can also influence parasite *in vitro* susceptibility to multiple antimalarial drugs including quinine, halofantrine and artemisinin (Johnson *et al*., 2004).

2.9.3 Kelch 13 gene

Plasmodium falciparum kelch 13 (Pf3D7_1343700) gene codes for a putative kelch protein (Adams *et al*., 2000). This gene is located on chromosome 13, and has a predicted 3-domain structure (Figure 2.6.2), with an approximate 225 residue long; *Plasmodium*-specific and well conserved N-terminal domain, followed by a BTB/POZ domain and a 6-blade C-terminal propeller domain formed of canonical kelch motifs (Li *et al*., 2004) .The propeller domain harbors multiple protein-protein interaction sites and mediates diverse cellular functions, including ubiquitin-regulated protein degradation and oxidative stress response (Adams *et al*., 2000).

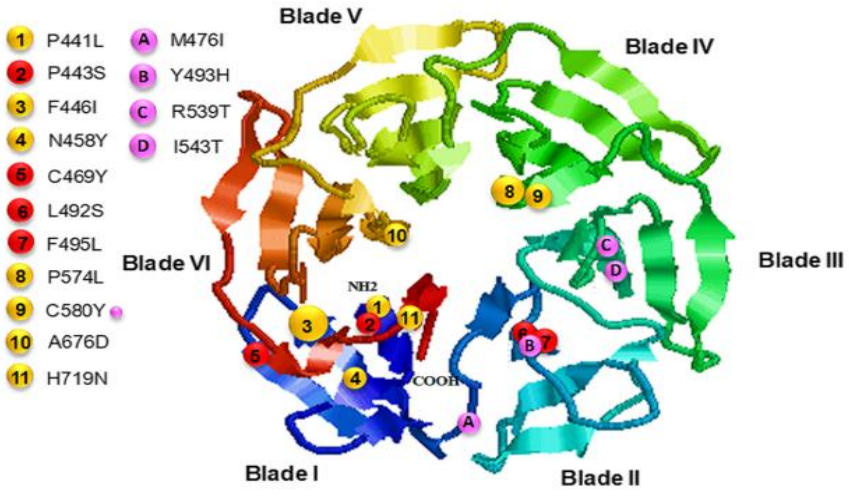


Figure 2.6.2: Distribution of mutations in the predicted 3D structure of K13-propeller domain.

The 3D' model of propeller domain consist 6 Kelch blades (I-VI), from N to C terminus. Positions of various mutations are indicated by spheres. Red color represent new mutations while the mutations reported by Ariey *et al*., 2014 are shown in pink.

K13 gene has been recently proposed as a molecular marker of artemisinin resistance following whole-genome sequencing of an artemisinin-resistant Tanzanian parasite line and clinical isolates from Cambodia (Ariey *et al*., 2014) Single nucleotide polymorphism (SNP) M47I was first observed in F32 Tanzanian parasites that were cultured under escalating concentrations of ART for 5 years. Further, subsequent genomic analysis of Cambodian isolates identified four mutant alleles Y493H, R539T, I543T, and C580Y within the kelch repeat motif of the C-terminal propeller domain. These mutations were associated with elevated $RSA_{0\text{-}3hours}$ survival rates *in vitro* and delayed parasite clearance half-life (>5hrs) in patients (Ariey *et al*., 2014; Amaratunga *et al*., 2014). Polymorphisms associated with ART resistance in Southeast Asia were however not detected in studies across sub-Saharan Africa (Kamau *et al*., 2014; Ashley *et al*., 2014)

The role of K13 gene in ART resistance was further elucidated in allelic replacement experiments. Straimer *et al., 2015* used zinc finger nuclease technology to edit the K13 gene in contemporary isolated Cambodian parasite (Straimer *et al*., 2015).Parasites engineered to express K13 mutations showed an increase in $RSA_{0\text{-}3hrs}$ survival rates, as well as a loss of resistance in parasite whose mutant K13 gene was reverted back to wild type. Additional studies revealed non-synonymous mutation in the propeller and BTB-POZ domains (KPBD) that were associated with artemisinin resistance *in vivo* (Miotto *et al*., 2015).C580Y mutation emerged independently in Cambodia and shared a common

background consisting of four SNPs in genes encoding apicoplast ribosomal protein 10 (*arps10* V127M), *ferrodoxin* (D193Y), *Mdr2* T484I, and chloroquine resistant transporter (N326S). These mutations were hypothesized to increase fitness by compensating for putative deleterious effects of K13-propeller mutations.

Although the molecular mechanisms by which K13-propeller mutations mediate artemisinin resistance remains unclear, several models have been proposed to account for the exact mechanism. It was initially postulated that the wild-type K13 constitutively binds to a putative transcription factor in the parasite cytosol and delivers it to ubiquitin ligase, which polyubiquitinates the transcription factor and targets it for proteosomal degradation (Fairhust, 2015). Kelch 13-propeller mutations on the other hand prevents the binding of K13 to the transcription factor, leading to a base line gene expression pattern that prepares parasites to withstand the oxidative damage caused by artemisinins. It has also been proposed that artemisinin targets *P.falciparum* phosphatidylinositol-3-kinase (P13K), which is the binding partner of K13 (Mbengue *et al*., 2015). The interaction between wild-type K13 and P13K targets the latter for proteosomal degradation. This lowers the basal levels of P13-phosphate, the product of P13K activity, needed for parasite growth. This model speculated that mutant K13 will not bind to P13K and P13K will accumulate and produce high levels of P13P enabling the continuous P13P-dependent growth of ART-resistant parasites even under artemisinin drug pressure.

2.9.4 Ubiquitin specific protease-1 (*Ubp-1*)

Ubp-1 is a 3.3 kb gene (Pf3D7_0104300) located on chromosome 1, and encodes a deubiquitinating enzyme (Imwog *et al*., 2010). A genetic cross between artemisinin resistant and *Plasmodium chabaudi* sensitive clone AJ identified two candidate mutations V739F and V770F that were shown to confer resistance to artesunate (Hunt *et al*., 2007). The causative association of this gene and ART resistance was however uncertain, as no mutation in the *ubp-1* gene encoding the *P.falciparum* orthologue of deubiquitinating enzyme was observed. Additionally, other studies did not show any association between these mutations with artemisinin resistance in laboratory selected *P.falciparum* parasites (Imwog *et al*., 2010; Chavchich *et al*., 2010)

2.9.5 *PfATPase6*

PfATP6 gene (Pf3D7_0106300) encodes sarco-endoplasmic reticulum calcium dependent ATPase (SERCA) protein. This gene has been proposed as the main target for artemisinin action (Eckstein-Ludwig *et al*., 2003). Several single nucleotide polymorphisms in *Pfatpase6* gene have been reported, whereby L263E, E431K, A623E and S769N are the most common (Jambou *et al*., 2010). The *Pfatpase6* S769N mutation is associated with an increase in artemether IC50, while mutation at L263E codon have been reported to confer reduced susceptibility to artemisinin (Valderramos *et al*., 2010).

2.10 Strategy against resistance

2.10.1 Artemisinin combination therapy (ACT)

The world health organization have recommended use of ACTs as the preferred first-line antimalarials against *falciparum* malaria (WHO, 2010). Artemisinins combination therapy comprise artemisinin derivatives with very short elimination half-life and long elimination half-life drug (Djimdé *et al*., 2008). Artemisinin derivatives are the most potent antimalarial drugs, reducing parasite biomass as well as gametocyte carriage while the long acting partner drug achieves effective clinical and parasitological cure. The principle underlying the use of drug in combination is based on the assumption that drug resistance essentially depends on DNA mutations. Therefore, if two drugs used have different mode of action, then the probability of parasite developing resistance to both drugs would be reduced compared to developing resistance to one drug (White, 2004). Moreover, ACTs would have similar pharmacokinetic properties so that no drug is left unprotected.

Artemisinin combination therapy has been adopted by more than 60 countries as a first-line therapy for treating uncomplicated malaria (Brasseur *et al*., 2007). Increasing body of evidence have shown the safety and efficacy of dihydroartemisinin piperaquine (Myint *et al*., 2007; Grande *et al*., 2007). *In vivo* studies done in Asia have reported dihydroartemisinin piperaquine efficacy of about 90% over 28-63 days. Other studies in Africa have also shown dihydroartemisinin piperaquine to be as efficacious as artemether-lumefantrine (Bassat *et al*., 2009).

2.11 Monitoring of drug resistance

Drug resistance in *Plasmodium falciparum* has become an issue of utmost concern over the past few decades. Resistance has been implicated in enhanced mortality for malaria in hyper and haloendemic areas (Trape *et al*., 2002). Thus, tracking of evolving resistance patterns is essential for proper management of clinical cases and for determining thresholds for revising national malaria treatment policies. The assessment of *P.falciparum* drug susceptibility involves various approaches, *in vivo* tests, *in vitro* susceptibility assays and identification of genes and mutation giving rise to resistance (Plowe, 2003).

2.11.1. *In vivo* tests

In vivo tests are the primary source of information used by policy makers to shape recommendations for malaria chemotherapy and prophylaxis (Plowe, 2003). They are the "gold standard" for monitoring antimalarial drug efficacy. Key characteristic of these tests is the ability to evaluate the actual clinical and epidemiological responses involving an interaction between the drug, parasites and the host. *In vivo* tests involve treatment of a group of symptomatic individuals with known dosage of drug and the subsequent monitoring of parasitological and clinical response over time (Basco, 2007). *In vivo* tests reflect actual clinical situation. The WHO recommends that the standard methods for measuring anti-malarial drug efficacy use a 14 day follow-up period in areas of intense transmission and 28 days in areas of low transmission (WH0, 2010c). All *in vivo* tests have to be carried out with set, standard therapeutic doses of drugs within the limits of general tolerability.

In vivo tests are however limited, they do not permit a quantitative assessment of the drug sensitivity of individual parasite beyond the question of treatment failure or success. Additional pharmacokinetics are therefore recommended to identify host related factors such as accelerated gastrointestinal passage of test drug, poor absorption, or metabolic peculiarities of some patients (Plowe, 2003).

2.11.2 *In vitro* assays

In vitro tests involve measurements of the intrinsic sensitivity of malaria parasites (Basco *et al*., 2007). These tests use *Plasmodium falciparum* parasites obtained from the patients' venous blood or laboratory-adapted isolates, cultured *in vitro.* The isolates are then used for quantitative assessment of anti-malarial activity *in vitro*. The inhibition concentration ($IC_{50)}$ values are calculated and compared with those obtained from reference strains whose genotype and phenotype are known. The *in vitro* methods can be used to confirm, characterize resistance and to monitor sensitivity to candidate drugs (WHO, 2003). These tests also allow for exclusion of host related factors, such as drug failure and host immunity.

Most common assays for measuring the drug sensitivity include microscopic examination of blood film for the WHO mark III test, radioisotopic test and fluorometric assay with DNA binding fluorescent dye (Corbett *et al*., 2004).

2.11.3 Molecular monitoring of parasite resistance

Advances in understanding the molecular mechanism of drug action allowed identification of the putative molecular markers of drug resistance. For example, *Pfcrt* which modulates resistance to CQ (Fidock *et al*., 2000) and *Pfmdr1* which confers resistance to multiple antimalarials (Sidhu *et al*., 2005). Most molecular techniques use polymerase chain reaction to detect mutations or duplications in the parasite genes (Plowe, 2003). For instance, under drug pressure, the genetic determinants of drug resistance may leave a selection signature in the genomes of parasites. Samples are then screened for single nucleotide polymorphisms associated with drug resistance. Detection of molecular markers of drug resistance offers rapid and affordable options for monitoring parasite resistance in the fields (Price *et al.,* 2006). These techniques include, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the selected loci (Ranford-Cartwright *et al*., 2002), pyrosequencing (Nair *et al*., 2002), molecular beacons (Durand *et al*., 2000) or real-time PCR (Alker *et al*., 2004).

2.12 Genotyping methods

2.12.1. DNA sequencing

DNA sequencing has been considered the "gold standard" procedure for characterization of specific nucleotide alterations in many organisms. The method enables accurate determination of alleles and their exact position within a locus. Whole genome sequencing of malaria parasites has been used to track clonal diversity within individual infections

(Juliano *et al*., 2010; Manske *et al*., 2012), and to correlate population genetic patterns with resistant phenotypes (Miotto *et al*., 2013). The common approaches used are whole genome sequencing (Manske *et al*., 2012) and amplicon sequencing (Gandhi *et al*., 2012; Rao *et al*., 2016). Recently whole genome sequencing was used to locate mutations in K13 propeller gene that were associated with artemisinin resistance *in vitro* and *in vivo* (Ariey *et al*., 2014).

2.12.2 Merozoite surface protein genotyping

Merozoite surface protein, *MSP-1* and *MSP-2* are the most commonly used markers for genotyping *Plasmodium falciparum*. The markers exhibit both length and sequence polymorphism. Length variations of these genes are due to tandemly repeated sequences and alleles can be distinguished following electrophoresis of PCR amplified products. *MSP-1* gene, repeats occur in region denoted "block2". *Msp2* alleles are classified into two groups denoted FC-27 and IC3D7.

The technique involves use of PCR to detect alleles of *MSP-1* and *MSP-2* genes. Primary PCR uses primers that target conserved sequences spanning polymorphic regions of *MSP-1* gene block 2 and *MSP-2* gene block 3. Nested PCR use gene specific primers to amplify alleles, K1, MAD20, and RO33 of *MSP-1* gene and FC-27 and IC3D7 of *MSP2* gene. PCR products from Primary PCR are used as template for the secondary reaction. PCR amplicons are run in agarose gel electrophoresis against a molecular ladder of known size. Migration in the gel is dependent on fragment size. Further analysis can be done for adequate discrimination of alleles using capillary electrophoresis (LIljander *et al*., 2009).

2.12.3 Microsatellite marker analysis

Microsatellite markers are simple sequence repeats occurring every 2-3kb in both coding and non-coding sequences. They demonstrate a high degree of allelic variation and have advantages for population studies (Anderson *et al*., 2000). Multiple alleles of a microsatellite marker make it valuable for searching signatures of drug selection between parasite populations (Roper *et al*., 2004). Detection of microsatellite markers involves the use of primers to amplify the repeat locus by PCR. Multiplexing can be accomplished through co-amplification of multiple microsatellites in a single PCR procedure.

2.12.4 Restriction fragment length polymorphism

PCR-restriction fragment length polymorphism is a DNA fingerprinting technique for genotyping SNPs (Ranford-Cartwright *et al*., 2002). The target SNP is discriminated by digestion with specific restriction enzymes. Primary PCR is carried out to amplify the locus under investigation. The amplicons of primary PCR are used as template in the nested PCR. The final PCR products are then digested with restriction enzymes and SNP type is easily identified by using agarose gel electrophoresis (Ota *et al*., 2007). Although RFLP is an excellent tool for malaria genotyping, it is not able to access the actual number of parasite genotype in a single field isolate. This necessitates other techniques like sequencing.

2.13 High-throughput next generation sequencing-a method of mutation discovery

The phrase 'next generation sequencing' is a general term applied to sequencing platforms that use post-Sanger technology to sequence large numbers of DNA fragments in parallel (Glenn, 2011). One important advantage of whole genome sequencing is the capacity to understand the evolutionary history of genome organization and structural variation caused by chromosome rearrangements (Pollard *et al*., 2006). The principle behind next generation sequencing (NGS) technology is similar to capillary sequencing, DNA polymerase catalyzes the incorporation of fluorescently labelled deoxyribonucleotide triphosphates (dNTPS) into a DNA template strand during sequential cycles of DNA synthesis. The DNA template is immobilized to an acrylamide coating on the surface of a glass flow cell and during each cycle, at point of incorporation, the nucleotides are identified by fluorophore excitation. The Illumina Solexa genome analyser allows rapid and accurate sequencing on a genome scale and can generate billions of bases in a single run (Illumina, 2014). It is based on reversible terminator technology which employs a sequencing-by-synthesis concept (Bently *et al*., 2008). Illumina sequencing-by-synthesis delivers the high accuracy, high yield of error-free reads, and high percentage of base calls above a phred score of 30 (Nakazato *et al*., 2013; Ross *et al*., 2013). The newly identified sequence read are then aligned to a reference genome during data analysis to identify any variations such as single nucleotide polymorphisms, indels, read counting for RNA methods, phylogenetic and metagenomic analysis.

2.14 Overview of genome assembly

2.14.1 *De novo* assembly

Advancement of next generation sequencing has led to the generation of hundreds of millions of sequence reads in a single run (Soon *et al*., 2013). The number of reads generated varies between 1 million for longer reads generated by Roche/454 sequences (~400bps) and 2.4 billion for short reads generated by Illumina/Solexa and ABI/SOLID/TM sequencers (~75bps). From practical point of view, the large amount of data generated tells almost nothing about the DNA (Flicek and Birneym, 2009), this is due to the lack of proper analysis tools and algorithms. Reconstructing a genome sequence from whole-genome shotgun reads depends on the depth of sequence coverage generated (Lander and Waterman, 1988).

Currently, numerous *de novo* assembler tools have been developed, such as ALLPATH-LG (Gnerre *et al*., 2011), SOAPdenovo (*Li et al*., 2010), and Velvet (Zebrino *et al*., 2008). De novo assembly involves grouping the reads into contigs based on their overlaps. The most widely used approaches are based on either the overlap graph, in which the nodes represent the reads, and the edges connect overlapping reads. In *de Bruijn* graph method (Pevzner *et al*., (2001), the nodes are fixed-length sequences (K-mers), and edges indicate the predecessor and successor relationships of the K-mers. The contigs generated in assembly step are further assembled into scaffolds by using paired-end reads. Paired-end reads can also be used in contig production step to correctly extend contigs through repetitive regions (Zebrino, 2008). Scaffolds are the final output of assembly algorithms, and further assembly of the scaffolds to generate chromosome sequence is usually done

by integrating with genetic or physical maps (Lewin *et al* ., 2009). Longer paired-end reads produced by Pac Bio sequencers are more beneficial for correctly assembling the repetitive region.

2.14.2 Reference-assisted assembly (Mapping)

Read mapping is the alignment of sequence reads to a reference genome. It is a common first step during genomic data analysis and plays a critical role in medical and population genetics. Numerous short-read alignment programs, such as burrow-wheeler aligner (Li and Durbin, 2009), Bowtie (Langmeead *et al*., 2009), and SOAP2 (Li *et al*., 2009), have been developed in the past years. Read mapping is often the most computationally intensive part of the analysis. Read aligners take algorithmic shortcuts because the computational cost of comparing every read to every possible position in the genome is prohibitively expensive. First, the program maps a shorter part of the read (seed) to the reference genome (Li and Durbin, 2009) and only a small number of mismatches are permitted. The aligners then work out from the location that the seed mapped to, trying to match the remainder of the read to genome surrounding the original location, a process called the extension step. Since a short read will map to multiple locations in the genome, the seed extends at multiple locations before it can be settled to which of the original locations has the best overall match to the complete read. The extension step usually involves Smith-Waterman dynamic programming (Li *et al*., 2008; Langmead and Salzberg, 2012), backtracking (Langmead *et al*., 2009) or Needleman-Wunsch dynamic programming (Misra *et al*., 2011).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Study site

Experimental procedures of this study were carried out at the Centre for Biotechnology Research and Development (CBRD), Kenya Medical Research Institute (KEMRI) Headquarters, Nairobi, Kenya.

3.2 Experimental design

3.2.1 *Plasmodium falciparum* lines

Two *Plasmodium falciparum* lines, W2 (CQ resistant clone from Indochina) and D6 (CQ-sensitive clone from Sierra-Leone) were used in the study. The parasite lines (Table 3.1) were obtained from a continuous study by Kang'ethe *et al* 2016. Parasites were selected under pure artemisinin and *Artemisia annua* crude extract for 3 years as follows; one year old *Artemisia annua* plants obtained from Tanzania highlands (2000-2200 m altitude) in Arusha was used. The leaves were harvested just before flowering, dried for approximately 3 weeks under shade, and then crushed, powdered and homogenized. Samples of dried, whole-leaf uniformly powdered *A. annua* were extracted sequentially with solvents of increasing polarity (hexane, Dichloromethane, ethanol, and water).

The starting concentration of artemisinin was 10 ug/ml for the two parasite clones (W2 and D6). Then increments were in escalations of 10, 20, 30, 50 and 50 ug/ml. The starting

concentration for Artemisia annua was 250ug/ml. Parasites were cultured in RPMI 1640 medium supplemented with 15% human serum in citrate-dextrose anticoagulant (ACD), 25mM HEPES (N-2-hydroxyethyl piperazine-N'-2-ethylsulfonic79acid) and 25 mM $NaHCO_3$, and were incubated at 37^0C in an atmosphere of 3% CO_2 ,5% oxygen and 92% Nitrogen. Drug pressure cycle started with the addition of artemisinin to cultures with 4-8% parasitemia. W2 and D6 plain (cultured for the same period without drug exposure) were used as controls.

The choice of the W2 and D6 parasite lines was made in the anticipation that different genetic backgrounds of the parasite may provide a wide span of genetic determinants during the evolution of artemisinin and *Artemisia annua* resistance.

Table 3.1: *P. falciparum* W2 and D6 parasite lines used in the study

Study ID	Parasite line	Number of drug pressure cycles	Drug selecting
W2	W2	-	-
D6	D6	-	-
1L	D6-A	C17	Artemisinin
2L	D6-B	C20	Artemisinin
3L	D6-C	C20	*Artemisia annua*
4L	D6-D	C20	*Artemisia annua*
6L	W2-1	C36	Artemisinin
7L	W2-2	C42	Artemisinin
8L	W2-3	C37	*Artemisia annua*
9L	W2-4	C45	*Artemisia annua*

D6-A, D6-B =D6 parasites exposed to artemisinin at IC_{50} and IC_{90} respectively. D6-C and D6-D =D6 parasites exposed to *A.annua* at IC_{50} and IC_{90} equivalents. W2-1, W2-2= W2 parasites exposed to ART at IC_{50} and IC_{90}. W2-3, W2-4 =W2 parasites exposed to *A. annua* at IC_{50} and IC_{90} respectively.

3.3 Genotyping

3.3.1 Genomic DNA extraction

1 to 2 ml aliquots of the parasite culture was used for DNA extraction and each sample was diluted in one volume of PBS. In preparing genomic DNA, leucocytes were first depleted using Plasmodipur filtration columns (Euro-Diagnostica) (Auburn *et al*., 2011). Spinning was then done at 2000/rpm for 20 minutes to pellet the parasite. Genomic DNA was extracted using the QIAamp blood mini kit (Qiagen, Valencia, CA) as described by Miotto *et al*., 2015 .Briefly, 200/µl blood sample was added into 1.5/ml microcentrifuge tube followed by addition 200/µl lysis buffer. Proteins were digested by adding 20/µl proteinase K. The mixture was mixed by pulse-vortexing for 15 seconds followed by incubation at 56 ^{o}C for 10 minutes to ensure efficient lysis of *Plasmodium* pellet. 200/µl of 96% ethanol was then used to purify the DNA. Purification was achieved by micro-centrifugation at 8000/rpm for 1 minute. Filtrate was discarded and spin column was transferred into a sterile 2ml collection tube. DNA was washed using 500/µl wash buffer and centrifuged at 8000/rpm for 1 minute, and filtrate was discarded. This was followed by addition of 500/µl buffer AW2 into the spin column and the mixture was centrifuged at 14,000/rpm for 3 minutes. DNA was eluted from the spin column by adding 100/µl elution buffer and the preparation was centrifuged at 8,000/rpm for 1 minute. Purified genomic DNA was stored at -20 ^{o}C to -80 ^{o}C until use.

3.3.2 Genomic DNA quantification

Parasite genomic DNA quantities were determined by florescence analysis using Qubit 3.0 flourometer (Thermo Fisher Scientific).

3.3.3 Whole genome sequencing

3.3.3.1 Library preparation

Paired-end sequencing libraries were prepared for the 10 samples using Nextera XT DNA library preparation Kit according to the manufacturer's recommended protocol (https://www. Illumina .com). Briefly, 1ng of genomic DNA was fragmented by Nextera XT transposome to a mean fragment distribution of 300-500bp, and sequencing adapters were ligated to the fragments. The adapter ligated fragments were amplified using index 1 (i7) and index 2 (i5) primers. The reaction was carried out using the following PCR protocol: initial denaturation at 95 ^{o}C for 30 seconds, followed by 12 cycles at 95 ^{o}C for 10 seconds, 55^{o}C for 30 seconds, 72 ^{o}C for 30 seconds with final elongation step at 72 ^{o}C for 5 minutes. Fragments were then size selected and purified using Agencourt AMpure XP beads (Beckman Coulter) allowing removal of very short library fragments <200bp from the population. This was followed by normalization of the library quantities. The libraries were further pooled to contain equal amount of enriched DNA from each of 10 samples. The final library pool were quantified by quantitative PCR.

3.3.3.2 Cluster generation and sequencing

Samples underwent whole genome sequencing on an Illumina platform as previously described (Manske *et al*., 2012), using 151 cycles. Each library was hybridized onto the

flow cell surface bound with oligos complementary to the library adapters. Each bound fragment was then amplified through bridge amplification into distinct clonal clusters. The paired-end sequencing allowed each DNA fragment to be sequenced in both forward and reverse direction onto Miseq platform (https://www.illumina.com). After sequencing was complete, image analysis, base calling and error estimation was performed using Illumina Analysis Pipeline. Short reads generated (~151bp) were mapped to the pre-existing *P.falciparum* v3.1 genome scaffold (ftp://ftp.sanger.ac.uk/pub/project/pathogens/plasmodium/falciparum/3D7/3D7.latest_version/version3.1/2016/), and difference between the assembled sequence and the reference sequence was used to identify candidate mutations associated with artemisinin and *Artemisia annua* resistance.

3.4 Bioinformatics data analysis

Sequence read data were analyzed using a pipeline based on GATK best practices recommendations (Van der Auwera *et al*., 2013; DePistro *et al*., 2011). The pipeline was designed with three distinct phases (Appendix 1): (1) data preprocessing, (2) variant discovery and filtering and (3) analysis of clinically important SNPs in Artemis.

3.4.1 Quality control

Sequence read data (fastq files) obtained from 10 *P. falciparum* samples was subjected to standard Illumina quality control procedures. Sequence adaptors and low quality reads were filtered by Trimmomatic v0.35 software (Bolger *et al*., 2014), with the following stringent parameters: adapter trimming; LEADING- cutting bases at the start of a read if

below a threshold quality of 20; TRAILING- cutting bases at the end of a read if below threshold quality of 25; SLIDING WINDOW- scanning the read with a 4-base sliding window and cutting when the average quality per base drop below 15; MINLENGTH- dropping a read below 35 base pairs long. The quality control checks were then done using FastQC software.

3.4.2 Mapping

Each dataset was aligned separately against the *P. falciparum* 3D7 reference sequence version 3.1 (ftp://ftp.sanger.ac.uk/pub/project/pathogens/plasmodium/falciparum/3D7/3D7.latest_version/version3.1/2016/), using burrow-wheeler aligner, as previously described by Manske *et al*., 2012. The alignment files were generated in SAM format. Each sample SAM files were then converted to BAM files (binary file of tab-deliminated format of SAM) using Samtools (Li et *al*., 2009).

3.4.3 Pre-processing of BAM files

Pre-processing was done separately for each dataset. First, Sorting and indexing of BAM files was performed using Picard tool version 1.106 (http://picard.sourceforge.net). Sorted BAM files were then processed using Picard FixMateInformation tool (http://picard.sourceforge.net). Next, potential PCR duplicates which arise during the PCR amplification step of the library preparation were mapped and marked using Picard MarkDuplicates tool .The resulting BAM file was indexed using Picard tool and a visual inspection of mapped reads was done in Artemis (Rutherford *et al*., 2000).

3.4.4 Mapping quality control

A preliminary quality control of the BAM/BAI files produced in the mark duplicate step was done using FastQC and Qualimap software (Garcia-Alcalde *et al*., 2012), to check whether duplicates were in fact removed. The number and proportion of reads mapped by BWA was computed using Samtools flagstat and Samtools stats (Li *et al*., 2009). The genome wide coverage and loci covered to a certain percentage were calculated using GATK DepthOfCoverage (McKenna *et al*., 2010).

3.4.5 Local realignment around possible indels

The initial mapping algorithm tend to produce various type of artifacts, such as reads aligned on the edge of insertions/deletions (indels). Local realignment of reads around possible Indels and areas of high entropy was done using GATK IndelRealigner (Mckenna *et al*., 2010). The BAM/BAI files generated from mark duplicates step were provided as input.

3.4.6 Base quality score recalibration

Base quality score recalibration was applied to each sample realigned BAM files using GATK BaseRecalibrator (McKenna *et al*., 2010), and known variants from *Plasmodium falciparum* crosses 1.0 data as a training set (ftp://ngs.sanger.ac.uk/production/malaria/pf-crosses /1.0 / 7g8 _gb4.combined.final.vcf.gz, ftp://ngs.sanger.ac.uk/production/malaria/ pf-crosses/1.0/hb3_dd2. combined.final.vcf.gz). The recalibration process was divided

into two phases. First, the GATK program was used to build a model of covariation based on the data and sets of known variants. A second pass was then done to analyze the covariation remaining after first recalibration and R software (R Core Team., 2013) was used to generate before and after plots.

3.4.7 Variant calling and filtering

The processed BAM files from the recalibration step were provided as the starting point for this analysis. Variants calls were generated on each sample using GATK HaplotypeCaller (McKenna *et al*., 2010), with the following parameters; -stand_emit_conf 10 and -stand_call_conf 30. The resulting variant call format files (VCF) were merged using VCF tools (Danecek *et al*., 2011).

After merging the VCF files, single nucleotide polymorphisms (SNPs) were selected from the VCF file using GATK SelectVariants tool (McKenna *et al*., 2010). The initial quality filtering step was carried out using quality parameters provided by GATK developers (McKenna *et al*., 2010) as follows; minimum Quality by depth (QD) of 2.0, P-value of Fisher exact test of detecting strand bias <0.001, Minimum Mapping quality phred score (MQ) of 40 (mapping quality is the probability that the read is incorrectly aligned), Minimum depth of coverage at every SNP position of 10.0, Z-score of rank sum test for mapping qualities <-12.5 (mapping quality of all reads with alternate versus reference allele), and Z-score for rank sum test for relative positioning of reference versus alternative alleles within the reads of 8.0. Further, heterozygous were filtered using Vcf filter tool. Heterozygous calls are identified when

two possible bases are called in a particular position in the genome. The "recessive" allele constitute 20% of the calls while the major base is always identical to the reference base.

SNPs positions located within the sub-telomeric regions and hypervariable multi gene families (Var, stevor, and rifin) were also excluded from the analysis. Finally, functional annotation was produced with snpEFF tool (Cingolan *et al*., 2012), using Ensembl functional annotation of the *Plasmodium falciparum* as input.

3.4.8 Analysis of drug resistant SNPs in Artemis

The exonic sequences for the parental and selected strains were compared and SNPs analysed in Artemis (Rutherford *et al*., 2000). Further, the DNA and predicted amino acid sequences from each locus were analyzed by alignment in Muscle (Edgar *et al*., 2004) against a 3D7 reference sequence.

3.4.9 *Pfcrt* haplotype determination

The core *Pfcrt* haplotype coding for five amino-acid substitutions at position 72-76 was genotyped using a procedure previously described (Srimuang *et al*., 2016). Briefly, Samtools (Li *et al*., 2009) was used to extract all sequence reads containing two invariant flanking sequences, TATTATTTATTTAAGTGTA (upstream of the core *Pfcrt* haplotype and ATTTTTGCTAAAAGAAC (downstream) from each sample's alignment. Low quality reads (Phred scores < 20) were discarded using Fastq_quality_filter tool. The reads were then aligned against the *Pfcrt* reference sequence V3 (Plasmodb Pf3D7_0709000), and the core haplotype was read directly from the resulting alignment.

3.4.10 Calling of copy number variation in *Pmdr1* gene

The method used to detect copy number variation (CNV) in this study was based on read-depth strategy. The underlying concept of read depth method is that mapping depth across the whole genome follows a random Poisson distribution, and the number of reads expected to map within a region is proportional to the number of times that region appears in the sequenced genome (Teo *et al*., 2012). A region that is duplicated will display increase in signal or higher than expected coverage while a region that is deleted will display lower than expected depth of coverage intensity (Zhao *et al*., 2013).

Recalibrated BAM files were used as input for this analysis. Each dataset was evaluated for copy number variation (CNVs) relative to the parental strains (W2 and D6) using the R-package CNVseq tool (Xie and Tammi *et al*., 2009). Briefly, Samtools was used to generate the read count for each sample. CNV-seq Perl script was then used to calculate sliding window size, number of mapped hits in each window, and to call CNVs using the following parameters; significance threshold of $P>0.001$ and $\log 2> 0.6$.

CHAPTER FOUR

RESULTS

4.1 Quantitative analysis of *P. falciparum* genomic DNA

Extracted *Plasmodium falciparum* genomic DNA was quantified using Qubit fluorometer. The DNA concentration ranged from 1.51ng/ul (W2-1 C36) to 14.4ng/ul (D6-B C20) (Table 4.1). The DNA volume for all the samples was 100/ul. The yield was sufficient for whole genome sequencing.

Table 4.1: Table showing Qubit readings of genomic DNA extracted from *P.falciparum* parasite samples.

Parasite line	% Parasitemia	Concentration ng/ul
D6	1%	2.20
D6-A C17	6%	9.86
D6-B C20	8%	14.4
D6-C C20	7%	12.7
D6-D C20	2%	3.86
W2	2%	3.40
W2-1 C36	0.5%	1.51
W2-2 C42	3%	4.62
W2-3 C37	5%	5.52
W2-4 C45	5%	5.90

D6-A C17, D6-B C20 = D6 parasites exposed to ART. D6-C C20, D6-D C20 =D6 parasites exposed to *Artemisia annua* extract. W2-1 C36, W2-2 C42 =W2 parasites exposed to ART. W2-3 C37, W2-4 C45 = W2 parasites exposed to *Artemisia annua* extract. D6 =D6 control. W2 = W2 control.

4.2 Genome sequencing of *Plasmodium falciparum* parasite lines

4.2.1 Sequencing and initial data analysis

Sequencing generated between 2.6 - 6.3 million paired-end reads per sample (Table 4.2), and the mean read length was 151bp. After quality filtering, 2.3 to 5.8 million reads were obtained (Table 4.3).

Table 4.2: Summary of whole genome sequencing

Study ID	Parasite line	Raw read pairs
D6	D6	6,354,848
1L	D6-A C17	3,883,688
2L	D6-B C20	6,052,700
3L	D6-C C20	3,876,570
4L	D6-D C20	3,973,492
W2	W2	2,933,446
6L	W2-1 C36	5,011,184
7L	W2-2 C42	5,541,234
8L	W2-3 C37	2,620,124
9L	W2-4 C45	5,128,674

Table 4.3: Total number of filtered reads for the 10 *Plasmodium falciparum* samples

Study 1D	Parasite line	Total number of filtered reads
D6	D6	5,857,358
1L	D6-A C17	3,487,744
2L	D6-B C20	5,631,426
3L	D6-C C20	3,603,698
4L	D6-D C20	3,667,966
W2	W2	2,699,322
6L	W2-1 C36	4,591,450
7L	W2-2 C42	5,107,176
8L	W2-3 C37	2,372,582
9L	W2-4 C45	4,741,238

The quality control checks were done using FastQC software. Quality scores, expressed in phred scale (a logarithm function of base-calling error probabilities) verses position in read are shown in figure 4.1. As indicated, the phred scores were above 30 (less than 1 error in 1,000 bases) for all the samples. This showed that the base calls were of good quality and could be used for downstream analysis.

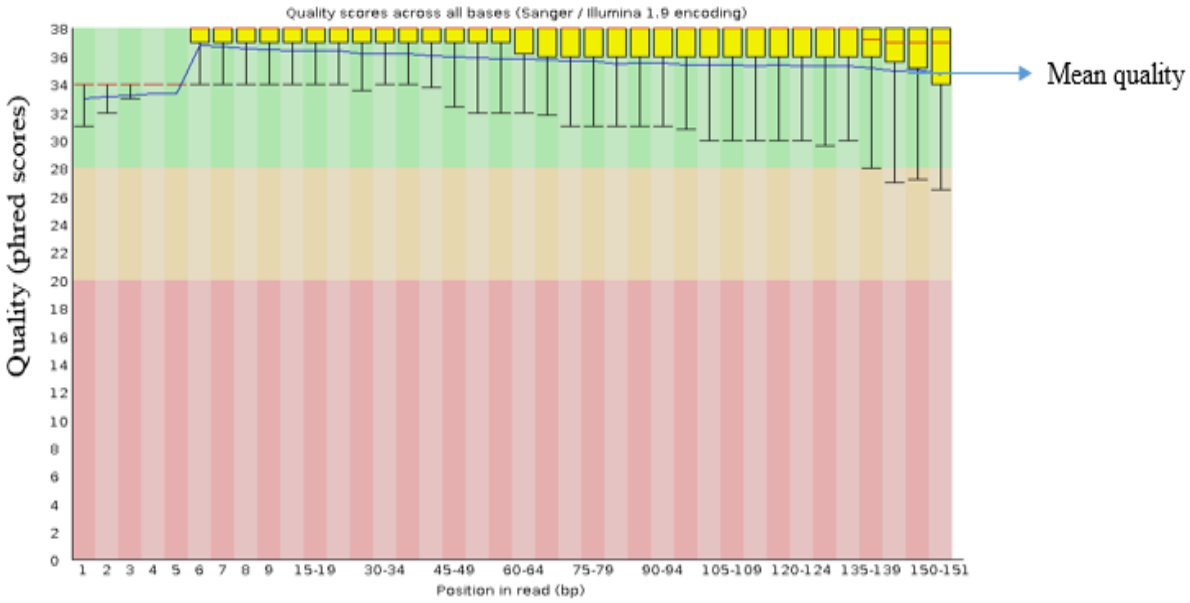


Figure 4.1: Quality scores across all bases as observed with FastQC software

Similarly, the average quality per read (phred scores) was roughly 37 (**Figure 4.2**), indicating that the probability of an incorrect base call was less than 1 in 1,000 bases, of an average (>99.9% base call accuracy).

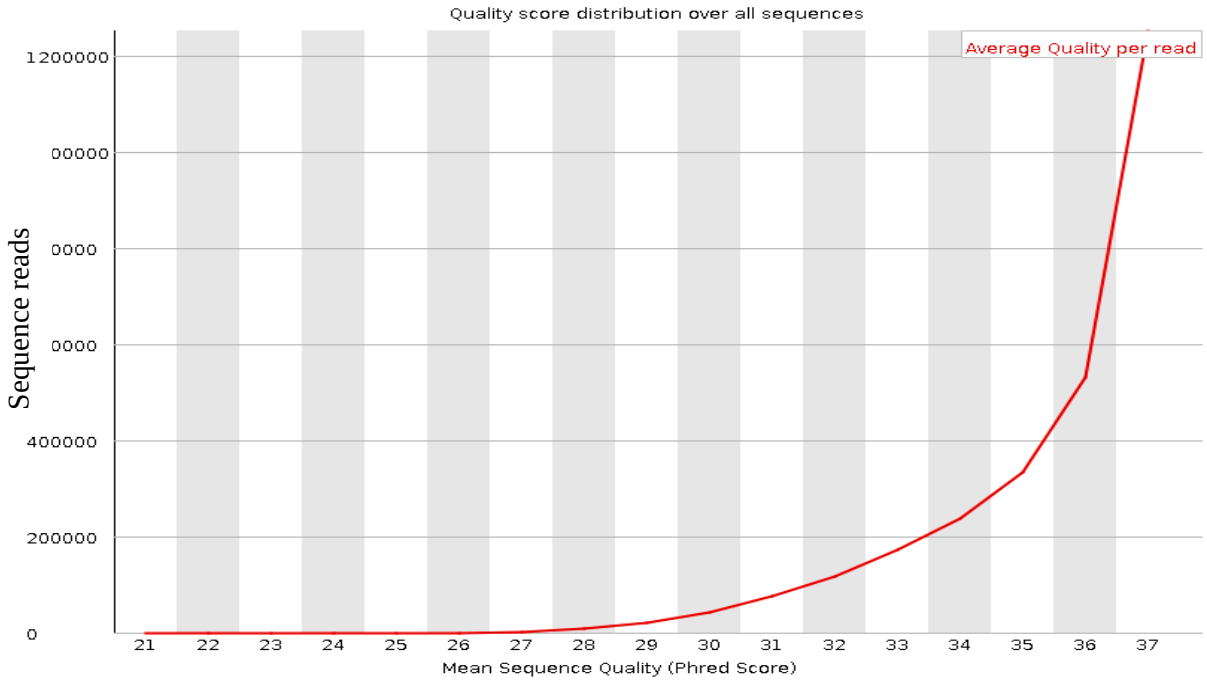


Figure 4. 2: Quality score distribution over all sequences as observed with FastQC software

4.2.2 Mapping of sequence reads using Burrow-wheeler aligner (BWA)

The initial data analysis showed that with BWA majority of the reads could be mapped against the *P. falciparum* reference sequence. The average alignment rate was ~ 95.8% (Table 4.4). After read alignment, PCR duplicates were marked using Picard tool, to reduce false coverage which could bias variant identification process. The percentage of PCR duplicates ranged from 2.0 % (W2-2 C42) to 4.3 % (D6), with an average of 2.8% (Table 4.4).

Parental strains D6 and W2 were sequenced to a genome wide coverage of 21X and 12X respectively (Table 4.4). The average coverage for the resistant parasites ranged from 9X

(W2-3 C37) to 24X (D6-B C20) although it was variable in the subtelomeric regions. Moreover, the majority of the genome was covered with reads with the coverage of at least 5X (Table 4.4), and this was directly correlated to genome fraction coverage (Figure 4.3).

Table 4.4: Summary of the sequencing metrics and average coverage of the parental strains and the resistant selections.

Parasite line	Mapped reads	% PCR duplicates	Genome wide coverage	%genome covered by at least 5 or more reads
D6	5,700,867 (97.37%)	4.3%	21X	84.7%
D6-A C17	3,393,981 (97.38%)	2.1%	15X	78%
D6-B C20	5,531,750 (98.23%)	2.4%	24X	85%
D6-C C20	3,442,943 (95.67%)	2.9%	14X	77%
D6-D C20	3,618,676 (98.69%)	3.0%	14X	75%
W2	2,585,551 (95.94%)	2.2%	12X	70%
W2-1 C36	3,784,220 (82.84%)	3.0%	15X	79%
W2-2 C42	4,941,512 (96.85%)	2.0%	21X	86%
W2-3 C37	2,344,031(98.83%)	3.2%	9X	65%
W2-4 C45	4,568,484(96.46%)	3.2%	19X	84%

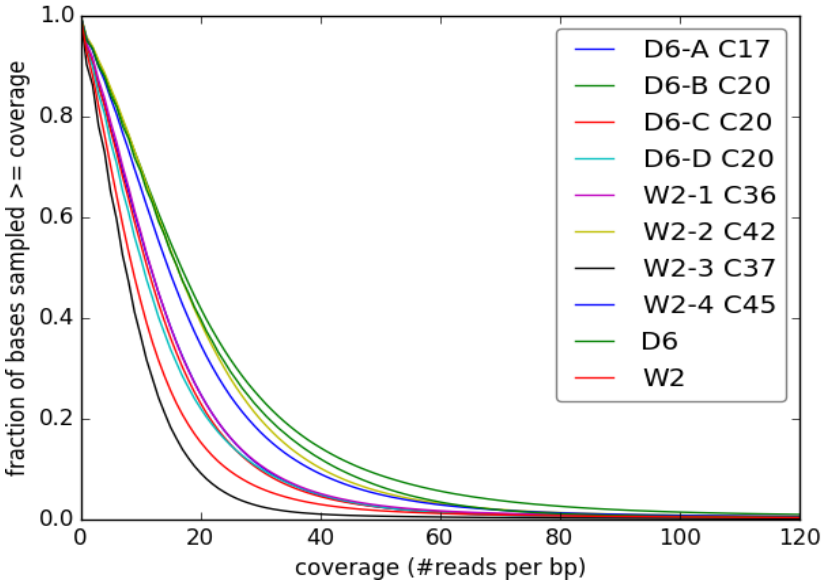


Figure 4.3: Genome fraction coverage: percent of *P.falciparum* genome covered by various coverage threshold for the 10 samples.

The figure shows the fraction of the coverage sampled. Apart from sample W2-3 C37, 70% of the genome was covered by 5X.

Mapping quality as assessed by Qualimap software is shown in Appendix 2. Despite the left tail of the distribution of the number of genomic locations, most of the reads presented mapping scores of around 60. This implied that the best alignment had very few mismatches (>99.99 accuracy).

4.3 Discovery of potential SNPs

Single nucleotide polymorphisms were called from high quality datasets obtained from the 10 *P. falciparum* samples. In total, 120,561 variable positions were identified between the strains and the reference sequence. This list of candidate SNPs was further filtered

based on the individual properties of SNPs (mapping quality, depth of coverage and quality by depth). The quality filtering produced a list of 34,520 biallelic SNPs.

4.3.1 *PfK13* propeller polymorphism.

One non-synonymous mutation was identified in the Apicomplexa/specific domain of K13 gene. Mutation K189T corresponding to nucleotide position A566C (Appendix 3), was found in 7 out of the 10 samples (Appendix 6). The D6 parental strain and its resistant progenies (D6-A C17, D6-B C20, D6-C C20 and D6-D C20) had the K189T mutation. Two W2 parasites (W2-2 C42 and W2-4 C45) had the 189T mutation, while the W2 parental parasite and two samples of the four sequenced (W2-1 C36 and W2-3 C37) harbored the wild type allele K189 (Table 4.5).

Table 4.5: Polymorphism observed in K13 gene

Parasite line	Nucleotide position 566	Amino acid position 189
W2	A	K
W2-1 C36	A	K
W2-2 C42	C	T
W2-3 C37	A	K
W2-4 C45	C	T
D6	C	T
D6-A C17	C	T
D6-B C20	C	T
D6-C C20	C	T
D6-D C20	C	T

4.3.2 *Pfmdr1* polymorphism

This study identified one allelic variation in *Pfmdr1* gene (Table 4.6). The N86Y mutation corresponding to nucleotide position A256T was observed in one sample, W2-1C36 parasites exposed to artemisinin at $1C_{50}$ equivalents (Appendix 4). The parasites exposed to *Artemisia annua* extracts harbored the wild type allele N86.

Table 4.6 Polymorphisms observed in *Pfmdr1* and *Pfcrt* gene

Gene	Codon Position	Reference sequence		Mutant Sequence		Total number of samples %
		Amino acid	Nucleotide	Amino acid	Nucleotide	
Pfmdr1	86	N	**A**AT	Y	**T**AT	1/10 (10)
Pfcrt	76	K	A**A**A	T	A**C**A	2/10 (20)
Pfcrt	220	A	**G**CC	S	**T**CC	1/10 (10)
Pfcrt	271	Q	AA**C**	E	AA**G**	2/10 (20)
Pfcrt	356	I	A**T**A	T	A**C**A	1/10 (10)
Pfcrt	371	R	A**G**A	I	A**T**A	1/10 (10)

Nucleotide changes from reference to mutant are shown in bold color

4.3.3 *Pfcrt* allelic types

The *pfcrt* wild-type CVMNK haplotype at codons 72-76 of CRT protein was confirmed present in 80% (8/10) samples. The K76T mutation was identified in W2 parental strain and W2-1 C36 parasites (Table 4.6). This study also identified 4 additional mutations distinct from the CVIET genotypes commonly assessed (Table 4.6). Mutations A220S, 1356T and R371I were observed in W2-1C36 parasites exposed to artemisinin at IC_{50} equivalents (Appendix 5). The Q271E mutation corresponding to nucleotide position

C811G was observed in W2 parental strain and its resistant progeny (one sample of the five sequenced, W2-1C36).

4.4 Detection of copy number variation in *Pfmdr1* gene

Each dataset was evaluated for presence of copy number variations in *Pfmdr1* gene relative to the parental strains using CNVseq tool. Deletions of significance ($P<0.001$) were detected at nucleotide position 1952 of the *Pfmdr1* gene (Appendix 7). Furthermore, no duplicated regions were observed in any of the selections analyzed here indicating that the *Pfmdr1* gene had only one copy.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Resistance associated mutations within the D6 and W2 lineages

Clinical artemisinin resistance has been confirmed in Southeast-Asia, manifesting itself as delayed parasite clearance half-life > 5hrs following treatment with ACTs (Dondorp *et al*., 2009; Ashley *et al*., 2014). This is an incoming threat to malaria control and treatment as there are no alternative treatment options for malaria. Deducing the molecular genetics of artemisinin resistance would be the first step toward containing it and determining treatment strategies (Anderson *et al*., 2010; Witkowski *et al*., 2010). Although *in vivo* studies are a vital tool for assessing antimalarial drug efficacy, they are prohibitively expensive, thus a limitation to conducting huge number of cases. This study filled this gap by analyzing mutations acquired specifically by laboratory lines selected under artemisinin and *Artemisia annua* extract. This provided a useful tool for studying the mutations acquired through the drug selection period.

In this study, Illumina whole genome sequencing was used to define the genetic changes that occurred in the W2 and D6 lineage during the selection of artemisinin and *Artemisia annua* resistance. High quality sequence data (>Q30) obtained from sequencing allowed assembly of almost the whole genome.

Point mutations in the propeller domain of K13 gene (PF3D7_1343700) were recently associated with slow parasite clearance (>5h), and *in vitro* artemisinin resistance (Ariey *et al*., 2014; Ashley *et al*., 2014). These studies reported an association of prolonged parasite *ex vivo* survival with the Y493H, R539T, C580Y, and I543T mutations and C580Y, R539T and Y493H mutations were linked to delayed parasite clearance. K13 propeller polymorphisms were also found to generate an ART-resistant phenotype *in vitro*, where M476I mutation was linked to artemisinin tolerance in African parasites (F32ART5). Although the role of K13 gene in artemisinin resistance is yet to be defined, transfection studies inducing genetic modification of K13 locus of the parasites have confirmed it as the major gene conferring resistance to artemisinin drugs (Ghorbal *et al*., 2014). The above research demonstrated that the introduction of C580Y mutation into artemisinin sensitive *P. falciparum* strain caused a significant increase in ring-stage parasite survival in presence of artemisinin. In other study, Straimer *et al* 2015 modified the *K13* gene locus using zinc finger nucleases (Straimer *et al*., 2015) and demonstrated the importance of C580Y, 1543T, Y493H, and R539T in mediating *in vitro* artemisinin resistance.

In the current study, there was a very limited variability within the coding sequence of K13 gene. Polymorphisms previously associated with artemisinin resistance in Southeast Asia were not identified nor the M476I mutation associated with artemisinin tolerance *in vitro*. Only one non-synonymous mutation (K189T), located in the *Plasmodium*/Apicomplexa-specific domain was detected. The K189T mutation was present in 70% of the samples. This mutation was found in 42.2% (27/64) isolates from

Dakar, Senegal (Torrentino-madamet *et al*., 2014), and in 4.7 % (8/169) isolates from Bangladesh (Mishra *et al*., 2016). In another study, (Takala-Harrison *et al*., 2015), the isolate harboring K189T mutation in Bangladesh was associated with parasite clearance half-life >5hrs. Although, K189T allele has been found at a comparatively high frequency in Africa (Talundzic *et al*., 2017), it is not associated with prolonged parasite clearance or confirmed artemisinin resistance, suggesting that this polymorphism may be a part of naturally-evolving parasite population.

Polymorphisms in *Pfmdr1* at codons 86, 184 and 1246 have been suggested as possible markers of changes in parasite susceptibility to various drugs, including amodiaquine and artemether-lumefantrine (Humphreys *et al*., 2007). Amodiaquine treatment has been found to select parasites with 86Y, 184Y and 1246Y haplotypes, whereas artemether-lumefantrine selected parasites harboring 86N, 184F and 1246D genotypes. In the two resistant parasite lines used in the present study, only one allelic variation was identified in *Pfmdr1* gene. The N86Y mutation was found in parasites exposed to artemisinin drug at IC_{50} equivalents. The parasites exposed to *Artemisia annua* extract did not harbor mutant allele in *Pfmdr1* gene.

Although, *Pfmdr1* polymorphisms have been associated with reduced susceptibility to ART (Veiga *et al*., 2011), other studies found no association between this gene and prolonged parasite clearance rates in patients from Cambodia (Imwong *et al*., 2010). Importantly, no *Pfmdr1* selection was shown in parasites cultured under artemisinin drug

pressure for 5 years (Ariey *et al*., 2014). This suggests that *Pfmdr1* action may be dependent on other genetic factors

Studies have reported that ACT treatment selects for particular alleles in *Pfcrt* gene, which are firmly associated with resistance to chloroquine and amodiaquine (Sondo *et al*., 2016). *Pfcrt* encodes a 424-amino acid integral protein and is involved in drug transport from the food vacuole lumen to cytoplasm, an action important in the development of drug resistance (Ibraheem *et al*., 2014). The present study shows that *Pfcrt* CQ sensitive CVMNK haplotype was the most favored allele. The *Pfcrt* K76T mutation, was observed inW2 parental line and W2 parasites exposed to artemisinin. This mutation is however suggested not to be linked to artemisinin resistance.

Antimalarial drug resistance is accompanied by reduced replicative fitness in *P. falciparum* parasites. A recent study reported that there is an interplay between K13 mutations and six background mutations; D193Y in *ferrodoxin* gene (*fd*), T481I in *mdr2*, V127M in *apicoplast ribosomal protein10* (*arps10*), N326S and 1356T in *Pfcrt*, V1157L in *protein phosphatase* (pph) and C1484F in *phosphoinositide-binding protein* (Miotto *et al*., 2015). In this study, all parasites harbored wild type alleles in *mdr2*, *arps10, fd, pph* and *pibp* genes. *Pfcrt* 1356T mutation was observed in parasites exposed to artemisinin at IC_{50} equivalents. This compensatory mutation may be required in the evolution of artemisinin resistant phenotype. Notably, the cysteine protease *falcipain* 2a gene, previously associated with *in vitro* response to artemisinin (Klonis *et al*., 2011, Ariey *et al*., 2014), remained unaltered during the 3 year selection.

Pfmdr1 copy number variations have been associated with accumulation of lumefantrine in the digestive vacuole (Rohrbach *et al*., 2006). Additionally, its amplification has been linked to mefloquine tolerance (Price *et al*., 2004), and reduced efficacy to the widely used ACTs, artemether-lumefantrine (Srimuang *et al*., 2016). In this study there was no *Pfmdr1* gene duplication. This conforms with other study that reported lack of *Pfmdr1* amplification in parasites cultured for 5 years under artemisinin derivatives *in vitro* (Ariey *et al*., 2014).

Artemisia annua extract has been found to be more effective at least five times than a comparable dose of pure artemisinin in *Plasmodium chabaudi* (Elfawal *et al*., 2012. It has also been shown to overcome existing resistance to pure artemisinin in rodent malaria parasites (Elfawal *et al*., 2015). The current study did not detect any polymorphism associated with *Artemisia annua* resistance. A possible explanation is that the likelihood of *Plasmodium* parasites developing resistance to a single compound is greater than for a combination of compounds. *Artemisia annua* consist of a large phytochemical repertoire of small compounds like flavonoids, that have antimalarial activity but weaker than artemisinin (Willcox, 2009). Chrysoplenetin, chrysoplenol-D, and eupatorin flavonoids have showed a significant improvement in the parasites IC50 in the presence of artemisinin (Ferreira *et al*., 2010). Further a recent study showed that dried *Artemisia annua* leaf extract treated patients with *Plasmodium* parasites resistant to artemether-lumefantrine (Daddy *et al*., 2017)

In Africa, Only a few cases of reduced parasite clearing infections or of prolonged $RSA_{0\text{-}3hours}$ survival rates have been reported to date (Ashley *et al*., 2014; Menrad *et al*., 2016), suggesting that the drug pressure has not yet selected for K13 mutations. A plausible explanation would be absence of a favorable genetic background that predispose the parasite populations to the emergence of artemisinin resistance. Polymorphisms in *Pfmdr2*, ferrodoxin, apicoplast ribosomal protein 10 form a background on which K13 mutations arise independently, and they were also associated with slow clearance in Southeast Asia (Miotto *et al*., 2015). These mutations may compensate for the reduced fitness accompanied by mutation of the highly conserved K13 protein. Resistance-conferring K13 mutations appear to have multiple geographical origin (Takala-Harrison *et al*., 2015), and it is clear from a recent work that *PfK13* alleles reported in Southeast Asia may not be the same alleles selected in Africa (Talundzic *et al*., 2017) . Therefore, there is need to characterize the functional significance of novel K13 mutations circulating in Africa.

5.2 Conclusions

1. This study identified the genetic changes that occurred in the D6 and W2 *P. falciparum* lines after the selection of artemisinin and *Artemisia annua* resistance. The results showed that there was selection of *Pfmdr1* N86Y variant in parasites exposed to pure artemisinin at IC_{50} equivalents. However, no mutation was identified in the propeller domain of K13 gene. *K13* K189T mutation may not be associated with artemisinin resistance.

2. The exonic sequences of both parental and parasites exposed to *Artemisia annua* extracts were similar, thus no polymorphisms were associated with *A. annua* resistance.
3. Further, there was no increase in *Pfmdr1* copy number in any of the selection analyzed here. This indicates that *Pfmdr1* amplification may not be associated with artemisinin resistance.

5.3 Recommendations

1) The role played by *Pfmdr1* mutations in artemisinin resistance need to be further studied.
2) High artemisinin resistant lines which can grow continuously under artemisinin pressure should be selected.
3) *Artemisia annua* extract should be incorporated into the antimalarial regimen and this could reduce the cost of health care.
4) There is need for continued surveillance of molecular markers underlying artemisinin resistance in the field.

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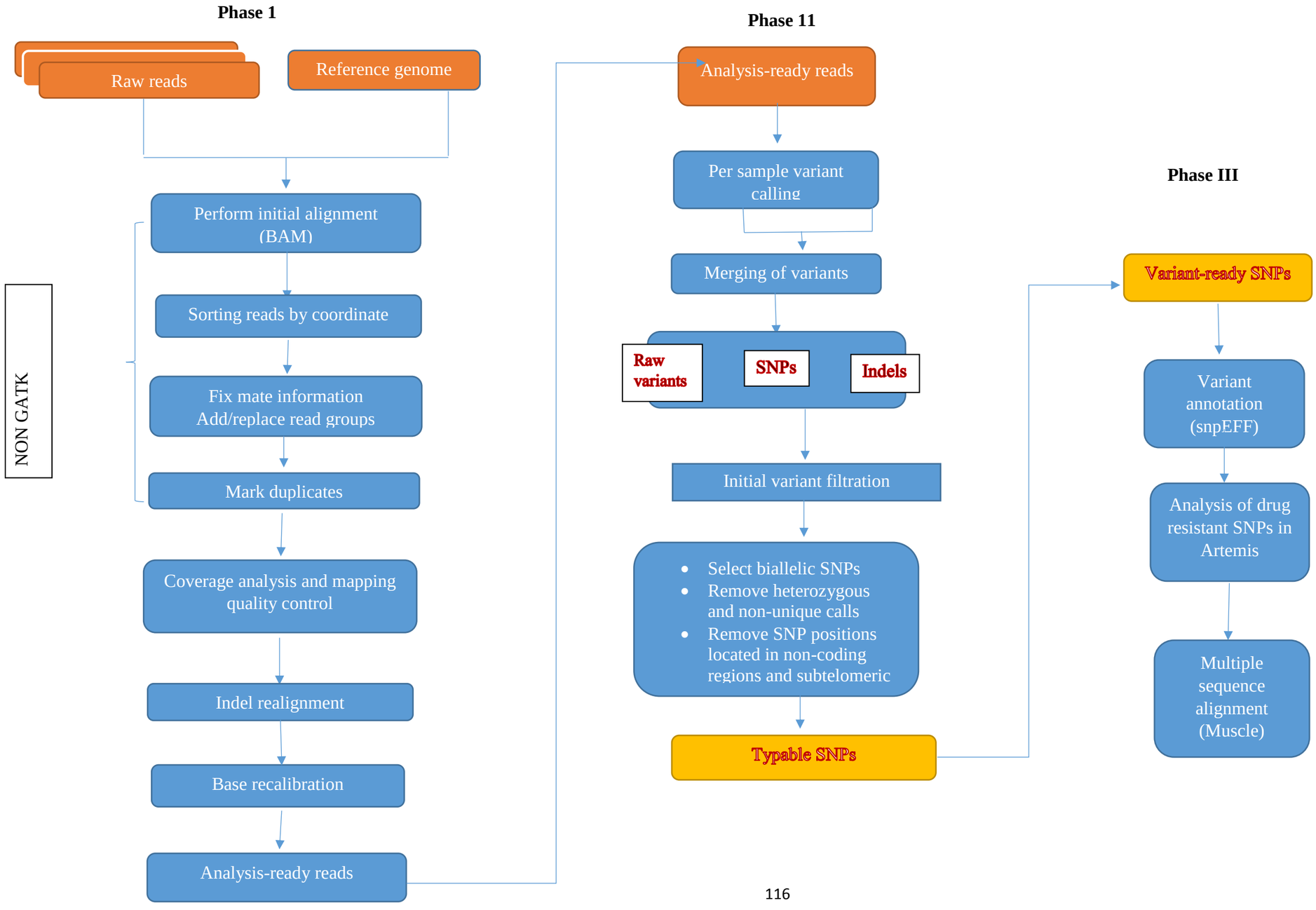
APPENDICES

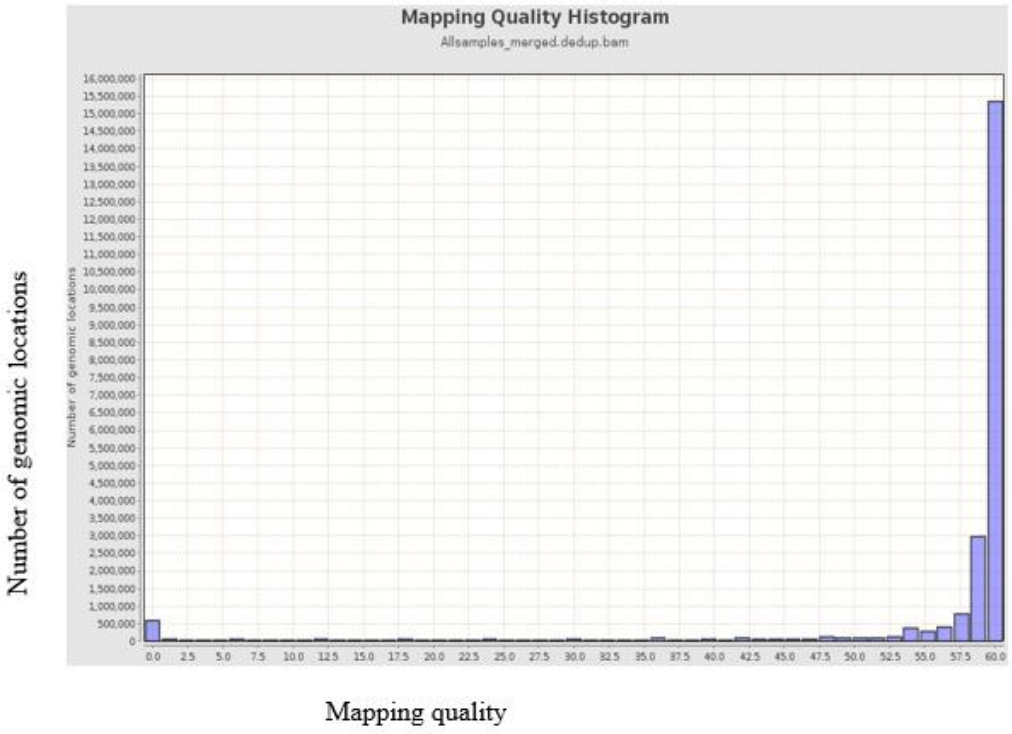
Appendix 1: Variant calling pipeline

Diagram showing the workflow leading to analysis of typable SNP dataset. Raw inputs workflow are shown in brown boxes, analysis tasks in blue and yellow boxes indicate final dataset products

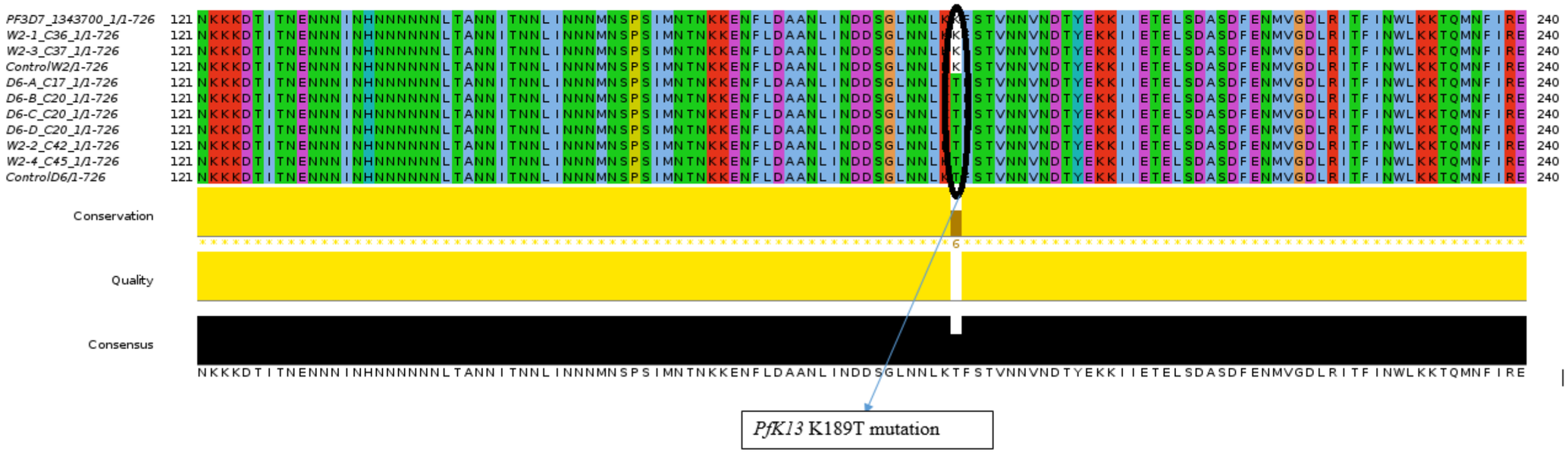


Appendix 2: Mapping quality histogram

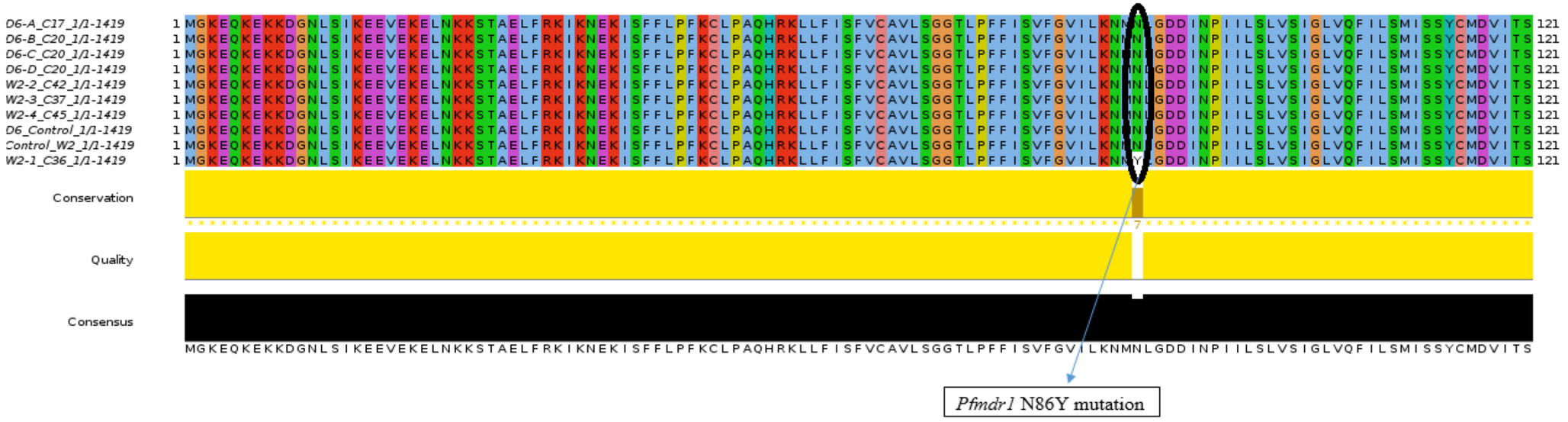
Mapping quality histogram for all the samples as observed by Qualimap software.



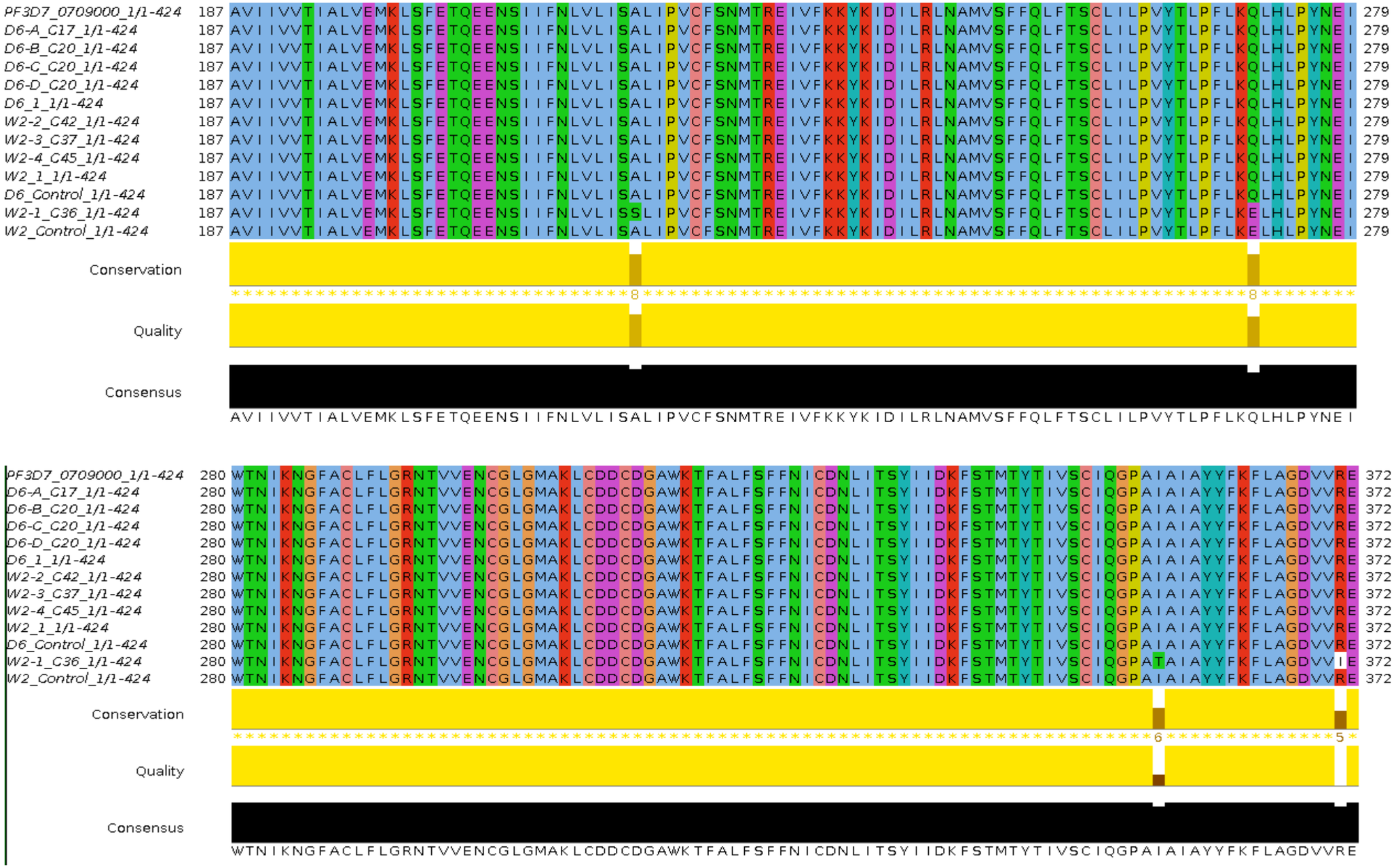
Appendix 3: Multiple sequence alignments showing *kelch 13* K189T mutation



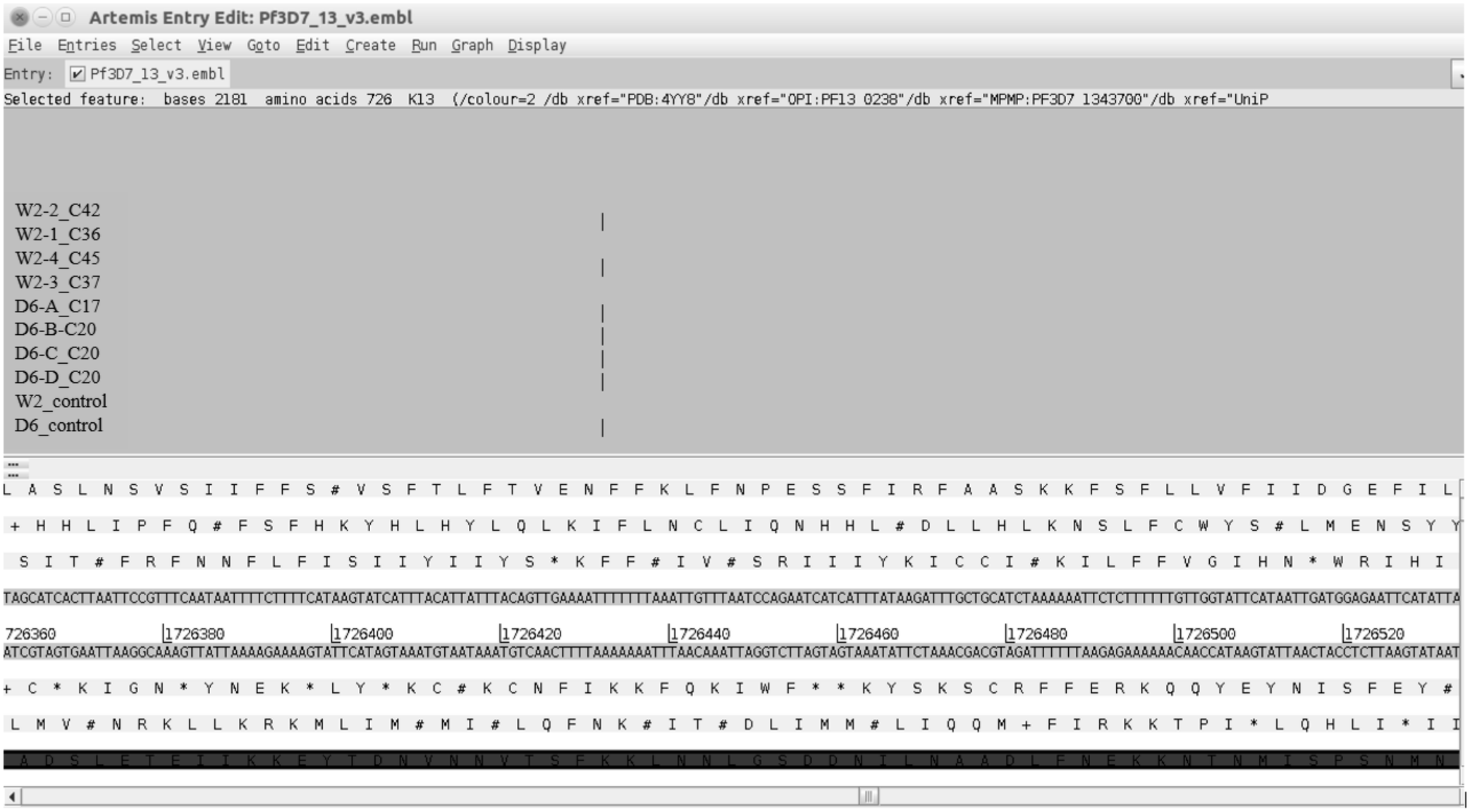
Appendix 4: Multiple sequence alignment showing *Pfmdr1* N86Y mutation



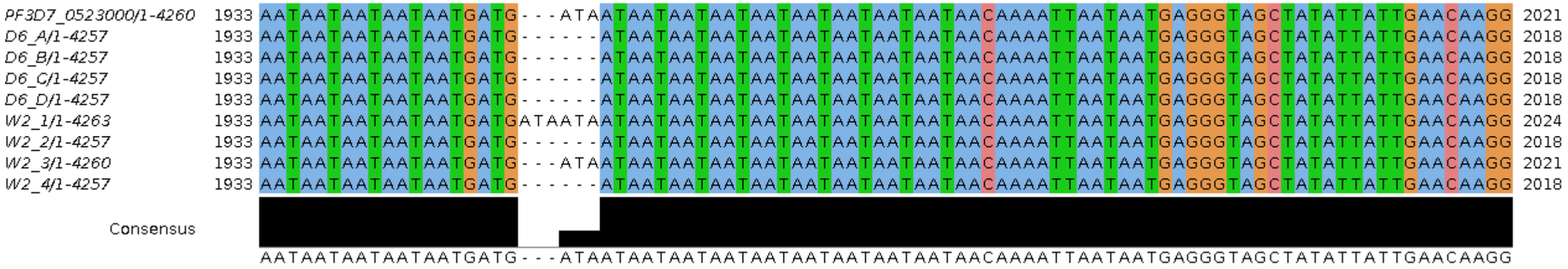
Appendix 5: Multiple sequence alignments showing *Pfcrt* mutations



Appendix 6: Artemis screenshot of *K13* K189T mutation



Appendix 7: *Pfmdr1* Indels (deletion/insertion) at nucleotide position 1952



Appendix 8: Publication

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Limited Polymorphisms in *Plasmodium falciparum* Lines Exposed to Pure Artemisinin and *Artemisia Annua* Extracts

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Abstract: ***Artemisinin-based combination therapy has been a vital tool in malaria control and elimination programmes. However, artemisinin resistant P. falciparum parasites have emerged in Southeast Asia, posing a major threat to the effectiveness of ACT. Resistance results in prolonged parasite clearance in vivo and enhanced survival of ring-stage parasites in vitro. Therefore, understanding the genetic basis of resistance would be critical to the success treatment and intervention strategies. This study aimed at identifying single nucleotide polymorphisms associated with artemisinin and Artemisia annua resistance. Genetic analysis was done on P. falciparum lines W2 and D6, previously selected under pure artemisinin and Artemisia annua extracts. Genomic DNA was extracted using QIAamp blood mini kit. Libraries were sequenced on Illumina Miseq platform using 151bp paired-end chemistry. Sequencing read data from each sample was mapped against P.falciparum reference sequence version 3.1. One non-synonymous (NS) mutation K189T was identified in K13 gene. The Pfmdr1 mutation N86Y was detected in W2 parasite exposed to pure artemisinin at IC_{50} equivalents and notably, the Pfcrt CQ sensitive CVMNK genotype was retained. The study also identified one background mutation in Pfcrt (I356T) in W2 parasites exposed to artemisinin at IC_{50} equivalents. In conclusion, K13 mutation described here has not been linked to reduced parasite clearance or in vitro artemisinin tolerance. Pfmdr1 gene may putatively play a role in artemisinin resistance.***

Keywords: Artemisinin-resistance, K13 gene, malaria, *Plasmodium falciparum*