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**PfK13-Independent determinants of susceptibility of African *Plasmodium falciparum* to artemisinin and partner drugs *in vitro***

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Thesis submitted in accordance with the requirements for the degree of  
Doctor of Philosophy

**University of London**

**January 2017**

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LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE

## **DEDICATION**

For my father,  
Brigadier General Augustine Aniebo,  
Who is the reason I am the woman I am today, and who I lost to the cold hands of death  
during this PhD journey.

## ACKNOWLEDGEMENTS

I have been on this journey for the past four years and I would like to thank a few people who have supported me throughout. Special thanks to my supervisor Dr Colin Sutherland, who has been crucial to the successful completion of this PhD and has been a constant source of support and encouragement throughout this time. I would also like to thank my Co-supervisor Prof. Taane Clarke, who has always been a source of encouragement and support throughout the duration of the PhD. I am deeply grateful for the support I received from my colleagues at the Sutherland Lab. I would like to especially thank Don van Schalkwyk, who has been a constant source of support and who was always been happy to answer all my numerous questions and take time out to teach me. To my other colleagues, Mary, Khalid, Inke and Ryan, thank you all so much for your support and friendship. I would like to thank Eloise for her laboratory support and training.

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## **DECLARATION**

‘I, Ifeyinwa Aniebo, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.’

## ABSTRACT

Artemisinin-based combination therapy (ACT) is currently the most effective treatment strategy against *Plasmodium falciparum* malaria. There have been reports from Southeast Asia of parasite resistance to artemisinin and its derivatives, and there are fears that resistance to this class of drugs will spread to Africa. Artemisinin resistance is recognised by a relatively slow parasite clearance rate in patients receiving an ACT or artemisinin. *In-vitro* or *ex-vivo* methods are conventionally used to determine the EC<sub>50</sub> value – the effective concentration of drug that inhibits parasite growth by 50% for parasite lines in the laboratory. To date, there have been no consistent correlations between the estimated *in-vivo* half-life of *P. falciparum* treated with artemisinin *in-vivo* and *in-vitro* EC<sub>50</sub> estimates from standard 48hr artemisinin susceptibility assays.

This study investigates alternative methods of assaying artemisinin resistance by *in vitro* exposure to dihydroartemisin (DHA) - the major metabolite of all artemisinins. DHA has a short half-life (1-2hr) *in-vivo* and so short drug pulses were applied to parasites in an effort to better mimic *in-vivo* conditions. A 6hr drug pulse assay and a standard 48hr assay were used to observe any differences that may exist in EC<sub>50</sub> values among field isolates from Kenya (HL1204), Ghana (HL1210) and Nigeria (HL1212). In all three isolates tested, the mean DHA EC<sub>50</sub> values for the 6hr pulse assays were higher than estimates from standard 48hr assays. This trend was seen as an elevated EC<sub>50</sub> value in the dose response curve, allowing discrimination of DHA EC<sub>50</sub> 6hr values among the isolates. Field isolates from Ghana (6 fold higher than 3D7 lab strain) and Nigeria (3.4 fold higher) showed elevated mean DHA EC<sub>50</sub> 6hr value compared to that from Kenya (1.7 fold higher than 3D7).

When this assay was applied to parasites from Cambodia with known *PfK13*-determined artemisinin resistance, an elevated EC<sub>50</sub> value in the 6hr pulse assay was again observed, but discrimination between Cambodian resistant and sensitive isolates was not achieved. We present results from a new DHA *in vitro* susceptibility assay format we have devised to overcome this problem. Experiments suggest this format can identify both *PfK13*-dependent Asian phenotypes and *PfK13*-independent phenotypes in African parasites.

Susceptibility to partner drugs lumefantrine and piperaquine was assessed with standard 48hr assays for all isolates.

The genetic basis of the susceptibility phenotypes observed was explored by whole genome sequencing for each African isolate. Mutations present in 11 genes of interests for drug resistance loci were identified. As these isolates are multiclonal, all haplotypes present in selected genes of interests for every isolate were described. Implications of this work for continuing studies of parasite susceptibility of ACT in Africa are considered. This includes a new method that can be used to monitor and control the spread of drug resistance on the African continent.

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## ABBREVIATIONS

3D7	CQ Sensitive isolate
HB3	CQ sensitive isolate from Honduras
AP	Alkaline phosphate
ACRP	Adequate Clinical and Parasitological Response
ACT	Artemisinin combination therapy
AL	Artemether lumefantrine
AQUAMAT	Africa Quinine Artesunate malaria trial
ATPase	Adenosine triphosphatase
AL	Artelinic acid
ART	Artemisinin
CVIET	Chloroquine resistant
CVMNK	Chloroquine sensitive
CQ	Chloroquine
DNA	Deoxyribonucleic acid
Dd2	CQ resistant isolate
DHA	Dihydroartemisinin
DMSO	Dimethyl sulphoxide
EC50	Half maximum effective concentration
EDTA	Ethylene-diamine-tetraacetic acid
G6PD	Glucose-6-phosphate dehydrogenase
HTD	Hospital for Tropical Diseases
HPLC	High performance liquid chromatography
EC <sub>50</sub>	50% Inhibition concentration
IPT	Intermittent preventative therapy

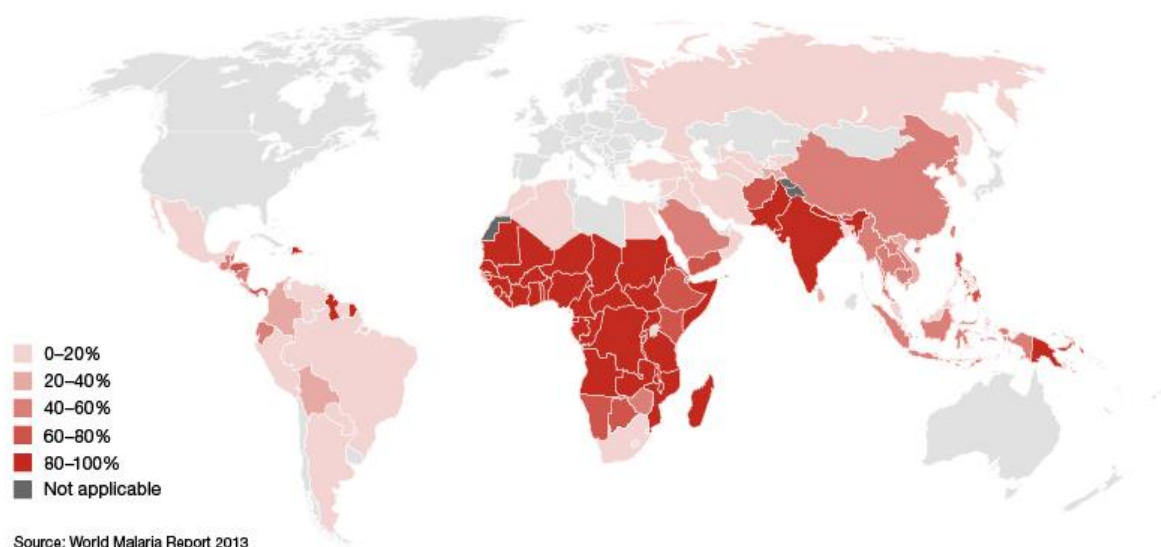
MOI	Multiplicity of infection
MSA	Mature stage survival assay
MSP	Merozoite surface proteins
PCR	Polymerase chain reaction
PABA	P-aminobenzoic acid
PCT	Parasite clearance time
<i>Pcubp1</i>	<i>Plasmodium falciparum</i> de-ubiquitinating enzyme gene
<i>Pgh1</i>	<i>P-glycoprotein homolog 1</i>
<i>PfCRT</i>	<i>Plasmodium falciparum</i> chloroquine resistance transporter
<i>Pfmdr1</i>	<i>Plasmodium falciparum</i> multidrug resistance 1
<i>Pfmrp</i>	<i>Plasmodium falciparum</i> multidrug resistance –associated protein
<i>PfATP6</i>	Sarco/endoplasmic reticulum $Ca^{2+}$ -ATPase orthologue
<i>Pfk13</i>	<i>P.falciparum</i> kelch 13 gene
<i>PfP13K</i>	<i>Plasmodium falciparum</i> phosphatidylinositol-3-Kinase
<i>Pfdhfr</i>	<i>P. falciparum</i> dihydrofolate reductase gene
<i>Pfdhps</i>	<i>P. falciparum</i> dihydropteroate synthase gene
<i>Pfap2mu</i>	<i>P. falciparum</i> mu chain of the AP2 adaptor protein complex gene
<i>Pfnhe1</i>	<i>P. falciparum</i> sodium/hydrogen exchanger gene
qpcr	Quantitative polymerase chain reaction
RSA	Ring stage survival assay
SMC	Seasonal malaria chemoprevention
SNP	Single Nucleotide polymorphisms
SP	Sulfadoxine-pyrimethamine
SEAQUAMAT	Southeast -Asia quinine artesunate malaria trial
WHO	World Health Organization

## **CHAPTER 1: Literature Review**

## 1.1 Malaria: A brief history

Malaria is one of the world's most deadly diseases affecting approximately 5% of the world's population. Although the disease has been eradicated in most temperate regions, it continues to be endemic throughout much of the tropics and subtropics. In 2015, 95 countries and territories had ongoing malaria transmission and it was estimated that about 3.2 billion people are at risk of malaria (WHO, 2015).

Malaria is a highly preventable and treatable disease with children and pregnant women bearing the greatest burden. However, increased efforts are reducing the burden of the disease. Between the year 2000 and 2015, it was estimated that the incidence of malaria amongst at risk populations fell by 37% globally and the death rate among at risk populations fell by 65% globally amongst children under the ages of 5 and by 60% among all age groups (WHO, 2015). At present 90% of cases and deaths occur in Africa (Figure 1.1) and 85% of malaria-related deaths occur in children under 5 years of age. According to the WHO, malaria is both a disease of poverty and a cause of poverty ([www.who.com](http://www.who.com))



**Figure 1.1** Areas at risk of *Plasmodium falciparum* malaria transmission in 2013. Graph shows percentage population at risk (World malaria report, 2013).

Malaria has been described since antiquity and Hippocrates, amongst occidental writers, is usually accredited with the first clear description. He differentiated patterns of fever in epidemics and he described the regular paroxysms of intermittent in his aphorisms. Seasonal periodic fevers were particularly common in marshy areas of Europe and were often referred to as 'paludial' (Bruce-Chwatt, 1988). Italian writers thought malaria was caused by the offensive vapours emanating from the tiberian marshes. The cause of the seasonal periodic fevers remained a continuous source of debate until the late nineteenth century. The work of Meckel, Frerich and Virchow showed that the pigment observed in the blood of some patients that had periodic fever resulted from the destruction of red blood corpuscles. The same pigment was also found to cause the characteristic grey discolouration of the internal organs in patients that were dying from the disease (Foster *et al.*, 1965). In 1880, Charles Alphonse Laveran identified moving bodies whilst examining the blood of a patient with ague and he deduced correctly these were parasites of red blood cells (Laveran, 1880). The transmissibility of the infection in blood was proven by Gerhardt four years later and, by the next decades the route of natural infection was discovered (Bruce-Chwatt, 1988).

In 1897, Ronald Ross started investigating the possibility that malaria could be transmitted by mosquitoes. He then reported the presence of pigmented bodies in the gut of a species of mosquito after many months of failure. This mosquito had fed on patients with malaria so he suggested the pigmented bodies might represent the parasite stage in the mosquito. After many years of study, Ross characterized the complete lifecycle. He also identified the anopheles mosquito as the vector of human malaria (Ross, 1897). Both Ross and Laveran received Nobel Prizes for their respective discoveries in malaria.

The understanding of the biology of malaria was further advanced by the Viennese psychiatrist Julius Wagner-Jauregg, who won the third Nobel Prize for his discovery. He became interested in the relationship between fever and mental illness and, between 1888 and 1917 he experimented on a few methods to induce fever to treat patients with general paralysis of the insane, which is a form of neurosyphilis. In 1917, he inoculated blood from a malaria infected soldier into two patients that had neurosyphilis and from then on, began the

era of malaria therapy of neurosyphilis. This therapy became the standard practice for treating neurosyphilis all over the world until the introduction of penicillin thirty years later (Bruce-Chwatt, 1988).

Until the 19<sup>th</sup> century, Malaria was found in northern Europe, North America and Russia. There was also intense transmission in South America. It has been eradicated from many of these areas since then and the number of cases in China, India and the Middle East has significantly reduced (Bruce-Chwatt, 1988). However, in Africa, malaria continues with much morbidity and mortality. In recent times, due to interventions like insecticide treated bed nets and indoor residual spraying, coupled with effective case management, morbidity and mortality of malaria have been significantly reduced.

## 1.2 Phylogeny

Malaria is caused by protozoan parasites of the genus *Plasmodium*, which are inoculated into humans by biting female Anopheles mosquitoes. There are six known human malaria parasites namely *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale curtisi*, *Plasmodium ovale wallikeri*, and *Plasmodium knowlesi* (Singh *et al.*, 2004; Cox-Singh *et al.*, 2008; Sutherland *et al.*, 2010; WHO 2013c). Of all the strains, *P. falciparum* is the most virulent causing about 85% of all malaria cases in humans and almost all mortality (Rich *et al.*, 2009). It has been suggested that the relative pathogenicity of *P. falciparum* is as a result of recently being acquired by transfer from a non-human host (Rich *et al.*, 2009). Molecular phylogenies carried out a few years ago, were consistent with this hypothesis as they showed that *P. falciparum* was more closely related to *Plasmodium gallinaceum*, a chicken parasite, than it was to other human parasite species (Waters *et al.*, 1991). A considered explanation was that *P. falciparum* evolved from an avian parasite through a horizontal transfer, probably through the Neolithic domestication of chickens (Rich *et al.*, 2009). However, with the increasing availability of genomic DNA sequence data, the closest relative of *P. falciparum* was shown to be *P. reichenowi*, a malaria parasite found in chimpanzees which was not included in earlier studies (Escalante and Ayala, 1994).

There are differences in the morphology, immunology and geographic distribution of these species. There are also differences in drug response and the ability to relapse (Greenwood et al. 2005).

### **1.3 Geographical aspects**

#### **1.3.1 Distribution**

Malaria was eradicated in the United States in the 1940s after widespread spraying of dichlorodiphenyltrichloroethane. Europe, Central and South America and other parts of the world have been successful in eradicating malaria but the tropics still bear the burden. *P. falciparum* is common in areas like Africa, Haiti, and Papua New Guinea while *P. vivax* is predominant in North Africa, the Middle East, central and parts of South America and the Indian subcontinent. In South America, Oceania and East Asia, the prevalence of both species is approximately equal. *P. vivax* is very rare in sub-Saharan Africa while *P. ovale* spp. are uncommon outside the tropical belt of Africa. *P. malariae* is less common outside Africa but is found in most areas. In China and neighbouring countries, *P. vivax* strains are very much present with long incubation periods (White, 2011).

#### **1.3.2 The vector**

Certain species of Anopheline mosquitoes are responsible for the transmission of malaria. Transmission does not occur at temperatures below 16°C or above 33°C, it also doesn't occur at altitudes greater than 2000m above sea level. This is because development in the mosquito (sporogony) cannot take place. High humidity and an ambient temperature between 20°C and 30°C are optimum conditions for transmission to take place. Rainfall provides mosquitoes with breeding sites but also, too much rainfall may wash mosquito larvae and pupae away (Gillies et al., 1998).

Malaria epidemiology may vary considerably even within small geographic areas. The transmission of malaria to humans is dependent on many interrelated factors but the most important concerns the anopheline mosquito vector and its longevity. The development of the sporozoite parasites in the vector (sporogony) takes over a week and this is dependent on ambient temperature. If malaria is to be transmitted, the mosquito must survive for longer than this after feeding on a gametocyte-carrying human (Mollineaux *et al.*, 1988).

#### **1.4 The human host**

There has to be an accessible human reservoir of viable gametocytes to transmit malaria infection. As a result, the behaviour of humans also plays an important role in malaria epidemiology. Infants and young children are more susceptible to malaria in areas of high transmission rates than the more immune older children and adults. In these areas, the densities of parasites are higher and the presence of gametocytes in the blood of children is detected more frequently. Traditionally, the endemicity of malaria has been defined in terms of the spleen rates or the rate of parasite clearance in children aged between 2 and 9 years (Mollineaux *et al.*, 1988).

Areas like tropical Africa or coastal New Guinea are holoendemic or hyperendemic for *P. falciparum* and people are repeatedly infected throughout their lives. A lot of morbidity and mortality occurs during childhood. For example, in the Gambia, where people were infected once each year on average, which is a relatively low figure for the African continent (Marsh K *et al.*, 1999), malaria has been estimated to cause 25% of deaths in children between 1 and 4 years of age (Greenwood *et al.*, 1987). If the child survives eventually, a state of 'premunity' is achieved where infections cause little or no problems in the (Baird *et al.*, 1991; Perignon & Druilhe, 1994). This means a form of immunity develops which becomes sufficient enough to control the infection but not to prevent it. Premunity occurs at a slow rate and acquisition of this state is age dependent. Non-immune adults entering an area of intense transmission acquire premunity much faster than children (Aponte *et al.*, 2007; Baird *et al.*, 1991).

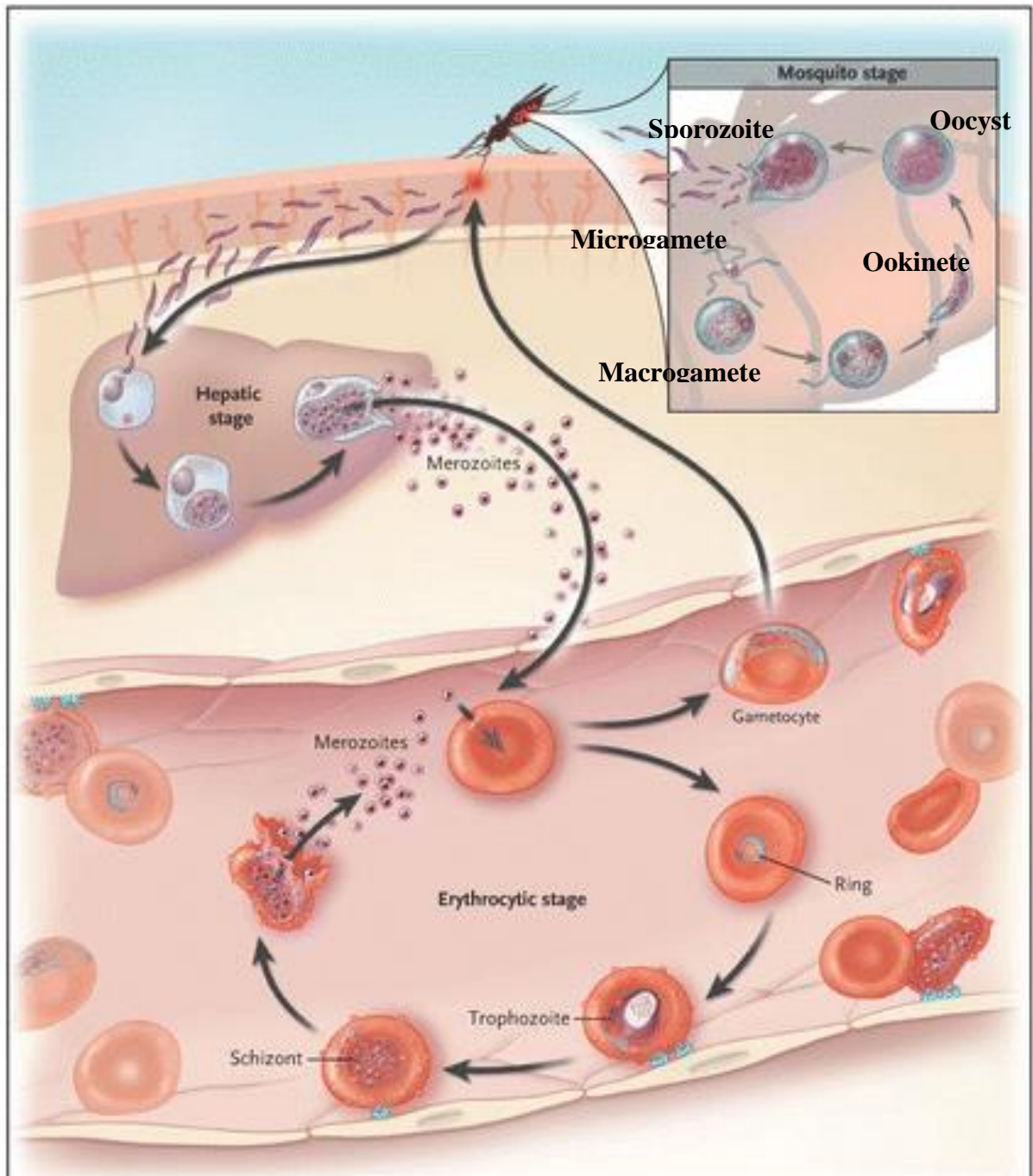


## 1.5 Life cycle

The life cycle of *Plasmodium falciparum* is a complex one that involves transmission between a mosquito vector and a human host, and sexual and asexual stages of development (Fig 1.2). The injection of haploid sporozoites into the human host by an infected female Anopheles mosquito during a blood meal begins the lifecycle. Sporozoites are motile forms of the parasite which gain access to the liver through the blood stream. In the liver, it undergoes asexual multiplication that results in tens of thousands of merozoites. This pre-erythrocytic parasite development stage is short and the release of merozoites from the hepatocyte (hepatic stage) to the bloodstream occurs after 6-8 days (Hermsen *et al.*, 2004) where red blood cell (erythrocytes) invasion occurs. Once merozoites are in the erythrocyte (erythrocytic stage), *P. falciparum* develops into a ring stage, a trophozoite stage which has high metabolic activity and finally, into schizonts where asexual replication occurs. In the schizont, new merozoites are formed and released into the blood stream when erythrocytes erupt. This is the stage where the clinical symptoms of malaria (e.g. fever) are manifested. Free merozoites go on to invade new erythrocytes which then develop into schizonts and continue the asexual life cycle. However, a fraction of released merozoites produce sexual gametocytes, which are the forms transmitted from humans to the mosquito. This occurs after differentiation into haploid male and female gametocytes occur. Gametocyte maturation in *P. falciparum* occurs in the bone marrow and requires 10-12 days (Eichner *et al.*, 2001) and mature gametocytes (stage V) appear and persist in the peripheral blood with a mean circulation time of 3.4-6.5 days per gametocyte (Eichner *et al.*, 2001; Bousema *et al.*, 2010). Immature gametocytes (stages I-IV) sequester in the bone marrow (Drakeley *et al.*, 2006).

When a human host is bitten by a female anopheline mosquito, the macro and micro gametocytes are taken up during a blood meal. Gametocytes develop into gametes and sexual reproduction occurs in the midgut of the mosquito. These forms are motile and long lived and the process (gametocytogenesis) takes about 7-10 days in *P. falciparum*. The male gametocyte undergoes rapid nuclear division and each of the nuclei formed is associated with a flagellum that is approximately 20-25µm long. Fusion and meiosis (sexual reproduction) takes place after the motile male microgamete seeks the female macrogametes (Read *et al.*, 1992). This sexual reproduction stage is diploid and allows recombination of genetic material to take place. The diploid zygote matures into an ookinete and then develops in an oocyst

after penetrating the midgut epithelium cell wall (Fig 1.2). Each oocyst produces haploid sporozoites that migrate to the salivary gland of the mosquito to await inoculation into the next human host during a blood meal thereby completing the life cycle. The development of the parasite in the mosquito is known as sporogony and this usually takes between 8 and 35 days depending on the species of mosquito and parasite and also the ambient temperature. (Stone *et al.*, 2013; Miurhead-Thomas, 1954).



**Figure 1.2** The life cycle of *P. falciparum* (adapted from (Rosenthal 2008)). The parasite cycle involves both an anopheline mosquito and a human host with sexual reproduction taking place in the mosquito and asexual reproduction takes place in the human host.

## **1.6 Genetic complexity of *P. falciparum* infections**

### **1.6.1 Estimation of multiplicity of infection (MOI)**

In malaria endemic areas, MOI can be a useful indicator of the transmission level, where the latter is positively correlated with the average number of malaria parasite strains in an individual (Babiker *et al.*, 1994). The merozoite surface proteins (MSPs (MSP1, MSP2) in *Plasmodium falciparum* are involved in invasion of erythrocytes. Genotyping of the MSP1 and MSP2 genes is a method used to assess MOI in *P. falciparum* because both genes are highly polymorphic in sequence and in length (Ntoumi *et al.*, 1995). MSP genotyping can distinguish between infections and clones by detecting the presence of different alleles at a polymorphic marker (Snounou *et al.*, 1999).

Malaria clinical isolates are made up of a mixture of different parasite strains (and species) in the form of multiple haploid clones. An individual can simultaneously be parasitized by many genetically distinct infections and the multiplicity of such infections in a host may be related to malaria endemicity, the risk of clinical malaria and the degree of immunity against *P. falciparum*. Different parasite clones may vary significantly in immune-avoidance mechanisms, immunogenicity, and drug susceptibility (Meyer *et al.*, 2002; Nsobya *et al.*, 2008).

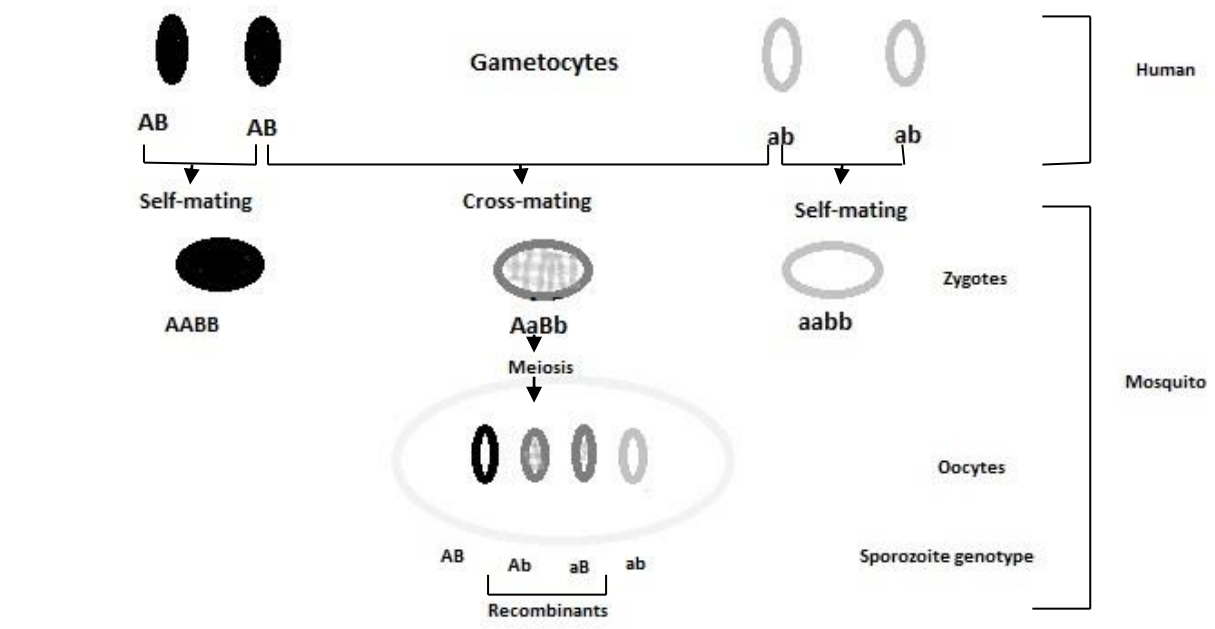
### **1.6.2 Genetics of *Plasmodium***

In the human host, the malaria parasite is haploid and an individual may be infected with a multiplicity of genetically diverse clones (Barbiker *et al.*, 1994). The zygote (ookinete) phase is the only diploid phase in the parasite lifecycle and is produced through fertilization of the micro and macro gamete in the midgut. After zygote formation, meiosis occurs rapidly which gives rise to haploid nuclei in the ookinete. In eukaryotic organisms, meiosis creates diversity

by genetic recombination, gene re-assortment on different chromosomes and by cross-over events.

During mosquito transmission, the gametes of a single haploid parasite clone taken up in the mosquito midgut undergoes self-mating to produce homozygous zygotes which contain identical alleles at all loci (Freitas-Junior *et al.*, 2000). Heterozygotes are expected if gametocytes of two or more genetically distinct clones are taken up into the mosquito and then a cross over event or selfing event occurs amongst gametes. This recombination (during meiosis) gives rise to parasites with novel gene combination among the haploid progeny (Babiker *et al.*, 1993; Walliker *et al.*, 1987).

### 1.6.2 Genetic recombination



**Figure 1.3:** The process of genetic recombination in malaria parasites when 2 clones undergo cross-mating in mosquitoes. A and a are alleles of one gene, and B and b are alleles of a second gene. One parent clone has genotype AB and the other ab; both produce gametocytes, capable of infecting mosquitoes, between which mating occur. Recombinants Ab and aB are produced following meiosis of diploid zygotes AaBb (Beale *et al.*, 1978; Walliker *et al.*, 1987; Babiker *et al.*, 1999). The diagram was drawn from findings from the three published papers.

## **1.7 Antimalarial drugs**

### **1.7.1 Quinine**

Quinine is an alkaloid which was first isolated by French chemists Pierre Pelletier and Joseph Caventou in 1820 (Greenwood 1992; Meshnick and Dobson, 2001). It was isolated from the bark of a cinchona tree and has been used in Western medicine since the early 17<sup>th</sup> century for its medical properties, together with related alkaloids (WHO 1986; Meshnick and Dobson 2001).

The need to treat soldiers infected with malaria during World War I gave rise to chemical research on antimalarial compounds and resulted in the synthesis of quinine. In 1920, Plasmoquine (8-aminoquinoline pamaquine) was the first drug synthesized; it was active against the liver stage parasites (hypnozoites) of *P. ovale* and *P. vivax* infections but use was discontinued as it was found to be very toxic (Greenwood, 1995; Sa *et al.*, 2011).

Quinine has a half-life range of 11-18 hours and is active against the Schizont stage of malaria parasites (Jamaludin *et al.*, 1988). It was used to treat severe malaria until research published from the AQUAMAT and SEAQUAMAT study trials showed that the use of artesunate was effective in treating severe malaria (Dondorp *et al.*, 2010). Quinine was later replaced with artesunate for treatment of severe malaria under the recommendation of the WHO, although the use of quinine is recommended in places where artesunate is unavailable (WHO 2010).

### **1.7.2 Chloroquine (CQ)**

A 4- aminoquinoline compound called resoquine was developed in Germany in 1934 but was not used due to concerns about toxicity (Loeb, 1946). The interest in resoquine was later sparked during World War II when a modified compound, santoquine, was captured from the Germans. In 1946, resoquine was renamed to chloroquine (Loeb, 1946) and is one of the most successful drugs ever produced. Chloroquine was the most widely used antimalarial drug until recently. It is easy to administer, inexpensive and well tolerated and kills all blood stages of malaria parasite (WHO 1986, Meshnick and Dobson 2001; Sa *et al.*, 2011). In the

1990s, its use was replaced with other regimens as a result of parasite resistance (White, 1998), which is thought to have led to the increase in disease burden on the African continent in the 1980s (Trape *et al.*, 1998).

Chloroquine works by interfering with hemozoin detoxification of plasmodium, which is a toxic by-product of haemoglobin degradation in the food vacuole of *Plasmodium*. Chloroquine is no longer recommended for the treatment of *P. falciparum* however, it is still used to treat infections caused by *P. vivax* and *P. ovale* which are less severe forms of malaria (Ratcliffe *et al.*, 2007). It is recommended for *P. vivax* infections in combination with primaquine due to the fact that resistance has not fully developed (Price *et al.*, 2009; WHO 2010).

### **1.7.3 Amodiaquine**

American scientists carried out a screening programme of antimalarials which led to the discovery of amodiaquine, a synthetic 4-aminoquinoline (Greenwood, 1995), but its use was discontinued after reports of long term prophylaxis side effects like hepatotoxicity (Ringwald *et al.*, 1998; Taylor and White, 2004). It is highly efficacious in treating cases involving chloroquine resistance and was very potent in some African countries where chloroquine had failed. Although it is more active than chloroquine, it rapidly metabolizes to desethylamodiaquine and shows cross resistance to chloroquine.

Currently, amodiaquine is used in combination with artesunate as first line treatment of uncomplicated *P. falciparum* because the side effects do not occur when shorter treatment courses are given (Olliaro *et al.*, 1996).

### **1.7.4 Pyronaridine**

Pyronaridine has been used to treat malaria in China for more than 30 years (Croft *et al.*, 2012). It was first synthesized in the Chinese Institute of Parasitic Diseases in 1970 (Zheng *et al.*, 1979) and has a similar structure to chloroquine. It is active against erythrocytic stages of *P. falciparum* (Ringwald *et al.*, 1996; Vivas *et al.*, 2008) and has been shown in clinical

studies to be less toxic when compared to chloroquine (Chang *et al.*, 1992). In cases of chloroquine resistance, pyronaridine is highly effective (Ringwald *et al.*, 1996) and is combined with artesunate in the treatment of uncomplicated *P.falciparum* malaria (Croft *et al.*, 2012; Rueangweerayut *et al.*, 2012).

### 1.7.5 Primaquine

Primaquine was used during the Korean War and is still used today (Greenwood, 1995; Baird and Hoffman, 2004). It is used in the treatment of *P. vivax* and *P. ovale* hypnozoites and is also used as prophylaxis in regions that are endemic (Vale *et al.*, 2009). Primaquine is fully effective against all stages of gametocytes and was discovered when an alternative to pamaquine was sought after, specifically, an alternative drug that could prevent relapse. However, it causes haemolysis in patients who are glucose-6-phosphate dehydrogenase (G6PD) enzyme deficient (Howes *et al.*, 2013). According to the WHO, a single low dose of primaquine (0.25 mg base/kg) is administered to patients with uncomplicated *P. falciparum* malaria and this dose is effective in patients who have G6PD deficiency (WHO, 2012).

### 1.7.6 Piperaquine

First synthesized in the 1960s, piperaquine was developed in China where it was used as prophylaxis and treatment of malaria (O'Neill *et al.*, 2012). It consists of two 4-aminoquinoline moieties (bisoquinoline) and is very active against chloroquine resistant *P.falciparum*. Piperaquine is relatively inexpensive, has low toxicity when compared to the other quinolone based compounds and is classified as safe and tolerable (Raynes, 1999; O'Neill *et al.*, 2012). It is used as a partner drug in combination therapy (dihydroartemisinin-piperaquine) by the recommendation of the WHO because it has a long elimination half-life (Davis *et al.*, 2005; WHO 2010). Reports of dihydroartemisinin-piperaquine treatment failure have emerged (Leang *et al.*, 2013; Saunders *et al.*, 2014; Spring *et al.*, 2015; Amaratunga *et al.*, 2016) in Cambodia, Vietnam, China, Myanmar, Thailand and Indonesia, areas where artemisinin resistance has emerged and spread (Dondorp *et al.*, 2009; Arie *et al.*, 2014; Ashley *et al.*, 2014; Takala-Harrison *et al.*, 2015).



### 1.7.7 Mefloquine

During the Vietnam War, the United States Army Antimalarial Drug Development Program discovered new chemical groups that were used as prototypes to develop new antimalarial compounds and mefloquine was one of such (Kitchen *et al.*, 2006). It is a 4-quinolinemethanol that was introduced in the mid-1970s (WHO, 1986) but was approved for use in 1989 (Shanks, 1994). As a result of its long half-life, it's used as prophylaxis with doses given once a week. It is also used in combination therapy with artesunate as first line treatment of uncomplicated *P. falciparum* (WHO 2010; Biamonte *et al.*, 2013). Unfortunately, reports of neurotoxicity and the emergence of drug resistance limits its use and so other less toxic and safer antimalarial drug are favoured (Overbosch *et al.*, 2001; Croft, 2007).

### 1.7.8 Lumefantrine

Lumefantrine, previously called benflumetol, was synthesized by the Beijing Academy of Military Medical Sciences in the 1970s (Bickii *et al.*, 1998). Earlier studies of the combination therapy using artemether and lumefantrine showed both drugs had synergistic activity and were highly efficacious (Hassan *et al.*, 1999). The WHO recommended artemether-lumefantrine (AL) in combination as the first fixed-dose artemisinin based combination therapy for the treatment of uncomplicated *P. falciparum* malaria (Nosten and white, 2007). It was earlier suggested that since lumefantrine had never been used as monotherapy prior to its combination with artemether, there will be a low risk of resistance emerging when compared to other drugs used in combination therapies (Premji, 2009). However, a recent study has shown artemether-lumefantrine failure in parasites isolates of African origin imported into the U.K (Sutherland *et al.*, 2016); although findings would have been strengthened if full regimen compliance was observed and if post-treatment lumefantrine levels were measured to rule out malabsorption (Laloo *et al.*, 2016). Another recent study showed evidence of increasing treatment failure with artemether-lumefantrine among malaria patients in Zambia (Cardol *et al.*, 2016).

### 1.7.9 Atovaquone

Atovaquone is a 2-hydroxynapthoquinone currently used in combination with proguanil (Meshnick and Dobson, 2001). It is used as prophylaxis in travellers because it readily clears liver schizonts, and is used in the treatment of uncomplicated *P. falciparum* malaria (Hudson, 1993; Biamonte et al, 2013).

### 1.7.10 Antifolates

In the early 1950s, British chemists synthesized antifolates which are derivatives of pyrimidine targeting the folate metabolism pathway (Schiltzer, 2007). One of such compounds is proguanil, which inspired further investigation on antifolates (WHO, 1986; Nzila, 2006) and pyrimethamine, a compound active against blood forms of malaria was found (Falco *et al.*, 1951). Other examples of antitfolates discovered include sulfadoxine, dapsone and sulfone. These drugs are used in combination and have a synergistic effect.

In 1971, two antifolate compounds, sulfadoxine and pyrimethamine (Fansidar <sup>TM</sup>) were the first available combination therapies. Sulfadoxine-pyrimethamine (SP) is affordable, effective, has a good compliance with a long half-life and can be taken in a single dose (Basco *et al.*, 1998). SP replaced chloroquine as first line treatment in many countries due to the emergence and spread of chloroquine resistance (White, 2004).

SP was changed as first line treatment of uncomplicated *P.falciparum* when resistance emerged in South America (Gregson and Plowe, 2005), and Southeast Asia (WHO 2001). Currently, artemisinin combination therapies (ACTs) are used as first line treatment of malaria. As a result, SP in combination with artesunate is recommended (WHO, 2010), although SP alone is used in areas where ACT cannot be accessed (Thriemer *et al*, 2006; Mkulama *et al.*, 2008).

An advantage of SP is that it has a good safety profile and as a result, it is recommended for preventative use in pregnant women and in children in Africa. It is also used in intermittent preventative therapy (IPT) programmes in areas that have low drug resistance and moderate

to high malaria transmission setting (Peters *et al.*, 2007; WHO, 2013; Beshir *et al.*, 2013). In children, SP in combination with amodiaquine is used as seasonal malaria chemoprevention (SMC) to reduce mortality caused by malaria infection in the Sahel sub-region of Africa (WHO, 2013).

The combination of pyrimethamine and sulfadoxine, have played a significant role in malaria therapy. However, development of resistance by mutations in dihydrofolate reductase gene has compromised the use of pyrimethamine. Likewise, mutations in dihydropteroate synthase gene have caused reduced sensitivity to sulfadoxine (Warhurst, 1998).

## **1. 8 Antimalarial drug resistance**

Antimalarial drug resistance has been defined as ‘the ability of a parasite strain to survive and /or multiply regardless of the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject’ (Bruce-Chwatt *et al.*, 1986). It is usual to also specify that the particular drug in question must be in contact with the parasite or the infected red blood cell for the duration of the time necessary for its normal action (Bruce-Chwatt *et al.*, 1986; WHO, 2010). This is often interpreted as referring only to the parasite’s persistence after treatment doses of an antimalarial rather than failure of prophylaxis (Lobel *et al.*, 1986). It is worth mentioning that the definition of drug resistance with regards artemisinin has been controversial particularly because of its mode of action. This will be discussed further in section 1.9.2

Antimalarial drug resistance could sometimes be mistaken for failure to clear malarial parasitaemia or failure to resolve the disease after treatment with an antimalarial drug. While it is true that drug resistance can cause treatment failure, not all treatment failure is as a result of drug resistance (Boland, 2001). It is worth mentioning that there are several other factors that can contribute to treatment failure including misdiagnosis, poor drug quality, incorrect dosing, non-compliance with duration of dosing regimen, poor absorption, and drug interactions (Boland, 2001). Each of these factors that contribute to treatment failure may enhance development of true drug resistance by increasing the exposure of parasites exposure to drug levels thereby increasing the chances of resistant parasites to emerge (White, 1998).

Antimalarial drugs are one of the most important tools in the fight against malaria and the emergence of drug resistance threatens malaria control and eradication programmes especially in Africa. Case in point was the emergence of chloroquine drug resistance in Africa in the 1980s, which was considered responsible for the dramatic increase in child morbidity and mortality (Trape, 2001). Currently, there have been reports on resistance to almost all antimalarial compounds established. In the last few years, ACTs have been used as first line treatment for malaria treatment (Dondorp *et al.*, 2011) and there are currently no alternative antimalarials with the same level of tolerability and efficacy (WHO, 2013). Unfortunately, resistance to ACTs have been reported in areas of South-East Asia and there are fears drug resistance will spread to Africa or rise *de-novo* (Dondorp *et al.*, 2009; Arie *et al.*, 2014; Ashley *et al.*, 2014; Takala-Harrison *et al.*, 2015). This could affect malaria eradication and elimination efforts. ACT drug resistance will be discussed in more detail in section 1.9.2

### **1.8.1 Mechanism of malaria resistance**

Resistance occurs through mutations that are spontaneous and confer reduced sensitivity to a particular drug or class of drugs (Thaithong S, 1983). A single point mutation is needed to confer resistance for some drugs while multiple mutations or copy number polymorphisms are required for other (White and Pongtavornpinyo, 2003).

Genetically determined resistance that occurs in a pathogen under drug pressure develops through *de-novo* emergence of new genotype and the subsequent spread of the new genotypes through the parasite population. Emergence of *de-novo* mutation is thought to be independent of drug selection pressure and is also thought to be a rare event (White and Pongtavornpinyo, 2003). Parasites with mutation can survive and expand in numbers in the presence of a drug to give rise to gametocyte densities sufficient for subsequent spread through the population (Barnes and White, 2005; Hastings, 2006). It is thought that drug pressure gives a selective advantage to resistant parasites by increasing their frequency in the population, thereby enabling transmission in mosquitoes and subsequently to the human population (Olliaro and Taylor, 2004).

After genetic recombination (as shown in figure 1.3), which takes place in the mosquito through fertilization of mosquito stages, a formation or breakdown of mutagenic resistance can take place (Barnes and White, 2005). This occurs if a mosquito infects a host carrying multiple parasite genotype or two hosts carrying different gametocyte (Barnes and White, 2005). Selection occurs in the parasite mutant alleles when antimalarial drug concentration are enough to repress susceptible parasites developing or not enough to inhibit new mutants, a phenomenon called ‘drug selection’ (White, 1998). The spread of resistance is difficult to avoid once drug resistant malaria parasites have emerged.

## **1.9 Resistance to chloroquine and ACT**

This section focuses on drug resistance to both chloroquine and ACT as both drugs are of interest in the work carried out in this thesis, particularly the latter. This is because the work carried out in Chapter 4 used chloroquine (see method section in Chapter 3), and the work carried out in both Chapters 4 and 5 made extensive use of dihydro-artemisinin.

### **1.9.1 Chloroquine resistance**

Chloroquine is classified as a diprotic weak base, which moves between the digestive vacuole, which is acidic and the extracellular matrix through diffusion. Once in the vacuole, protonation of CQ takes place which causes the CQ molecule to become trapped as the membranes are not permeable to charged species (Homewood *et al.*, 1972; Ferrari and Cutler, 1991). CQ accumulation occurs inside the digestive vacuole as a result of the ion-trapping (Hawley *et al.*, 1996). CQ binds to haem in the digestive vacuole which then leads to the inhibition of haemozoin polymerization, causing an accumulation of toxic free haem, membrane damage and oxidative stress in the parasite (Sullivan *et al.*, 1998; Warhurst *et al.*, 2007; Roepe, 2009).

There has been a history of improper use of chloroquine, despite this; there was approximately a 20 year lag between the initial introduction in the 1950s and the eventual

spread of resistance (Peters, 1987). As a result, it was suggested that CQ had a complex resistant mechanism that involved several genes and multiple mutations (Wernsdorfer, 1991). In the 1960s, the first CQ resistant case was reported in South America, specifically, Brazil, Venezuela and Columbia followed by reports in Southeast Asia, specifically Kampuchea and Thailand (Wernsdorfer, 1991). In the late 1970s, it was reported through epidemiological studies that CQ resistance had spread to Africa from Southeast Asia, where its emergence was reported in Tanzania and Kenya (Fogh *et al*, 1979; Campbell *et al*, 1979).

When a parasite becomes resistant to CQ, there is reduced accumulation in the digestive vacuole compared to a parasite sensitive to CQ (Saliba *et al*, 1998). As a result, the drug concentration in the digestive vacuole is kept below toxic levels (Saliba *et al*, 1998; Bray *et al*, 2005). Genetic cross studies carried out between Dd2 (CQ resistant) and HB3 (CQ sensitive) strains demonstrated that drug resistance occurs due to selection of a mutant *pfcr* protein (Wellems *et al*, 1991; Su *et al*, 1997; Fidock *et al*, 2000), which is located in the parasites digestive vacuole where it can control chloroquine access to haem (Fidock *et al*, 2000; Cooper *et al*, 2002). *Pfcr* belongs to the drug transporter superfamily (Martin and Kirk, 2004). The function of this protein is not well known however, it was proposed that it either allows protonated CQ to leave the digestive vacuole by acting as a channel, or it functions as an active transporter protein (Warhurst *et al*, 2002; Sanchez *et al*, 2007).

Linkage studies carried out by Fidock *et al*, (2000) showed that an association between an amino acid substitution of lysine to threonine at position 76 (K76T) causes CQ resistance. This mutation results in a loss of positive charge within the channel which alters the selectivity of the channel, causing it to transport the positively charged CQ and allowing it exit the digest vacuole thereby inhibiting it from its site of action (Warhurst *et al*, 2002; Lehane and Kirk, 2008).

Other mutations which have different drug resistant phenotypes and different geographic distributions also exist in combination with K76T (Bray *et al*, 2005; Sa *et al*, 2009). These mutations play a compensatory role for the unfavourable changes of the normal function of *Pfcr* (Hyde, 2007). Parasites that are CQ resistant are classified based on mutation at amino acid position 72-76, with mutations CVMNK being a sensitive haplotype, CVIET and SVMNT being a resistant haplotype (Cooper *et al*, 2005; Summers *et al*, 2012).

In Africa, CVIET is the most dominant haplotype but it's also found in South America and South East Asia while the SVMNT haplotype is more dominant in areas like the Philippines

(Chen *et al.*, 2005), Papua New Guinea (Mehlotra *et al.*, 2001) and South America (Fidock *et al.*, 2000; Cooper *et al.*, 2005; Gama *et al.*, 2010).

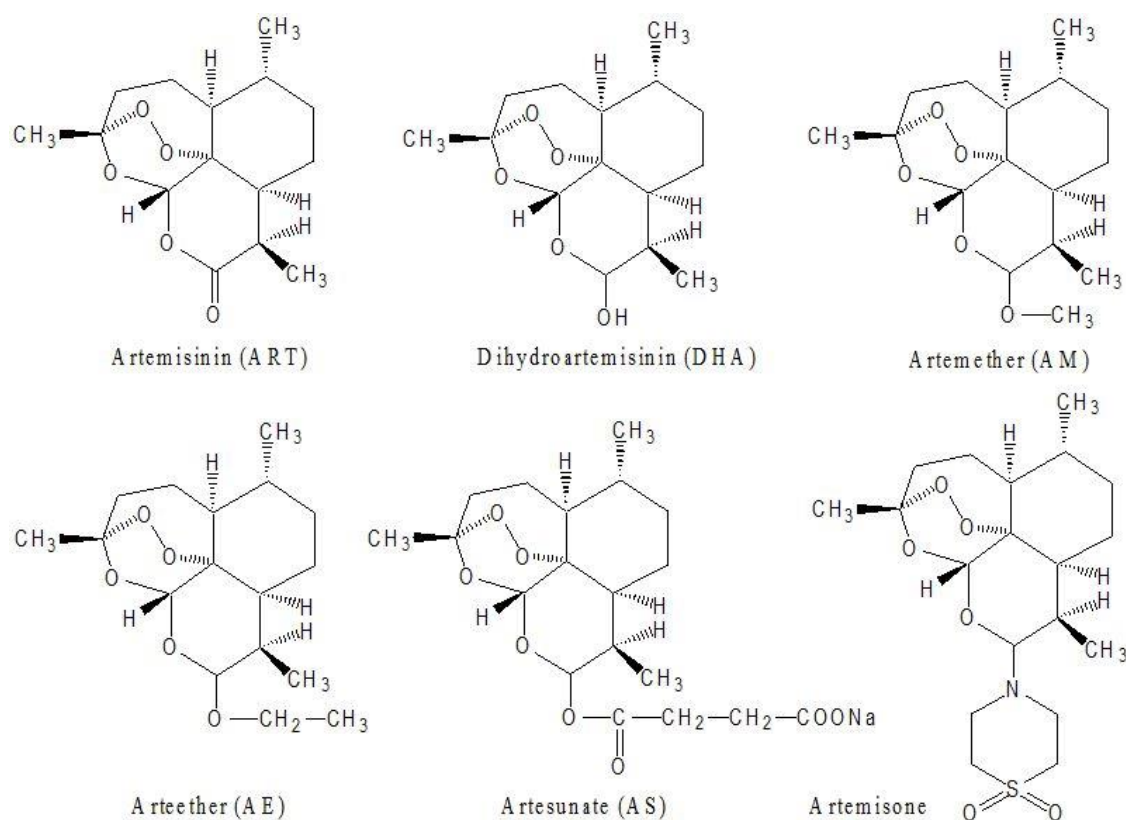
Other genes have been associated with CQ resistance such as *Pfmdr1*, a homologue of mammalian multidrug resistance gene (*mdr1*) called P-glycoprotein homolog 1 (Pgh1) (Foote *et al.*, 1990). This gene is discussed in more detail in section 1.16.1

### 1.9.2 Artemisinin

Artemisinins are derived from *Artemisia annua* and have been used in traditional Chinese medicine for centuries (Peters, 1987; Hsu, 2006; Cui and Su, 2009). The antimalarial properties of artemisinins were discovered by Chinese scientists in 1971 and are well established for the treatment of malaria, including drug resistant strains (Meshnick and Dobson, 2001). Through a collaborative effort known as ‘project 523’, the Chinese prepared dihydroartemisinin (DHA), artemether and artesunate (generically known as artemisinins) and these have had major contribution to the management of malaria (White, 2008).

Chemically, artemisinin is a sesquiterpene lactone containing a peroxide bridge which is believed to be responsible for the drugs mechanism of action. The active metabolite, DHA retains the potent activity of the parent compound against the erythrocytic stages of the parasite life cycle (Cui and Su, 2009). ART and its derivatives are an important class of antimalarials that have a number of advantages over other antimalarial compounds. These include its suitability for the treatment of severe malaria and the fact that it can be administered through several routes (Dondorp *et al.*, 2010).





**Figure 1.4:** Artemisinin derivatives: Chemical structure of artemisinin, dihydroartemisinin, artemether, arteether, artesunate and artemisone.

### 1.9.2.1 Pharmacological properties

Artemisinins kill nearly all of the asexual stages of a parasite development in the blood (Kuile *et al.*, 1993). Artemisinin is active against early and late ring forms, trophozoites, schizonts as well as early gametocytes (Krishna *et al.*, 2004). There are debates regarding artemisinin antimalarial action and although the endoperoxide bridge lies at the heart of antiparasitic activity, the chemical nature of this interaction is not fully understood. Ion-dependent alkylation of cellular proteins has been proposed as a likely mode of action because cations catalyse *in-vitro* reactions of some artemisinins, including their decomposition in aqueous solutions (Ter Kuile *et al.*, 1993).

Artemisinins have a broad stage specificity of antimalarial action that has two therapeutic consequences. First, the drug kills all young circulating ring-stage parasites in *P. falciparum* infections which give rise to a more rapid parasiteemia reduction compared to other class of antimalarials (Hien and White, 1993; White, 1997). Secondly, artemisinins reduce the

number of parasites that grow to mature stages and sequester in and block venules and capillaries of the host (Krishna *et al.*, 2004). Additionally, an advantage of artemisinin being active against early ring forms is that it can interrupt parasite transmission rates at a population level because inhibition of early ring forms can inhibit gametocyte development (Price *et al.*, 1996). Artemisinin is eliminated by metabolic bi-transformation, predominantly by CYP 2B6, to inactive metabolites (Kuile *et al.*, 1993).

Artemisinin derivatives are administered through various routes. Artemisinin, dihydroartemisinin, artemether (Intramuscular injections) and artesunate can be administered orally. For severe malaria, artesunate is used intravenously. Artesunate, arteether and artemether are metabolized *in-vivo* into dihydroartemisinin, which is the biologically active form of the drug (White, 1999). Artemisinin derivatives are most active against late rings to early trophozoite stages of *P. falciparum*, and they clear parasites faster than any other antimalarial medicines (McIntosh *et al.*, 1999; White N, 1999).

Within a single host, multiple stages may coexist and due to the fact artemisinin derivatives have a short half-life, short treatment regimens with artemisinins are inefficient in clearing all the parasites (White, 1999). This is why artemisinin monotherapy has very high recrudescence rate unless given for 7 days and is recommended that artemisinin derivatives be used in conjunction with other antimalarials with longer half-lives such as mefloquine or lumefantrine (White, 1998). Artemisinin derivatives are administered in the first day or two and rapidly decrease parasite biomass. Then the partner drug e.g. mefloquine is co-administered because it maintains therapeutic blood concentrations for several days (White, 1999). This allows the drug to kill the few remaining parasites that escape killing by artemisinin derivative. Artemisinin combination therapy (ACT) has an advantage over artemisinin monotherapy as it delays the appearance of resistance (White, 2008)

### **1.9.2.2 Toxicity**

In a prospective study carried out in Thailand, adverse events were rare in 3500 patients treated with artemisinin derivatives (oral artesunate) (Price *et al.*, 1999). The drug was reported to be safe in pregnant women (McGready *et al.*, 1999), however, another study

showed embryotoxicity of artemisinins in pregnant women in their first trimester (Clark *et al.*, 2009).

Several animal studies have shown however, that artemisinin derivatives cause neurotoxicity when given at high doses (Brewer *et al.*, 1998). There were two cases of patients who suffered from neurological conditions after receiving artemisinin treatments (Elias *et al.*, 1999), however, these two cases were isolated and could have been unrelated to the treatments received by the patients. Another study showed teratogenicity in rats (Chen, 1984). When compared to the very large number of patients treated safely with artemisinin's, the adverse neurotoxic effects could be said to be quite rare (Dayan, 1998).

### **1.9.2.3 Molecular targets of artemisinin's**

There has been continuous debate about the mode of artemisinin in *Plasmodium* and questions about being either a single or multiple targets. Artemisinin has as 1, 2, 4-trioxane core incorporating an endoperoxide bridge that is essential for activity (Avery *et al.*, 1993; Meshnick, 2002; Golenser *et al.*, 2006) (Figure 1.4). Some studies suggest that artemisinins become activated (Krishna *et al.*, 2004) inside the parasite, which leads to radical intermediates that reacts with parasite targets that are susceptible (Posner *et al.*, 1995; Olliaro *et al.*, 2001; O'Neill *et al.*, 2010). However, it is still not clear what the origin and nature of the artemisinin activator is. Some studies have proposed that parasite uptake of haemoglobin through haemoglobin derived haem or ferrous iron is important for artemisinin activity (O'Neill, 2004) while other studies have proposed a metal independent activation of artemisinins (Haynes *et al.*, 2010). Another hypothesis is that artemisinin works by directly binding to calcium ATPase (*Pf*atpase6), in a way that is haeme-independent and analogous to the inhibition of the sarco/endoplasmic reticulum calcium ATPase by thapsigargin (Krishna *et al.*, 2010). It is important to understand the mechanism of action of artemisinin so that the development of drug resistance by parasites can be fully elucidated. Proposed mechanisms of artemisinin action are discussed in more detail in section 1.14.

#### 1.9.2.4 Artemisinin combination treatment

*P. falciparum* has developed resistance to almost all classes of antimalarial compound (WHO, 2010). The WHO recommended artemisinin-based combination therapy (ACT) as first line treatment after *P. falciparum* developed resistance not only to chloroquine, but also to mefloquine, sulphadoxine-pyrimethamine, and other used antimalarial drugs. ACT is the combination of an artemisinin derivative such as arthemeter, artesunate or dihydroartemisin and a partner drug like mefloquine, piperaquine, amodiaquine or lumefantrine which have a longer half life in the bloodstream than artemisinin.

A 7 day regimen is required to maximize cure rates when artemisinins are given alone. There are issues with adhering to a 7 day treatment course and so a combination partner in ACT which has slower elimination allows a complete treatment to be given in 3 days (Nyunt *et al.*, 2007). Although a 3 day regimen of artemisinin reduces the parasite biomass, the longer acting drug, which is also less potent, kills any remaining parasites over a 1-2 week period. In order to avoid losing the potency of artemisinins to resistance development, the use of combined regimens was recommended for uncomplicated malaria. However, the use of artemisinin monotherapy continues to be common practice in areas that are malaria endemic (WHO, 2010). Unfortunately, impaired parasite response to artemisinin monotherapy has emerged and is very well established in the Thailand-Cambodia border region which is a historical epicentre for the development and spread of antimalarial resistance (Noedl *et al.*, 2008).

As a result of reduced sensitivity to artemisinin, the ACT partner drug becomes more vulnerable to the development of resistance (Spring *et al.*, 2015; Amaratunga *et al.*, 2016). Once resistance develops, current ACT regimens will begin to fail especially as previous emergence of parasite resistance to all currently used partner drugs have already developed (Leang *et al.*, 2013; Saunders *et al.*, 2014; Spring *et al.*, 2015; Amaratunga *et al.*, 2016). There is a current fear that resistance will either spread to Africa from Southeast Asia or independently arise and so it is crucial to fill in the current gaps in the understanding of how artemisinin resistance has emerged and also on the mode of action of the drug.

### 1.9.2.5 Artemisinin resistance: Definition

The WHO has working definitions of resistance in human populations which is based on clinical and parasitological outcomes observed during *in vivo* studies of ACTs or artesunate monotherapy (WHO, 2014).

Suspected resistance occurs when >10% of patients still have parasitaemia at least 72 hours after treatment initiation. This is during a standard oral regimen of ACT directly administered over 3 consecutive days. Parasitaemia is detected by examining a thick blood film of asexual stage *P. falciparum* parasites (WHO, 2011). Confirmed resistance occurs when standard oral regimens of artemisinin monotherapy (2-4mg/kg/day) are administered over 7 consecutive days, and adequate levels of drug are documented in plasma, the WHO confirms resistance if parasites are still present on day 7 (i.e., at 168 hours), or if parasites are present at 72 hours and then recrudescence i.e. initially clear but then reappear as late as day 42 (WHO, 2011).

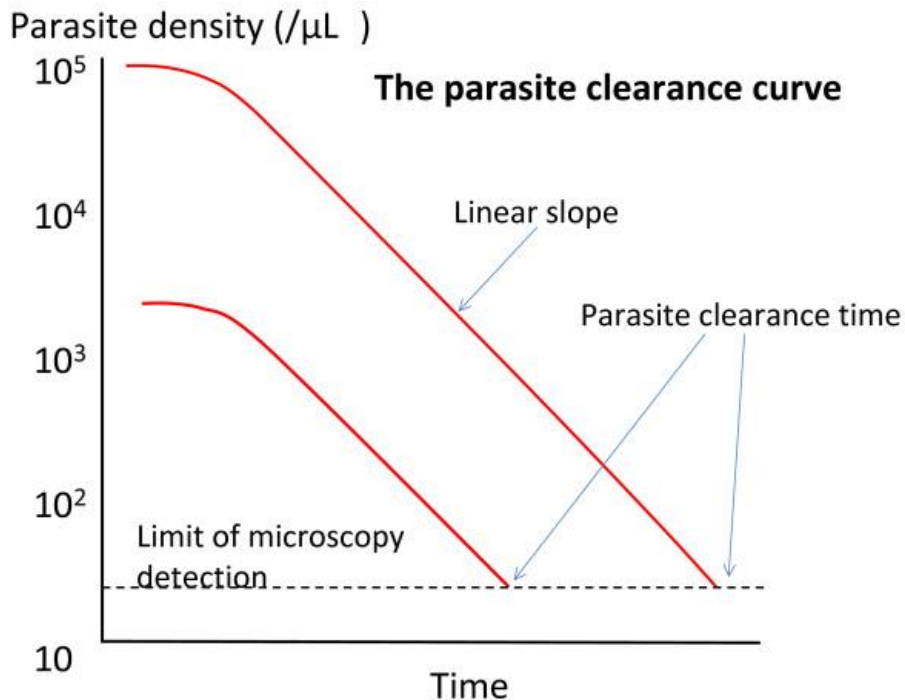
For *in-vitro* studies, there are conventional invitro tests that provide a quantitative measure of intrinsic susceptibility of *P. falciparum* parasites to antimalarial drugs (WHO, 2001). These were originally devised for monitoring CQ resistance. This is usually expressed as IC<sub>50</sub>, the concentration of drug that inhibits the growth of parasite by 50% (as determined by quantifying parasite DNA replication by radioisotope methods, monitoring the maturation of ring to schizont stage parasites by microscopy or flow cytometry, counting new ring stage parasites after one cycle of parasite growth and re-invasion, or quantifying the level of parasite specific proteins) (WHO, 2011).

Molecular marker studies can be more standardized and deployable compared to *in vivo* and *in vitro* studies and are very useful for large scale surveillance studies for monitoring antimalarial resistance (Plowe *et al.*, 2007). When molecular markers are identified, they reveal insight into the mode of action of artemisinin and drug resistance. The majority of studies in this area have deployed a candidate gene approach which focuses on genes that are known to play a role in parasite resistance, or to genes involved with proposed mechanism of drug action (Sisowath *et al.*, 2007; Humphreys *et al.*, 2007; Henriques *et al.*, 2014). Some other studies have focused on gene expression following exposure to high doses of artesunate (Deplaine *et al.*, 2011).

### 1.10 Parasite clearance

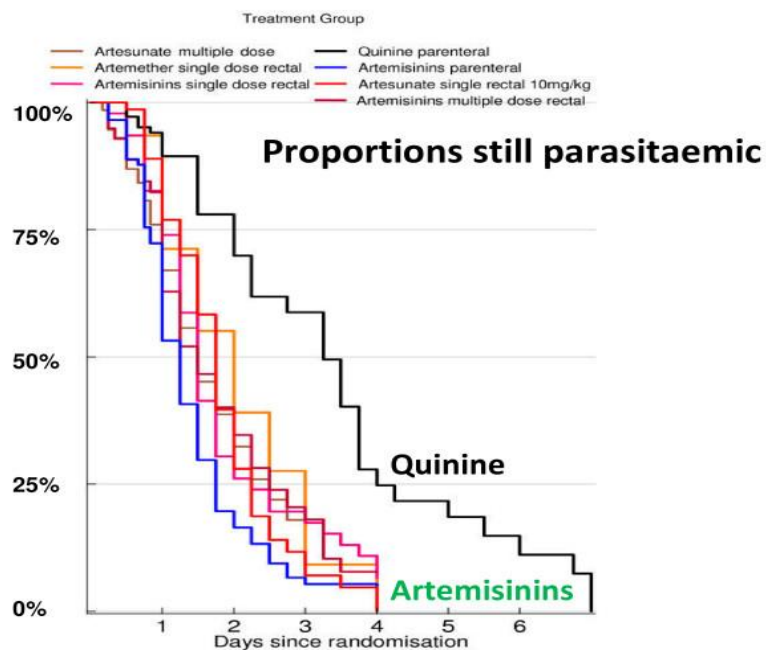
Antimalarial treatment reduces parasitaemia in patients and is essential for recovery from symptomatic malaria. A rapid decline in parasite density is expected provided a potent regimen is given in appropriate doses. The parasite clearance time is the frequently quoted measure of therapeutic response but it is an imprecise measure dependent on pre-treatment parasite density and parasitaemia (White, 2011). It is also worth mentioning that adequate clinical and parasitological response (ACRP) at day 28 and day 42 after treatment was the classic method used before parasite clearance times were used (WHO, 2006).

After the start of an anti-malarial treatment, the graphic plot of parasite densities that follows is known as the parasite clearance curve. This curve measures the therapeutic response of a drug (White, 2011). In a *P. falciparum* infection, the higher the initial parasite density, the longer counts will take to become undetectable (parasite clearance time). Factors which affect the parasite clearance time include frequency of sampling, parasitaemia estimate, stage of parasite development, drug effects and host defences (White, 2011).



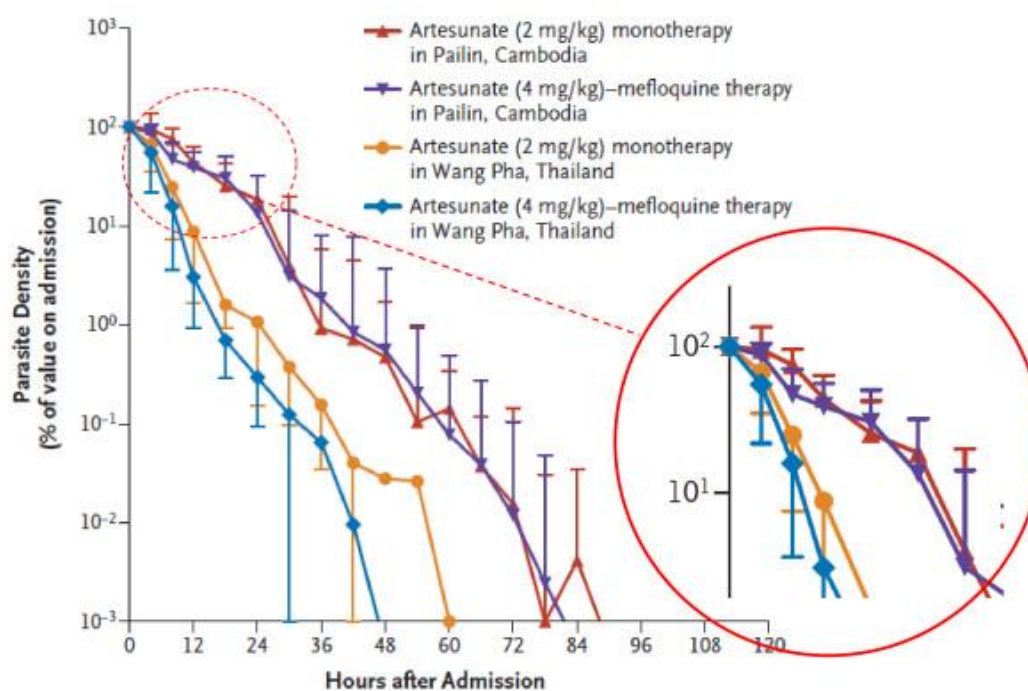
**Figure 1.5:** Graph showing the log therapeutic responses of two *P. falciparum* infections illustrating that parasite clearance time is dependent on pre-treatment parasite density, but for sensitive parasites, the slope is independent of the starting parasitaemia (White, 2011).

Artemisinin derivatives clear parasitaemia more rapidly than other malaria drugs and that is its pharmacological hallmark. In addition to this effect, they change the shape of the parasite clearance curve qualitatively, due to that, little or no initial plateau phase is visible and a log-linear decline in parasitaemia is reached almost as soon as parasitocidal drug concentrations are reached (White, 1999). Artemisinins create a steeper slope of the log-linear segment of the parasite clearance curve compared to any other antimalarial drug. This happens as a result of the killing of ring stage parasites, in addition to other stages which are then removed from circulation (Chotivanich et al, 2000). These parasites therefore do not mature or sequester (Udomsangpetch et al, 1996) and this explains the immediate rapid decline in parasitaemia observed consistently in all studies with artemisinin derivatives. The emergence of artemisinin resistance in *P. falciparum* in western Cambodia is manifest by marked slow parasite clearance *in-vivo* (Dondorp et al, 2009). Unfortunately, this *in-vivo* phenotype has not been reliably predicted *ex-vivo* and conventional in-vitro testing showed little difference in drug susceptibility between sensitive and resistant parasites (Dondorp et al, 2009).



**Figure 1.6:** The proportion of patients with severe *falciparum* malaria (defined by clinical or laboratory evidence of vital organ dysfunction) remaining parasitaemic after receiving rectal artemisinins or parenteral quinine in a series of randomized trials (Gomes *et al.*, 2008).





**Figure 1.7:** Normalized *P. falciparum* parasite clearance curves showing the fraction of initial parasitaemia versus time in patients treated with artesunate in Western Cambodia and Western Thailand. Parasite clearance was significantly slower in Western Cambodia very similar to clearance of quinine in figure 1.6 (Dondorp *et al.*, 2009).

Although most *P. falciparum* infections still eventually clear after treatment with artemisinin-based combination therapies in Cambodia, resistant parasites usually take 3 or 4 days to clear compared to 2 days for parasites which are artemisinin-sensitive. It has been suggested that this delayed clearance could be a step towards high level resistance and treatment failure (Dondorp *et al.*, 2011). The loss of potency to artemisinins renders the more slowly eliminated drugs which are part of the combination therapies, vulnerable to the development of resistance. This threat would have a huge impact on malaria eradication efforts worldwide and as such, an ambitious program was launched to contain artemisinin resistance under the guidance of the World Health Organisation (WHO).

### 1.11 *In-vitro* artemisinin drug response

Reduced artemisinin susceptibility in the greater Mekong sub-region is characterised by slow parasite clearance (Noedl *et al.*, 2008; Dondorp *et al.*, 2009) and for so long, there had been no consensus on the mechanism by which these phenomena occur. Mathematical modelling done Saralamba *et al* (2011) predicted that ring stage parasites in slow clearing infections had a reduced susceptibility to artemisinin. To explore genetic changes that could lead to resistance, Witkowski *et al* (2013) performed *in-vitro* experiments with artemisinin resistant parasites isolated from the F32- Tanzania clone and showed that parasites which were exposed to high doses of artemisinin undergo quiescence at ring stage. This was not considered to be responsible for resistance as evidence shows that artemisinin sensitive lines are also able to go into dormancy and this is dependent on the genetic background of the parasite (Tucker *et al.*, 2010). It was proposed that parasites that are resistant to artemisinin are able to tolerate more drugs by recovering from dormancy and resuming normal growth at a much greater rate than parental strains susceptible to artemisinin. This means that resistant lines which become dormant have a higher survival rate after dormancy (Tucker *et al.*, 2010). A study which investigated the link between dormancy and parasite resistance of a semi-synthetic derivative of artemisinin, artelinic acid (AL), showed the association between AL resistant parasites and a decreased sensitivity of mature-stage parasites, a decreased sensitivity of the ring stage to induce dormancy, and a faster recovery from dormancy (Teuscher *et al.*, 2012). Another study also reported that mature stage of artemisinin resistant laboratory lines had reduced sensitivity to artemisinin which also occurred in trophozoite and schizont stages and not only ring stages (Cui *et al.*, 2012). These findings are different from the artemisinin resistant F32-Tanzania mature stages, which are killed by artemisinin derivatives (Witkowski *et al.*, 2010).

To find out how these findings relate to reduced susceptibility of field isolates in Cambodia, *P. falciparum* field isolates from Pailin and Ratanakiri, which are areas of artemisinin resistance and susceptibility respectively (two geographically distant areas), were culture adapted and *in-vitro* susceptibility to DHA investigated using a ring stage survival assay (RSA) and a mature stage survival assay (MSA) (Witkowski *et al.*, 2013). The study showed that *P. falciparum* at ring stage from Pailin is able to withstand artemisinin toxicity through developmental arrest or quiescence, while mature stages are fully susceptible.

Artemisinin resistance has spread rapidly throughout Southeast Asia. Resistance has become prevalent in other Cambodian provinces (Amaratunga *et al.*, 2012; Arie *et al.*, 2012), Southern Vietnam (Ashley *et al.*, 2014; Hien *et al.*, 2012), Thailand-Myanmar border areas (Ashley *et al.*, 2014; Phyo *et al.*, 2012) and is emerging in Southern Laos (Ashley *et al.*, 2014).

### 1.12 Candidate molecular markers for Artemisinin resistance

Molecular markers of resistance are very useful tools that help monitor the evolution and spread of resistance, which could enable the establishment of national treatment policies and to design drug combinations that could delay drug resistance from evolving. For this, powerful new tools emerging from the fast growing field of genomics are being used to address this problem. Whole genome sequencing technology and computational methods which allow parasite genome variation to be studied are being utilised.

Until 2014, in *P. falciparum* there were no universally validated and accepted molecular markers of artemisinin resistance in the field. Amplification of the gene encoding for the multidrug resistance protein, *Pfmdr1* (Reed *et al.*, 2000; Sidhu *et al.*, 2006) and polymorphisms in *pfatpase6* (Jambou *et al.*, 2005) have been investigated but there was no correlation found between variants of these genes and *in-vivo* responses to artemisinin derivatives in the first study which described resistance along the Thai/Cambodian border.

Experimental studies have also been done to elucidate an area of the genome that could be associated with artemisinin resistance. For example, parasites resistant to artemisinins have been generated *in-vitro* and *Pfmdr1* duplications were identified (Chavchich *et al.*, 2010). The phenotypes and genetic changes associated with it have been shown to be unstable in the absence of drugs (Beez *et al.*, 2011; Hunt *et al.*, 2007). Another study carried out a genetic linkage analysis of the experimental *P. falciparum* Hb2 x Dd2 cross and showed that three loci, *Pfmdr1* and two on chromosome 12 and 13 (Beez *et al.*, 2011), were associated with artemisinin response and had the potential to evolve to artemisinin resistance. However, neither of these parental parasite lines had a distinct artemisinin resistant phenotype.

Another candidate from *P. chabaudi* *Pcubp1*, which encodes ubiquitin carboxyl-terminal hydrolase 1, and *Pcap2mu*, encoding clathrin vesicle-associated adaptor 2,  $\mu$  subunit, have

both been implicated in laboratory-derived artemisinin resistance in the rodent parasite *P. chaubaudi*. Both genes have polymorphic homologues in *P. falciparum* (Hunt *et al.*, 2007; Henriques *et al.*, 2013). These polymorphisms have not yet been completely validated *in-vivo* as markers in parasites that have been established to have reduced artemisinin sensitivity. Although a study in Kenya using genome wide association study, found an association between *Pfubp1* variant, E1528D and reduced artemisinin susceptibility *in-vitro* (Borrmann *et al.*, 2013).

Genome wide single nucleotide polymorphism (SNP) typing of *P. falciparum* parasites from Thailand, Laos and Cambodia was used for analysis of genetic haplotype variation and conservation and also geographic differentiation of parasites. Resistance to artemisinin had started emerging when candidate regions associated with slow parasite clearance rates after drug treatment was found in the chromosome 13 region (Cheeseman *et al.*, 2012). Since then, the Kelch propeller region has been identified as the candidate gene that influences artemisinin response (Ariey *et al.*, 2014).

### 1.12.1 Artemisinin resistance associated mutations in the *Pf* Kelch13 propeller region

Genome wide association studies of slow clearing and fully susceptible parasites from the tracking resistance to artemisinin collaboration (TRAC) study (Ashley *et al.*, 2014) were used to identify a region on *P. falciparum* chromosome 13 associated with artemisinin resistance (Takala-Harrison *et al.*, 2013). Resistance was independently linked with multiple SNPs in a gene known as kelch13 (K13) which is found in the  $\beta$ -propeller domain of the encoded kelch-like protein, PF3D7\_1343700 (Ariey *et al.*, 2014). K13 propeller domain mediates diverse cellular function, including ubiquitin-regulated protein degradation and oxidative stress responses (Adams *et al.*, 2000). A mutation in K13 was observed in the Tanzanian F32 parasite line that was exposed to escalating concentrations of artemisinin over a period of 5 years. This mutation known as M476I, gave rise to the F32-ART line which had other mutations (Witkowski *et al.*, 2010). Analysis of Cambodian isolates through genome analysis identified four prevalent K-13 mutations, I543T, Y493H, R539T and C580Y, which were also associated with long parasite clearance half-lives (>5 hours) in patients and high RSA<sup>0-3h</sup> survival rates in vitro (Ariey *et al.*, 2014; Amaratunga *et al.*, 2014).

A recent study deployed genome editing techniques to ascertain if K13-propeller mutations confer ART resistance in clinical isolates (Straimer *et al.*, 2014). Zing finger nucleases (Straimer *et al.*, 2012; Lee and Fidock, 2014) were developed to target genetic engineering of K13 into both older established reference lines and newly culture adapted Cambodian isolates. Plasmids were created to contain either wild type K13 allele or one of several already identified mutations found in F32-ART or ART-resistant Cambodian isolates.

A series of clones which had individual K13 mutations removed from ART resistant Cambodian isolates were generated using donor plasmids which contained a wild-type K13-propeller sequence and silent binding site mutations (Straimer *et al.*, 2014). One of such isolates, (Cam3.II) was a slow clearer following ART monotherapy and had an *in-vivo* half-life of 6 hours. The study showed that parental Cambodian lines Cam3.I<sup>R539T</sup> and Cam3.II<sup>R539T</sup>, both harbouring the R539T mutation had a survival rate of 40%-49% on the RSA0-3h assay while the edited Cam3.I<sup>rev</sup> and Cam3.II<sup>rev</sup> clones which had the reverted wild-type alleles only had 0.3%-0.7% survival rate (Straimer *et al.*, 2014). This experiment highlighted the importance of the mutation R539T in mediating in vitro artemisinin resistance

in Cambodian isolates. The same study also showed a similar trend in other resistant lines. The RSA<sup>0-3h</sup> survival rate was also reduced when the mutation I543T was removed. The parental line Cam5<sup>I543T</sup> had a survival rate of 43% compared to 0.3% of the revertant Cam5<sup>rev</sup> (Straimer *et al.*, 2014). Similarly, the mutation C580Y had a 13% survival rate in the parental line Cam2<sup>C580Y</sup> compared 2.4% in the revertant line Cam2<sup>rev</sup>.

The impact of introducing K13 mutations into a fast clearing line was also investigated. The survival rates of the edited clones in the RSA were higher compared to the parental wild-type lines (Straimer *et al.*, 2014). Using an alternative method of genome editing, the CRISPR-CAS9 system, Ghorbal et al (2013) generated transgenic, *P. falciparum* 3D7 parasites with enhanced RSA<sup>0-3h</sup> survival due to engineered expression of K13 C580Y.

The contribution of individual mutation to artemisinin resistance was investigated. Using the Dd2 reference line, it was found that the introduction of mutations M476I, I543T or R539T conferred higher levels of resistance compared to mutations Y493H and C580Y (10-30% compared to 2-4% survival). Of all the mutations, the C580Y mutation has rapidly become the predominant allele in western Cambodia (Ashley *et al.*, 2014). K13 mutations have also been observed in African isolates but none of the mutations observed are similar to the most prevalent mutations found in Southeast Asia. These data do not support the use of K-13propeller mutant genotypes as a marker for reduced parasite susceptibility to artemisinins.

## **1.13 Geographical distribution and spread of artemisinin resistance**

### **1.13.1 Southeast Asia**

Artemisinin resistance defined by *in-vivo* slow parasite clearance and RSA (marker prevalence) has spread rapidly throughout Southeast Asia. Resistance has become prevalent in other Cambodian provinces (Amaratunga *et al.*, 2012; Arieu *et al.*, 2012), Southern Vietnam (Ashley *et al.*, 2014; Hien *et al.*, 2012), Thailand-Myanmar border areas (Ashley *et al.*, 2014; Phyo *et al.*, 2012) and is emerging in Southern Laos (Ashley *et al.*, 2014).

### 1.13.2 Africa

Although none of the K13 mutations found in Africa correspond to the most prevalent mutations present in Cambodia, artemisinin and artemisinin combination treatments are still very efficacious in Africa (Ashley *et al.*, 2014; Menard *et al.*, 2016; Muwanguzi *et al.*, 2016). Taylor *et al.* carried out deep sequencing study of the K13 propeller domain in over 1,110 *P. falciparum* infections which were retrieved from 14 sites across sub-Saharan Africa and found a large reservoir of naturally occurring K13 propeller variations. The study identified one rare mutation, P553L which was previously found in Cambodia. Other mutations were also found such as A578S, which is close to known resistance-causing mutations in the propeller domain (Taylor *et al.*, 2014).

A study done in Dakar, Senegal analysed the K13 gene sequences of 138 malaria patient samples. Only two mutations, T149S (6.3%), and K189T (42.2%) were detected (Torrentino-Madamet *et al.*, 2014). However, when a subsequent study was done in the region from samples that were collected from patients in 2013 and 2014, the N554H, Q613H, and V637I mutations were identified in the propeller region of K13 in 92 (5.5%) isolates (Boussaroque *et al.*, 2016). Again, none of the polymorphisms associated with artemisinin resistance in Southeast Asia was detected in Dakar.

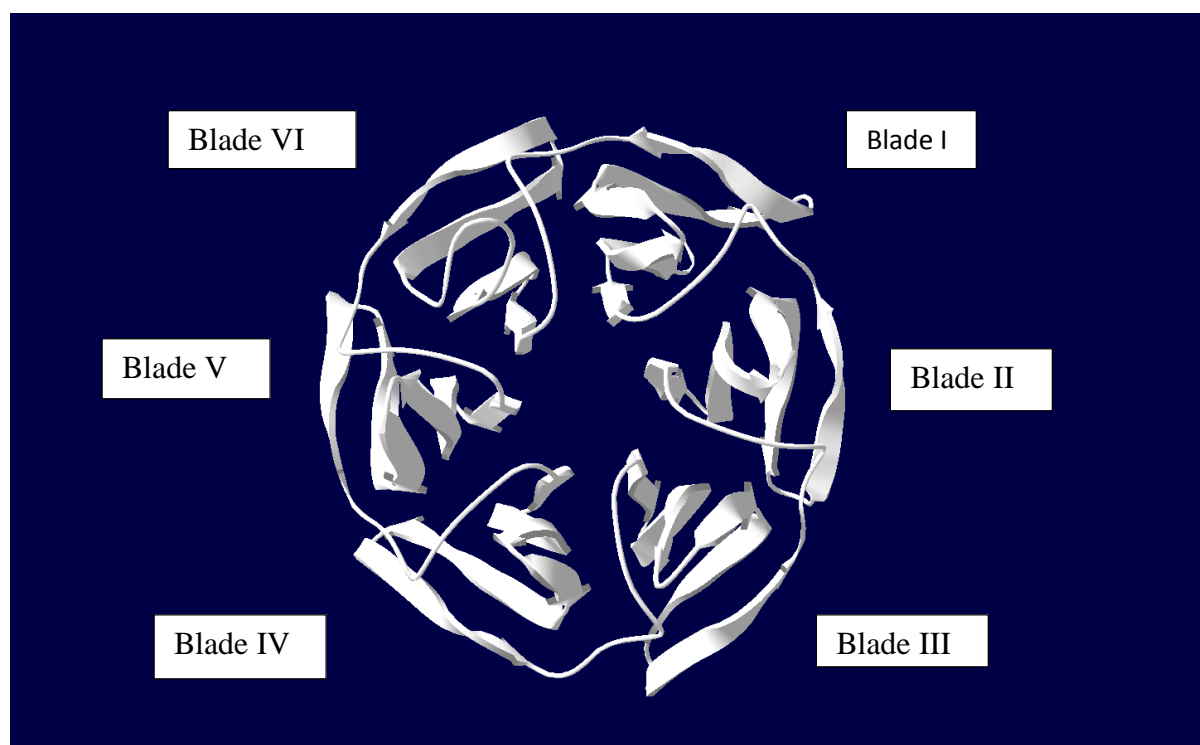
**Table 1.1:** Polymorphisms observed in the K13-propeller domain

Codon Position	Amino acid reference sequence	Nucleotide reference sequence	Amino Acid mutant-type sequence	Nucleotide mutant-type sequence
449	G	ggt	A	gCt
458	N	aat	Y	Tat
474	T	aca	I	aCa
476	M	atg	I	atA
481	A	gct	V	gTt
493	Y	tac	H	Tac
508	T	act	N	aCt
527	P	cct	T	Act
533	G	ggt	S	Agt
537	N	aat	I	aTt
539	R	aga	T	aCa
543	I	att	T	aCt
553	P	ccg	L	cTg
561	R	cgt	H	cAt
568	V	gtg	G	gGg
574	P	cct	L	cTt
580	C	tgt	Y	tAt
584	D	gat	V	gTt
612	E	gaa	D	gaT
623	S	agt	C	Tgt



### 1.14 New proposed molecular mechanism of artemisinin resistance

Parasite genetic loci associated with artemisinin resistance have been revealed through genome wide association studies (Takala-Harrison *et al.*, 2013; Arie *et al.*, 2014). Although there has not been any consensus on molecular targets of artemisinin, a study has provided biochemical and cellular evidence that artemisinins are potent inhibitors of *P. falciparum* phosphatidylinositol-3-kinase (*Pf*P13K) (Mbengue *et al.*, 2015). In strains that are artemisinin resistant, increased *Pf*P13K levels are associated with the C580Y mutation in *Pf*Kelch13 (Mbengue *et al.*, 2015).



**Figure 1.8:** Crystal model of the K13-propeller domain of *Plasmodium falciparum*. The data that feeds into the model was retrieved from rcsb.org and assembled using deep view software with direction from Prof. David Warhurst.

## 1.15 Current gaps in artemisinin efficacy and resistance

### 1.15.1 *In-vivo* studies

There are a few limitations with *in-vivo* classification of artemisinin resistance. Three doses of artemisinin in ACT therapy reduces parasite density to  $10^4$  fold. Resistance based on a parasite clearance time (PCT) to 0 by 72 hours will have limitations in situations where there are very high parasite densities eg  $>100,000/\mu\text{L}$  of whole blood. This means the drug may not effectively clear parasites. Another limitation would arise in areas of high endemicity where the immune system plays a role in acquired immunity by clearing parasites relatively fast compared to areas like Southeast Asia. In this instance, a delay in clearance time from 24-48 hours over time may indicate a decline in susceptibility of parasites to artemisinin and it may not meet the WHO standard 72 hours delayed clearance definition (Fairhurst *et al.*, 2012).

With the reduction in malaria transmission intensity in various regionites of Africa, and with changes in the rate of acquired immunity (O'Meara *et al.*, 2010; Barnes *et al.*, 2009), PCT trends are difficult to interpret (Borrmann *et al.*, 2011). Parasite clearance dynamics have to be monitored in detail after ACT is given to determine if parasite susceptibility to ACT is changing. *In-vivo* clearance dynamics can be analysed as parasite half life with blood obtained per day after treatment (Dondorp *et al.*, 2009; Amaratunga *et al.*, 2012), or by the absence or presence of parasite at a single time point after treatment initiation (Stepniewska *et al.*, 2010). These involve so much bleeding from patient and a lot of microscopy readouts. A simple duplex quantitative polymerase chain reaction (qPCR) method has been proposed as an alternative and sensitive method that involves analysing sequentially collected filter paper blood samples to detect and quantify parasites below the microscopic threshold of detection (Beshir *et al.*, 2010).

### 1.15.2 *In-vitro* studies

The standard *in-vitro* assays have produced  $\text{IC}_{50}$  values that have weak or no correlation to parasite clearance rate which is the current *in-vivo* parasite resistance phenotype. This means that a new type of *in-vitro* test, which can be validated, is needed. Two modified in vitro

assays have been used to assess resistance with some success with both assessing reduced susceptibility at the ring stage of development in erythrocytes (Tucker *et al.*, 2012; Witkowski *et al.*, 2013), however, these assays have not been validated elsewhere. These are the T<sub>0</sub> [<sup>3</sup>H] hypoxanthine assay and a ring stage survival assay. The latter assesses the survival of ring stage parasites exposed to a single 6-hour pulse of 700nM DHA. The percentage of normal parasites counted in blood smears stained with Giemsa at 72 hours is the endpoint. The RSA can be associated with clinical resistance due to a statistically significant correlation with in vivo clearance in the Greater Mekong region and with mutation in the K13 propeller domain (Ashley *et al.*, 2014; Arieu *et al.*, 2014).

### 1.15.3 Molecular markers

Previous studies using genome wide association identified genetic loci associated with *in-vitro* susceptibility of *P. falciparum* to artemisinins; none of these loci were associated with slow parasite clearance in malaria infected patients (Mok *et al.*, 2011). Delay in parasite clearance time usually correlates with an increase in IC<sub>50</sub> and putative molecular markers such as *pfmdr1*, *pfatpase6*, *pfcr1*, and *pfubp-1*. However Dondorp *et al* (2009) found no correlation between delayed clearance time and IC<sub>50</sub> and found no associated molecular markers (Dondorp *et al.*, 2009). More recent studies however showed that multiple point mutations in the propeller domain of Pf3D7\_1343700, the kelch gene on chromosome 13 (K13), have been associated with delayed parasite clearance phenotype in vivo and have provided a molecular marker for tracking the spread of artemisinin resistance (Straimer *et al.*, 2015; Arieu *et al.*, 2014).

## **1.16 Genetic molecular markers of *P. falciparum* drug resistance**

### **1.16.1 Multidrug resistance protein 1 (*Pfmdr1*)**

The *P. falciparum* multidrug resistance protein 1 (*Pfmdr1*) gene encodes a transmembrane protein present in the digestive vacuole of the parasite. It has a nucleotide binding fold region that binds ATP and thus belongs to the ATP-binding cassette (ABC) superfamily. It has a molecular mass of 162.25 KDa, an exon encoding for P-glycoprotein homolog 1 protein which comprises of 1419 amino acid (Duraisingh and Cowman, 2005). Variation in *Pfmdr1* mRNA expression level, *Pfmdr1* gene amplification and sequence polymorphism have all been involved in various antimalarial resistance and played a role in the emergence of multidrug resistance parasites (Duraisingh and Cowman, 2005).

Mutations in positions (N86Y, Y184F, S1034C, N1042D and D1246Y) of the *Pfmdr1* gene have been reported to be associated with drug susceptibility to quinine, chloroquine, amodiaquine, halofantrine, mefloquine, lumefantrine and artemisinin (Sidhu *et al.*, 2005; Humphreys *et al.*, 2005). Mutations at positions N86Y and N1042D are also associated with amodiaquine resistance (Sa *et al.*, 2009). Mutations in positions N86Y of *pfmdr1* gene together with mutations in K76T and A220S of the *pfcr1* gene have been associated with high resistance to chloroquine in field isolates (Sidhu *et al.*, 2006). Copy number variation present in *Pfmdr1* gene has been linked to quinine resistance, lumefantrine, mefloquine, halofantrine, and also resistance in artemisinin (Sidhu *et al.*, 2006).

### **1.16.2 Multidrug resistance-associated protein (*Pfmrp*)**

*P. falciparum* multidrug resistance-associated protein is a transmembrane protein located in the plasma membrane of the parasite. It encodes 1822 amino acid with a molecular mass of 214.44 kDa and has two nucleotide binding domains and two membrane-spanning domain with each made up of 6 helical transmembrane domains. *Pfmrp* belongs to the ABC transporter family, located on chromosome 1 (Raj *et al.*, 2009). The function of MRP is to

transport organic anionic substrates such as glutathione and is also involved in drug transport (Klokouzas *et al.*, 2004). Mutations at positions Y191H and A437S in *Pfmrp* are associated with resistance in quinine and chloroquine (Mu *et al.*, 2003). A high sensitivity to various antimalarial drugs such as artemisinin, piperazine, chloroquine and quinine were obtained when a genetic knockout of *Pfmrp* gene in resistant parasite was carried out (Raj *et al.*, 2009). As a result, *Pfmrp* is involved in changing the antimalarials response to drug resistance but not in determining the resistance in itself. It was hypothesized that *Pfmrp* pumps out various drugs and metabolites out of the parasite with the help of other transporters (Raj *et al.*, 2009).

### **1.16.3 *Plasmodium falciparum* sodium hydrogen exchanger (*Pfnhe1*)**

*Pfnhe1* is a transmembrane protein made up of 12 transmembrane domains and has a molecular weight of 226 kDA. It is located on chromosome 13 and has two exons that code for sodium hydrogen exchanger (Na<sup>+</sup>/H<sup>+</sup> exchanger or *pfnhe*) protein which has been reported to be associated with resistance in quinine (Ferdig *et al.*, 2004). *Pfnhe1* is localised in the plasma membrane of the parasite and is hypothesized to be involved in active efflux of protons to maintain a PH of 7.4 within the parasite as a result of a response to acidification by anaerobic glycolysis which is the main energy source for the parasite (Bosia *et al.*, 1993).

*In-vitro* culture studies have shown that polymorphisms in the microsatellite ms470 region showed a decrease in quinine susceptibility with an increase in DNNND repeat motif (Briolant, 2011). Subsequently, an increase in quinine susceptibility was observed with a rise in NHNDNHNNDddd motif (Briolant, 2011). Therefore, repeat polymorphisms present in *Pfnhe1* gene has been proposed as a useful genetic marker in determining quinine resistance in certain regions as well as resistance to quinine mediated by other genetic markers such as *Pfmdr1*, *Pfmrp*, and *Pfcrt* (Ekland and Fidock, 2007)

#### **1.16.4 *P. falciparum* bifunctional dihydrofolate reductase-thymidylase synthase**

The *Pfdhfr-ts* gene is located on chromosome 4 and encodes the *Pfdhfr-ts* protein. It is 608 amino acids long and has a molecular weight of 71.73kDa, a bifunctional enzyme involved in two main folate metabolic activity namely, the reduction of dihydrofolate into tetrahydrofolate by dihydrofolate reductase activity and the biosynthesis of dTMP by thymidylase synthase activity (Foote and Cowman, 1994). *Pfdhfr* is inhibited by the action of antifolate drugs such as cycloguanil and pyrimethamine and so reduces pyrimidine production needed for DNA replication (Hyde, 2005). Point mutations in the *Pfdhfr* protein associated with pyrimethamine resistance are present at positions S108D. Further mutations at positions N51I, C59N, and I164L increase the resistance to pyrimethamine other than gene amplification (Thaithong *et al.*, 2001).

#### **1.16.5 *Plasmodium falciparum* dihydropteroate synthetase**

The *pfdhps* gene is made up of 706 amino acid and has a molecular weight of 83.37kDa. It has three exons which encode for *pfdhps* and is located on chromosome 8. *Pfdhps* enzyme catalyzes the p-aminobenzoic acid (PABA) reaction with a pterin derivative in synthesizing dihydrofolate, which is a precursor of folate, needed for the synthesis of pyrimidine in the parasite (Foote and Cowman, 1994).

Mutations in *pfdhps* have been linked to sulfadoxine resistance in *P. falciparum*. There mutations include S436A/F, A437G, L540E, A581G, and A613T/S (Wernsdorfer and Noedl, 2003). At positions 436, 581 and 613, a higher level of resistance is associated with these codons while mutation at position 437 and 540 contribute to a low level of resistance but also having a modulation effect in association with other *Pfdhps* mutation (Hyde, 2007). Sulfadoxine is given in combination with pyrimethamine (SP) and resistance to SP have been associated with point mutation in both *Pfdhfr* and *Pfdhps* gene (Reed *et al.*, 2000)

### 1.17 Artemisinin resistance in Africa

With reports of artemisinin resistance in Southeast Asia (Dondorp *et al.*, 2014; Witkowski *et al.*, 2013), numerous studies have been carried out to investigate the spread of drug resistance in Africa for continuous monitoring of the therapeutic response of ACTs. This could aid in early detection and containment of drug resistance in endemic areas. To date, no study has found any delayed clearance of microscopically detectable parasites in Africa. Although there is limited information on the artemisinin *in-vitro* susceptibility of ring stage of *P.falciparum* development, there is also limited prevalence of mutations in *Pfk13* (Taylor *et al.*, 2015).

A few studies have found the presence of the A578S mutation of the propeller region in Africa (Ashley *et al.*, 2014; Conrad *et al.*, 2014; Taylor *et al.*, 2015; Hawkes *et al.*, 2015), but none of these studies suggest that A578S is an artemisinin resistant mutation. Another study was carried out to ascertain the prevalence of mutations in *Pfk13* in 14 sub-Saharan African countries and found no mutation associated with artemisinin resistance in Southeast Asia except one mutation at codon 543 (Kamau *et al.*, 2015). It has been suggested that the majority of rare K13 alleles are neutral with respect to artemisinin resistance in Africa and lack evidence of artemisinin-driven selection on the K13 locus (Menard *et al.*, 2016).

It was shown that residual sub-microscopic parasites were present in Kenyan children on day 3 after treatment with ACT and were associated with subsequent recrudescence and transmission (Beshir *et al.*, 2013). Another study showed that parasites that harboured certain mutations in *Pfmdr1*, *Pfcrt*, *Pfubp1*, and *Pfap2mu* genes showed a higher survival rate at a sub-microscopic level after treatment with ACT (Henriques *et al.*, 2014). To date, ACTs remains efficacious against malaria infections in Africa (Sawa *et al.*, 2013; Ashley *et al.*, 2014). More *in-vitro* phenotyping studies and allele editing studies (Straimer *et al.*, 2015) needs to be done to provide more information about the potential presence of drug resistance in Africa.

This thesis sets out to do just that by testing the hypothesis that differences in drug sensitivity ( $EC_{50}$ ) exist between African isolates and these differences are genetically determined. These differences, if found can be used to assign drug sensitivity phenotypes for African isolates *in-vitro*. To test this hypothesis, I will examine field isolates of African origin and test them for their response to artemisinin in vitro. I will also compare these directly against the DHA sensitivity profiles of artemisinin resistant isolates from Cambodia. To achieve this, I will

modify the current standard *in vitro* assay by exposing the parasite to short pulses of artemisinin and making comparisons to the standard 48 hours exposure. I will also employ a ring stage assay (RSA) to assess the DHA response of African parasite lines and to validate already existing methods. In this thesis, I will carry out next generation genome sequencing using the illumina platform on all African lines and elucidate genotypes at all candidate loci, in each line for which *in-vitro* DHA phenotype has been ascertained.



## **CHAPTER 2: Aims and Objectives**

## 2.1 Aim

The aim of this study is to identify a scalable assay able to discriminate between parasite lines with small differences in artemisinin sensitivity. The study also aims to identify phenotypes relevant to the genetic exploration of artemisinin resistance in African settings.

## 2.2 Hypothesis

This study hypothesizes that differences in EC<sub>50</sub> values exist within and between the African isolates and these differences are also detectable genetically. These genetic differences can be used to predict drug sensitivity phenotypes for African isolates *in vitro*.

## 2.3 Objectives

The specific objectives are as follows:

**Chapter 4:** To understand dihydro-artemisinin (DHA) sensitivity by using parasite lines that are lab adapted. To identify an assay able to discriminate between parasite lines with small differences in artemisinin sensitivity by using a 6-hour pulse assay. Currently, the standard 48 hr *in vitro* protocol is unable to discriminate between the response to artemisinin in parasite lines tested as they all appear sensitive to DHA (van Schalkwyk *et al.*, 2013). Short-term exposure (pulse assay) protocol will be used to compare sensitivity of different parasite lines to the active metabolite (DHA), based on recent publications. The pulse assay is used to simulate *in vivo* DHA exposure time. DHA drug sensitivity profiles of parasite lines from Kenya, Nigeria and Ghana will be assessed.

**Chapter 5:** To assess the DHA sensitivity profile of artemisinin resistant lines from Cambodia using the novel 6-hour pulse assay and an alternative assay protocol (RSA<sup>0-3h</sup>) by Witkowski *et al.*, To compare both assays to the standard 48 hr assay to see which assay best discriminates between the parasite lines. To use the ring stage assay to assess DHA response of African parasite lines and to validate already existing methods. This chapter aims to develop a methodology that works in both African and Cambodian setting.

**Chapter 6:** To produce full genomic sequences for each polyclonal line. To examine genomic data for evidence of polymorphisms at established candidate loci and to inform future functional studies deploying gene editing.

## **CHAPTER 3: Materials & Methods**

### **3.1 Reagents, Buffers and Solutions**

#### **3.1.1 Culture medium**

1. Albumax II, gentamicin sulphate (Invitrogen, Carlsbad, CA, USA)
2. HEPES, RPMI 1640, human AB serum, hypoxanthine, L-glutamine and D-glucose (Sigma-Aldrich, St Louis, MO, USA)
3. Antimalarial compounds supplied by Medicines for Malaria Venture (MMV, Geneva, Switzerland)
4. Dimethyl sulphoxide (DMSO)
5. Deionized water (Millipore, Watford, UK)

#### **3.1.2 PCR**

1. 10uM of each dNTP (Bioline, UK)

10ul of stock dNTP (100pM ) + 90ul water)

2. 10um of each primer (Eurofin, Germany)

10ul of stock primer (100pM) + 90ul water

3. 10um of each probe (Eurofin, Germany)

10ul stock probe (100pM) + 90ul water

4. 10x NH<sub>4</sub> buffer (Bioline, UK)

160mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 670mM Tris-HCl (pH 8.8 at 25°C) and stabilizers

5. MgCl buffer (Bioline, UK)

160mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 670mM Tris-HCl (pH 8.8 at 25°C), 15mM MgCl<sub>2</sub> and stabilizers

6. KCl buffer (Bioline, UK) 500mM KCl, 100mM Tris-HCl (pH 8.8 at 25°C), 15mM MgCl<sub>2</sub>, and stabilizers

### **3.1.3 Thawing solutions**

Thawing solution 1: 12% (w/v) NaCl (1.2g of NaCl, 10ml H<sub>2</sub>O)

Thawing solution 2: 1.6% (w/v) NaCl (0.8g of NaCl, 50ml H<sub>2</sub>O)

Thawing solution 3: 0.9% (w/v) NaCl + 0.2% glucose (0.45g of NaCl, 0.1g of D-glucose, 50ml H<sub>2</sub>O) (Sigma-Aldrich)

### **3.1.4 DNA separation**

1.2% (w/v) agarose gel

1.2g agarose (Sigma-Aldrich, UK) in 100ml 5X TBE

2. 5x TBE (Tris-Borate-EDTA) buffer

Tris (hydroxymethyl) aminomethane + Boric acid

1 sachet TBE (Sigma-Aldrich, UK) in 1L of water

### **3.1.5 PCR product purification**

1. FastAP (Thermosensitive Alkaline Phosphatase) (Fermentas, USA) supplied in 20mM HEPES-NaOH (pH 7.4), 1mM MgCl<sub>2</sub>, 0.1 mM ZnCl<sub>2</sub>, 0.1% Triton X-100 and 50% (v/v) glycerol.

2. Exo I (Exonuclease I) (Fermentas, USA) supplied in 20mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1mM DTT and 50% (v/v) glycerol.

3. 10X FastAP Buffer (Fermentas, USA) 100mM Tris-HCl (pH 8.0 at 37C), 50mM MgCl<sub>2</sub>, 1M KCl, 0.2% (v/v) Triton X-100 and 1mg/ml BSA.

### **3.1.6 DNA sequencing**

1. Big dye terminator (Applied biosystems, UK)

2. Big dye buffer (Applied biosystems, UK)

### **3.1.7 Sequencing reaction purification**

1. Ethanol/Sodium acetate

3M NaOAc + absolute ethanol + water

2. 3M NaOAc (Sodium acetate) (Sigma-Aldrich)

40.8g NaOAc + water made up to 100ml at 5.2 pH

3. 70% Ethanol

70ml absolute ethanol AnalaR (Fisher chemicals, UK) + 30ml water

4. HiDi (Applied Biosystems, UK).

### **3.1.8 DNA extraction**

QIAmp DNA blood midi kit (20) (Qiagen, Manchester UK)

1. QIAmp mini spin columns

2. Collection tubes (2 ml)

3. Buffers: Buffer AL, Buffer ATL, Buffer AWI, Buffer AW2, Buffer AE

4. Qiagen Protease

### 5. Proteinase K

6. 4.4ml distilled water added into the vial of lyophilized Qiagen protease provided.

7. Buffer AW1: 25 ml of 100% ethanol added to make a final volume of 44 ml

8. Buffer AW2: 150 ml of 100% ethanol added to make a final volume of 57 ml.

### 3.1.9 Limiting dilution

Malstat: 400  $\mu$ L Triton  $\times$ 100 (Thermofisher) + ~150 mL MilliQ H<sub>2</sub>O + 4 g Calcium L-Lactate, 1.32 g Tris Base (Sigma-Aldrich)+ 22 mg of APAD.

NBT/PES: 160 mg of Nitroblue Tetrazolium(Sigma-Aldrich) + 8 mg Phenazine Ethosulphate + 100 mL of MilliQ H<sub>2</sub>O.

### 3.1.10 Whole genome sequencing

1. NEBNext Sanger Sequencing sample Prep Kit I
2. MiSeq Reagent Kit
3. Qiagen Kit to clean up (mini elute and PCR clean up kits)
4. Agencourt Ampure XP beads (Beckman Coulter)
5. Kapa HiFi (KAPA Biosystems, South Africa)



## **3.2 Patient Sample**

### **3.2.1 African Samples**

Five African isolates were used in this study (HL1204, HL1210, HL1212, HL1402 and a Liberian isolate). Isolates HL1204, HL1210, HL1212 have been characterised elsewhere (Schalkwyk *et al.*, 2013) (Table 4.1). Four of the isolates were obtained from patients who presented with malaria at the Hospital for Tropical Diseases, London (HTD), or the Accident and Emergency Department of University College London Hospitals (UCLH). A 4mL Vacutainer tube containing EDTA (BD) was used to obtain venous blood sample, some of which was used for laboratory adaptation of the malaria parasites in the malaria culture laboratory at the London School of Hygiene and Tropical Medicine. Ethical approval was obtained from the Research Ethics Committee of the University College London Hospitals (Application number: 07/Q0505/60). The Liberian isolate was obtained from the Sanger Institute, Cambridge.

### **3.2.2. Cambodian Samples**

The Cambodian isolates (Cam 3.11wt, Cam 3.11C580Y and Cam 3.11R539T) were obtained from the Liverpool school of Tropical Medicine UK (Strainer *et al.*, 2015) (Table 5.6) while the KH2 line was obtained from the Sanger Institute via Cambodia (Miotto *et al.*, 2014). They were quickly adapted to complete medium supplemented with 10% (v/v) human AB serum, 25mg/L gentamicin, 147µM hypoxanthine, 23nM HEPES, and 25 mM Na<sub>2</sub>HCO<sub>3</sub>. After adaptation, isolates were cryopreserved in liquid nitrogen

### **3.3 Antimalarial drugs**

Two antimalarial drugs, namely chloroquine and DHA were used in this study. The drugs were supplied by the Medicines for Malaria Venture (MMV, Geneva, Switzerland). The first DHA stock solution was made by dissolving in dimethyl sulphoxide (DMSO) and subsequent dilutions were made using complete RPMI growth media (RPMI 1640 growth medium supplemented with 10% (v/v) human AB serum, 25 mg/L gentamicin, 147  $\mu$ M hypoxanthine, 25 mM HEPES, and 25 mM  $\text{Na}_2\text{HCO}_3$ ). The primary stock for chloroquine was made by dissolving in deionized water (Millipore, Watford, UK).

### **3.4 Laboratory adaptation**

#### **3.4.1 Parasite thawing**

1ml cryovials of liquid nitrogen frozen clinical isolate stock of 4 isolates (3D7, HL1204, HL1210, HL1212 and HL1402) were thawed using 3 thawing solutions (section 3.1.3). The vials were placed in a 37°C waterbath for 2 minutes and transferred to a 50ml tube. 1/5 of the volume of parasites of thawing solution 1 (200ul), was added dropwise while mixing gently. The mixture was allowed to stand for 2 minutes without agitation. 10x the original volume of parasites of thawing solution 2 (10ml), was added dropwise while mixing gently. The suspension was centrifuged at 500g for 5 minutes at room temperature. The supernatant was removed and discarded and 10ml of thawing solution 3 was added dropwise while mixing gently. The suspension was centrifuged at 500g for 5 minutes at room temperature. The supernatant was removed and discarded and the pellet was resuspended into complete RPMI media and added to culture.

### **3.4.2 Parasite culture and laboratory adaptation**

Complete media for culture adaptation of clinical isolates were prepared using reagents in section 3.1.1 and were put in a 37°C waterbath before adding to culture. Whole blood sample (A+) obtained from the blood bank (National Health Blood & Transplant, UK) was washed twice using RPMI-1640 (centrifuged at 2,000 x g for 10 minutes). The supernatant and buffy coat was removed and the red cell pellet was suspended in 25mL of RPMI. This process was repeated twice and the washed blood was then diluted to 50% haematocrit. Parasites were maintained in continuous culture in a sealed flask and incubated at 37°C under a gas mixture of 93% (v/v) N<sub>2</sub>, 4% (v/v) CO<sub>2</sub> and 3% (v/v) O<sub>2</sub>. The parasites were diluted in human A+ blood as the parasitaemia increased to maintain a parasitaemia of <4% and a haematocrit of 5%.

For all KH2 isolate adaptation and routine parasite cultivation the amount of human serum added was reduced to 2% (v/v) from 10% (v/v) and the culture media further supplemented with 5 g/L Albumax II, 10 mM D-glucose and 2 mM L-glutamine. The parasites were maintained in continuous culture with shaking initially, and then later maintained in static conditions. The parasites were stored in a sealed flask incubated at 37°C under a gas mixture of 93% N<sub>2</sub>, 4% CO<sub>2</sub> and 3% O<sub>2</sub>. As the parasitaemia increased the parasites were diluted in human A<sup>+</sup> blood (National Health Blood & Transplant, UK) to maintain a parasitaemia <4% and a haematocrit of 5%.

### **3.4.3 Parasite freezing**

Stocks of all 4 clinical isolates were frozen down at ring stage after culturing using glycerol solution and stored in -20°C, -80°C and liquid Nitrogen respectively for future experiments.

### **3.5 Anti-plasmodial activity assays**

#### **3.5.1 Synchronisation**

Parasites were synchronised at ring stage before any drug assay experiment were carried out. This was done with 5% Sorbitol (Sigma-Aldrich). 6ml of sorbitol was added to the parasitized RBC and was left to sit for 2 minutes. The solution was centrifuged at 600g for 2 minutes and the supernatant was removed. 25ml of RPMI was added and centrifuged as before and this wash was repeated twice.

#### **3.5.2 Drug dilution and 48hr assay ( $EC_{50}^{48h}$ )**

After synchronization at ring stage, parasites were diluted to a final parasitaemia of 0.5% and a haematocrit of 4% and left in the water bath at 37°C. A 2-fold drug dilution was carried out on drug compounds DHA and chloroquine, with starting concentrations at 1uM and 200nM respectively. A negative control of 20uM Chloroquine was used and a positive control (culture media and parasite solution) with no drug was also used. After the dilution, parasites were added to drugs in a 200ul volume within 96-well microtitre plates and incubated for 48 hrs at 37°C. Parasite viability is described in section 3.5.

#### **3.5.3 6 hour Pulse Assay ( $EC_{50}^{6h}$ )**

For a pulse assay, parasite and drug dilution was done in a 1ml eppendorf tube. The suspension was washed with RPMI on the 6<sup>th</sup> hour before transferring into a 96 well plate for 48 hour incubation. The starting concentrations were the same as previously described but the volume was 1ml in each tubes and a 1 in 2 drug dilution was done.

6 hours into incubation, 200ul of solution for both CQ and DHA were transferred into a 96 well plate in duplicate (400ul total). The remaining 600ul parasite solution was washed twice using 1ml RPMI and spun down for 1 minute. After the wash, 200ul of parasite solution for

both drugs were pipetted into the same 96 well plate, also in duplicate. The plate was incubated within a gassed container at 37°C for 48 hours.

#### **3.5.4 EC<sub>50</sub><sup>6h T0-T4</sup> assays**

The EC<sub>50</sub><sup>6h T0-T4</sup> assay had been carried out in the same format as the 6 hour pulse assay with a few adaptations. Parasites were tightly synchronised at 0.5% parasitaemia (two rounds of synchronisation to get the early ring stage (0-3hrs)). Parasites were exposed to DHA at two time points, Time 0hr and Time 4hr. The Time 0hr DHA exposure was carried out immediately after tight synchronisation and then plated out in a 96 well plate. In a different plate, parasites were plated out without drug for an added 4 hour growth time in readiness for the 4hr DHA exposure. 6 hours later, the time 0hr DHA exposure plate was washed off and incubated within a gassed container at 37°C for another 42 hours (48 hours in total). The Time 4hr exposure was exposed to DHA 4hr into the growth of its life cycle and incubated. The DHA drug was washed off 10 hours later and then incubated in the same gassed container as the first plate. A schematic diagram of this assay can be seen in appendix II, figure 1.

#### **3.5.4 Ring stage survival assay (RSA<sup>0-3hr</sup>)**

The ring stage assay was performed following the protocol provided by Witkowski and colleagues (Witkowski *et al.*, 2013). Tight synchronisation of parasites was carried out across two consecutive ring stage cycles using sorbitol treatment (Section 3.5.1). Enrichment of the late schizont stage was done using heparinised RPMI and 75% (v/v) percoll solution at room temperature and cultured for 3 hours at 37°C with fresh erythrocytes. The parasites were treated with sorbitol again to ensure no late stages were present. Parasites were in the early ring stage (0-3hr), at 0.5% parasitaemia and 2% haematocrit in a final 2ml volume. These were exposed to 700nM of dihydroartemisinin for 6 hours in 0.1% DMSO after which the cultures were washed and resuspended in drug-free culture medium. This was cultured at 37°C for another 66 hours. The susceptibility to DHA was assessed using microscopy read out on thin films. This was done by estimating the percentage of viable parasites that had

developed into a new cycle after DHA exposure by comparison with parasites exposed to 0.1% DMSO only (control).

### **3.5.5 Synchronisation and drug pressure**

Parasites were synchronized at ring stage and drug pressure was initiated with a final parasitaemia of 0.5% and a haematocrit of 2% in a 200uL volume within a 96-well microtitre plate. Two drugs were used in this study to carry out drug pressure and these are DHA and chloroquine. For DHA, parasites were exposed to 700nM for 72 hours after which the drug was washed off and parasites were allowed to recover in complete medium.

For chloroquine, a two-fold drug dilution was carried out with starting concentration of 100uM. After the dilution, 100ul of parasitized red cells were added to the 96 well plate and incubated for 72 hours. After the drug pressure, parasitized red cells were transferred to a 50ml tube and washed with 25ml RPMI at 2,000 x g for 10 minutes. Parasites were resuspended into a 10ml culture medium and incubated at 37°C under a gas mixture of 93% N<sub>2</sub>, 4% CO<sub>2</sub> and 3% O<sub>2</sub> and allowed to recover from the chloroquine drug pressure.

### **3.5 Viability of parasites**

Lysis buffer was made up using 29mM Tris, 5mM EDTA, 0.008% (w/v) saponin and 0.08% Triton X-100 (pH 7.5). Pico Green (Invitrogen, P7581) was diluted 1:200 (25ul Pico Green in 5ml lysis buffer). Afterwards, 50ul of the lysis buffer was pipetted into each well of a 96 well plate and then 100ul of parasite solution that had been resuspended was transferred from the original plate which was used for growth assay into the plate containing Pico green. The solution was mixed well and was read in a Spectramax M3 microplate reader (Molecular Devices). The excitation wavelength used was 485nm and the emission wavelength used was 538nm.

### **3.6 Limiting dilution**

The starting haematocrit for the limiting dilution experiment was 1.8%. The parasite stock was diluted in order to get 0.5 parasites per well or 5 parasites per 100ul, after counting using a haemocytometer. The parasites were plated out in a 96 well plate and 0.4% haematocrit of blood was added each week with fresh media change. The first row (row 1) was used as the positive control (100 uL of 1.8% haematocrit and 100 µL of complete medium). The plates were developed using Malstat and NBT/PES (Section 3.1.9). For development, each well was resuspended and 100ul of Malstat (room temperature) was added to a duplicate microtitre plate. 15 uL of the cell suspension was added and 25ul of NBT/PES was added after. Bubbles were removed using hair a hair dryer and the microtitre plate was stored in a dark cupboard to develop (NBT is light sensitive). After development (purper colour observed), the plate was read at an absorbance of 620nm (Molecular Devices).

### **3.7 Purification of DNA from parasite whole blood**

200ul Qiagen protease was pipetted into the bottom of a 15ml centrifuge tube containing 1.25ml of parasitized red blood cells (from culture-adapted parasites). 2.4ml buffer AL (Qiagen) was added and mixed thoroughly by inverting the tube 15 times, followed by additional vigorous shaking for 1 minute. When multiple tubes were used for multiple DNA extraction, they were inverted simultaneously by clamping them into a rack and inverting them together. The suspension was mixed thoroughly with buffer AL to yield a homogenous solution. The solution was incubated at 70°C for 10 minutes.

2ml of absolute ethanol (100%) was added to the sample and mixed by inverting the tube 10 times. This was then shaken vigorously in order to ensure efficient binding and to achieve a homogenous solution. Parasite genomic DNA was extracted from 200 µL of packed red cells using the QIAamp DNA Blood Mini Kit (Qiagen) as per the manufacturer's instructions. The DNA was stored at -80°C.

### **3.8 Assay data analysis**

Raw data from the Spectramax reader was transferred to a Microsoft Excel sheet and analysed to get percentage viability for each drug used and for all 4 clinical isolates. An average background figure for the negative control (20uM CQ) was calculated and subtracted from all 96 values. After this, the average control values (i.e. average of the values subtracted from the background) was used to calculate the percentage viability by dividing all values gotten after subtraction from the background value by the average control values.

Drug concentration for both CQ and DHA were logged and the average for quadruplet readings was calculated together with a standard deviation. Prism v4.02 (Graphpad software) was used to create a dose response curve showing percentage viability vs. drug concentration for each clinical isolate.

### **3.9 Whole genome sequencing**

1.25mls of parasitised red blood cells for all three isolates at, HL1204, HL1210 and HL1212 at schizont stage were stored in at 4°C for whole genome sequencing. The method for DNA extraction of parasites was adapted specifically for the parasite lines used in this thesis to obtain high quality of DNA and a high DNA library quality (see section 6.2.1). To obtain a fragment size of approximately 300 bp, all the isolates were sheared using a S2 series ultrasonicator (Covaris Inc.). The settings used to achieve a 300 bp size have been described in chapter 6.2.2 (Table 6.2). Paired end sequencing libraries were constructed using the NEBNext DNA sample preparation kit (Illumina). This was done following the standard illumine sample preparation protocol with slight modifications. End-repair reactions (100 µl) contained 75 µl of sheared DNA sample, 10ul of phosphorylation reaction buffer, 5ul T4 DNA polymerase, T4 polynucleotide kinase, 4ul dNTPs, 1ul Klenow DNA polymerase, and were incubated at 20°C for 30 mins. Resulting blunt phosphorylated DNA fragments (end-repaired) were cleaned using the Agencourt Ampure XP beads (Beckman Coutier) following the manufacturer's instructions. A single A base was added (A-tailing) to the 3' end of the end-repaired DNA fragments in a 50 µl reaction containing 32ul end repaired DNA, 5ul



NEB buffer, 10ul dATP, 3ul of Klenow fragment (3'-5') and incubated for 45 min at 37°C. A-tailed DNA fragments were cleaned using Ampure XP beads following the manufacturer's instructions and DNA eluted in 10 µl EB buffer.

Pre-annealed paired-end (PE) adapter oligonucleotides (Illumina) were ligated to the A-tailed fragments in a 50 µl reaction containing 10 µl of dA tailed DNA sample, 25ul Quick T4 DNA-ligase reaction buffer, 10 µl of NEBNEXT adapter, 5 µl of Quick T4 DNA ligase (NEB) and incubated at 20°C for 15 min. The ligation reaction was cleaned twice with 1× Agencourt Ampure XP beads (Beckman Coulter).

All PCR amplifications were performed with an MJ Research Thermo Cycler PTC-225. Illumina PE 1.0 and 2.0 primers were used to amplify adapter-ligated library fragments (10ng) by PCR. See section 6.3.2 for the PCR modifications. Before libraries were loaded on the Illumina whole genome sequencing platform, they were quantified using Qubit (Thermofisher Scientific).

### **3.10 Whole genome sequence analysis.**

The raw sequence data retrieved from the illumina platform was aligned against the 3D7 reference genome. SAM files were converted to BAM files and sorted. Reads were mapped using the aligner *bwa mem* (Li H & Durbin R, 2009) running on default parameters. The reference used was the *Plasmodium falciparum* 3D7 version 3 downloaded from PlasmoDB (<http://plasmodb.org/plasmo/>). The results were screened using the genome browser tooltablet (Milne *et al.*, 2013) and Geneious software version R9 (Biomatters Ltd).

### 3.11 Molecular analysis of drug-resistance associated genes

#### 3.11.1 Estimating Multiplicity of Infection

Multiplicity of infection (MOI) for *P.falciparum* isolates were assessed by nested PCR analysis of *msp1* block 2 (K1, MAD20, RO33) and *msp2* block 3 (FC27, 3D7/IC). These alleles comprise of conserved regions flanked by repeat sequences of different lengths. The length variation within the alleles can be used to discriminate different parasite clones by PCR fragment length polymorphism. The set of primers used were previously designed (Snounou et al, 1999), but with modified cycling conditions as follows: Nest 1 amplifications were performed in 25 µL reaction mixture containing 5 µL DNA, NH<sub>4</sub> buffer, 4 mM MgCl<sub>2</sub>, 1 mM deoxynucleotides (dNTPs), 0.2 µM forward primer, 0.2 µM reverse primer and 1 unit of BIOTAQ™ (Bioline, UK). The cycling conditions were: denaturation at 94°C for 3 min, 30 cycles of 94°C for 30 sec, 58°C for 1 min and 68°C for 1 min, followed by a final extension of 5 min at 68°C. Amplifications for the Nest 2 reaction was performed in a 25 µL reaction mixture containing 1 µL of Nest 1 product diluted 1/10, NH<sub>4</sub> buffer, 4 mM MgCl<sub>2</sub>, 1 mM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer and 1 unit of BIOTAQ. The cycling conditions were: denaturation at 94°C for 3 min, 30 cycles of 94°C for 30 sec, 61°C for 1 min and 72°C for 1 min, followed by a final extension of 5 min at 72°C.

The amplification products were separated and visualised using gel electrophoresis. Loading buffer was mixed with 15 µL of amplification products and was loaded onto 2% (w/v) agarose gels in 0.5% (v/v) TBE buffer, stained with 0.5 µg/mL ethidium bromide (EtBr). The minimum MOI was estimated to be the highest observed number of size variants for either *Pfmsp1* or *Pfmsp2*.

### 3.11.2 Genotyping of *pfcr* drug resistant marker

The multiplex real-time qPCR performed in a Rotorgene RG3000 thermocycler (Corbett Research, Australia) was used to assess the genotype of *pfcr*. The segment around codons 72-76 was amplified using three dual-labelled probes complementary to CVMNK (FAM fluorophore), CVIET (JOE fluorophore) and SVMNT (ROX fluorophore) as described (Gadalla et al, 2010; Sutherland et al, 2007). A threshold for each probe was set manually using the positive and negative controls for data analysis.

### 3.11.3 PfK13 Genotyping

The K-13 propeller was amplified by using primers used in previous studies (Menard et al, 2013). PCR reactions were carried out with the following conditions: Nest 1 amplifications were performed in 25 µL reaction mixture containing 5 µL DNA, 10 µM forward primer, 10 µM reverse primer and 5x HOT FirePol master mix. The cycling conditions were: denaturation at 95°C for 15 min, 30 cycles of 95°C for 30 sec, 58°C for 2 min and 72°C for 2 min, followed by a final extension of 10 min at 72°C.

Nest 2 amplifications were performed in a 22.5 µL reaction mixture containing 2.5 µL of nest 1 product, diluted 1/2 10 µM forward primer, 10 µM reverse primer and 5x HOT FirePol master mix. The cycling conditions were: denaturation at 95°C for 15 min, 40 cycles of 95°C for 30 sec, 60°C for 30 secs and 72°C for 1 min, followed by a final extension of 10 min at 72°C. 10ul of the PCR product was visualised using gel electrophoresis and was loaded onto 2% agarose gel in 0.5% TBE buffer, stained with ethidium bromide (EtBr) to confirm amplification.

### 3.11.4 Sequencing of PCR products

Exonuclease I (ExoI) and Thermosensitive Alkaline Phosphatase (AP) enzymes (Fermentas, UK) were used to purify PCR-amplified products. This ensured non-incorporated dNTPs and primers were eliminated. The enzymatic purification step was carried out in a final volume of 10  $\mu$ L containing 5  $\mu$ L of the PCR product, 3 U ExoI, 1 U AP and 1X AP reaction buffer. The mixture was incubated for 60 min at 37°C followed by 15 min at 72°C for enzyme denaturation.

The sequencing cycle reactions were carried out using 1  $\mu$ L ExoI/AP purified PCR amplicons, 0.5  $\mu$ L BigDye® Terminator v3.1 Cycle Sequencing Kit reaction mix (Applied Biosystems, UK), 0.2  $\mu$ M of each PCR primer, 1.75  $\mu$ L Big Dye Sequencing Buffer (Applied Biosystems) in a total volume of 10  $\mu$ L. Single-base extension was performed using the following conditions: one denaturation hold at 96°C for 1 min followed by 25 cycles of 96°C for 30 sec, 50°C for 15 sec, and 60°C for 4 min.

The ethanol/sodium acetate precipitation method was used to purify the sequence reaction fragments, where 3  $\mu$ L 3NaOAc (pH 4.6), 62.5  $\mu$ L 100% ethanol and 24.5  $\mu$ L of nuclease free water were added to each sample. The samples were mixed by inversion and incubated at -20°C for 25 min. Samples were centrifuged at 4°C at 3000 g for 30 min after incubation. The supernatant was discarded by inversion, 150  $\mu$ L ice cold 70% ethanol was added and the samples centrifuged at 4°C at 3000 g for 10 min. After centrifugation, supernatant was discarded by inversion and each sample was allowed to dry at room temperature for 30 min after which pellets were resuspended in 10  $\mu$ L Hi-Di formamide. Samples were loaded on an ABI prism 3730 Genetic Analyzer (Applied Biosystems). The sequence chromatograms data from the sense and antisense primers were edited, aligned and assembled into contigs using Geneious Pro software (version 5.5.3; Biomatters, New Zealand). Ambiguities were resolved by eye and single nucleotide polymorphisms were identified by comparing the sequence to that of the 3D7 reference genome.

**CHAPTER 4:** DHA sensitivity of recently adapted polyclonal African isolates of *P. falciparum*

## 4.1 Introduction

Artemisinin based combination therapy (ACT) was recommended by the WHO for the treatment of *Plasmodium falciparum* malaria and its use has contributed to reductions in malaria disease burden, contributing to an estimated 30% reduction in global mortality rates in the past decade (WHO, 2013). There have been reports from Southeast Asia of parasite resistance to artemisinin and its derivatives, similar to earlier antimalarials like chloroquine and sulphadoxine-pyrimethamine. Reports of resistance have emerged from Western Cambodia, Western Thailand, Southern Burma, and Southern Vietnam and there are fears that resistance will spread to Africa as it occurred in previous years (Wootton *et al.*, 2002; Roper *et al.*, 2003). Artemisinin resistance threatens malaria control, treatment and elimination efforts worldwide and the effect will be felt mostly in Africa which currently has the highest burden of the diseases and kills pregnant women and children (WHO, 2015). To prevent the spread of artemisinin resistant parasite to Africa, rapid detection of new artemisinin foci is important.

Artemisinin resistance is recognised as relatively slow parasite clearance rate in patients receiving an ACT or artemisinin (Dondorp *et al.*, 2009) and this is a major concern in the fight against malaria as highlighted by the WHO global plan for artemisinin resistance Containment (WHO, 2011). The parasite clearance half-life is estimated from frequent parasite density count from blood taken from patients who agree to several days of hospitalization. Such studies are both financially and logistically demanding and very inconvenient for patients and their families. So therefore, an alternative *in-vitro* method is used to ascertain parasite half-life by culture adapting parasite lines in the laboratory and determining the IC<sub>50</sub> value – the ‘effective concentration’ of drug that inhibits parasite growth by 50%. (EC<sub>50</sub> – will be used in this thesis)

Unfortunately, impressive gains are now being threatened by this emergence of resistance of *P. falciparum* to ARTs in South-East Asia and no alternative, fully effective first-line therapy is currently available to replace ACTs, should artemisinin fail globally.

Recently, a Vietnamese patient who was infected with malaria in Angola failed to respond to artesunate/clindamycin which was administered intravenously, followed by an oral ACT after

returning to Vietnam (Hong *et al.*, 2014). This type of incident makes it clear that emergence of resistance in sub-Saharan Africa is not unlikely and as a result, drug sensitivity profiles should be monitored. *In-vitro* sensitivity assays may play an important role in the control of drug resistance and an advantage to this method is that it prevents the interaction of host-related factors, thus, providing an objective insight into the intrinsic drug sensitivity of malaria parasites.

For a parasite to be considered resistant, slow parasite clearance time *in vivo* has to have *in vitro* correlates. For years, consistent and significant correlations between half-lives and readouts from any *in vitro* artemisinin susceptibility assay had not been demonstrated (Noedl *et al.*, 2008; Dondorp *et al.*, 2009; Amaratunga *et al.*, 2012).

A potential explanation for this observation was that parasites were exposed to very low concentrations of dihydroartemisinin (DHA, the active metabolite of all artemisinins *in-vivo*) for 48-72hr, while *in-vivo* parasites were exposed to much higher concentrations of DHA for only 1-2hr. It has been reported that artemisinin resistance in drug selected *P. falciparum* lines is associated with decreased susceptibility of ring-stage parasites (Witkowski *et al.*, 2010; Klonis *et al.*, 2013). As a result, a more recent study done in 2013 by a group of researchers demonstrated significant correlations between half-lives and readouts from an *in vitro* artemisinin susceptibility assay through a novel phenotypic assay known as the RSA<sup>0-3h</sup> (Witkowski *et al.*, 2013).

In this assay, long parasite clearance half-life (the time it takes for the peripheral blood parasite density to decrease by 50%) following treatment with ART monotherapy or an ACT, correlated with *in-vitro* studies. The *in vitro* studies showed that the percentage of early ring stage parasites during 0-3hr post invasion of human erythrocytes survive when exposed to a pharmacologically relevant exposure to dihydro-artemisinin (Witkowski *et al.*, 2013). Although the assay successfully discriminates between a DHA resistant and DHA sensitive parasites, it is laborious, expensive and time consuming. The standard EC<sub>50</sub> assays that have been used for decades (48hr assay) are not sensitive enough to discriminate between differences in EC<sub>50</sub> values that may exist between different isolates (Van Schalkwyk *et al.*, 2013, Witkowski *et al.*, 2013). Moreso, the most well studied parasites strains used in *in-vitro* assays have been in use for more than two decades and so were isolated years before the widespread artemisinin-based combination therapy era and the majority of these used strains

have originated from the Americas and Asia but Africa is where the greatest transmission intensity occurs and where the majority of deaths from malaria are reported (WHO, 2011).

As a result, there is an urgent need to develop an alternative assay that is less laborious, cheaper and developed specifically for African parasites which face the current ACT drug pressure. The standard 48-hour assay was designed for a longer acting drug, chloroquine which has a different pharmacological profile from ACTs. Artemisinin have a shorter half-life and recent evidence of reduced sensitivity to artemisinin *in-vivo* suggests that parasites have developed a mechanism that allows dormancy in the ring stage of their life cycle in order to evade the toxic effects of the short half-life artemisinin derivative (Teuscher *et al.*, 2010; Witkowski *et al.*, 2013). This has given rise to an altered phenotype which needs to be studied (Witkowski *et al.*, 2013).

This chapter aims to validate in vitro assays on parasite lines isolated since the use of ACT was widespread in Africa. The work in this chapter is to improve in vitro methodology using isolates from Ghana, Nigeria and Kenya.

The specific aim of this chapter is

1. To determine in vitro responses of African field isolates that have the current ACT exposure and are culture adapted.
2. To identify a scalable assay able to discriminate between parasites lines with small differences in artemisinin sensitivity.
3. To identify phenotypes relevant to the genetic exploration of artemisinin resistance in African settings.



## **4.2 Sample information of parasite lines used in this study**

The isolates used in this study were obtained from patients who presented with malaria at the Hospital for Tropical Diseases, London (HTD), or the accident and emergency department of the University College London Hospitals (UCLH). A 4mL vacutainer tube containing EDTA (BD) was used to obtain venous blood sample, some of which was used for laboratory adaptation of the malaria parasites in the malaria culture laboratory at the London School of Hygiene and Tropical Medicine. Ethical approval was obtained from the Research Ethics Committee of the University College London Hospitals (Application number: 07/Q0505/60).

### **4.2.1 Sample information for parasite lines**

#### **HL1204**

This isolate was collected from a Kenyan male who is resident in the UK and had spent three weeks in coastal Kenya and Nairobi. Intravenous artesunate was given to the patient with a full course of oral artemether-lumefantrine after being discharged and at ~72 hours on (Schalkwyk et al, 2013).

#### **HL1402**

This isolate was collected from an adult male patient who resided in Kenya. He presented with malaria at the Hospital for Tropical Diseases, while on a work-related trip to the UK.

#### **HL1210**

This isolate was collected from an Indian-born young adult male who studied in the UK but was resident in Singapore. The subject travelled to Ghana for two weeks and had a course of chloroquine-proguanil as anti-malarial chemoprophylaxis from a high street pharmacy in the UK before departure. A prescription (Chloroquine-proguanil), which is not available in the

UK to purchase, is no longer considered appropriate chemoprophylaxis for sub-Saharan Africa. After diagnosis at HTD, the patient was admitted to UCLH and prescribed oral artemether-lumefantrine (Schalkwyk *et al.*, 2013).

## HL1212

This isolate was collected from an adult UK resident female who recently travelled to Nigeria. A written permission was given by the parent's immediate family for any viable malaria parasites to be placed in culture for research (Schalkwyk *et al.*, 2013).

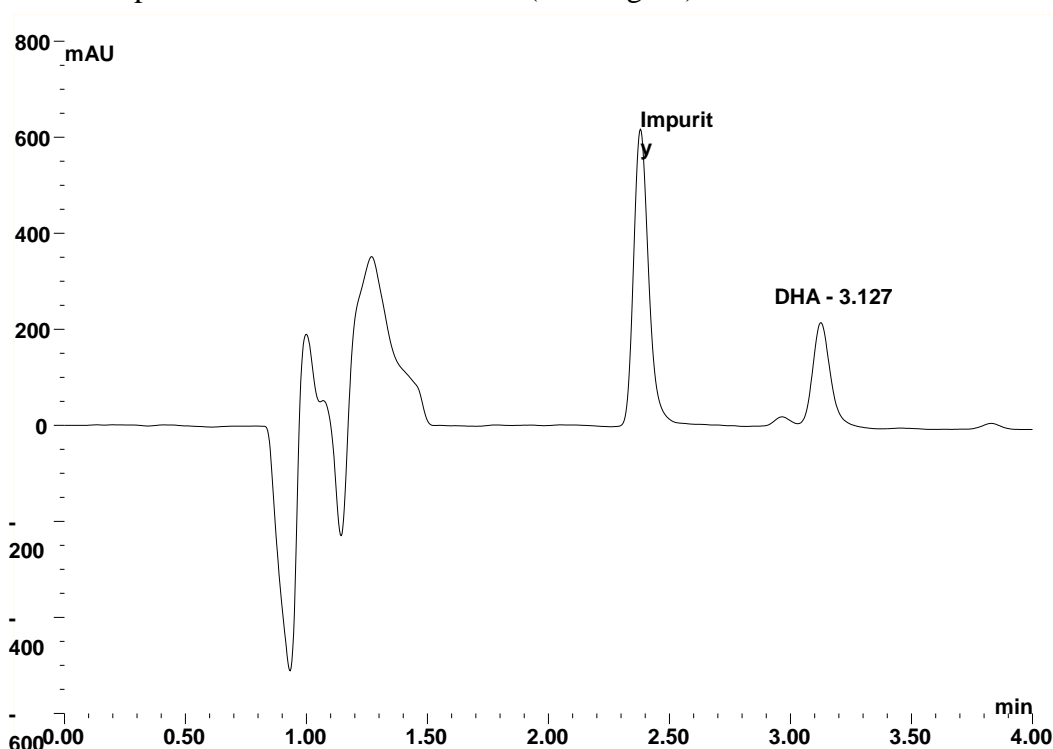
**Table 4.1 List of *Plasmodium falciparum* parasite lines, origin, characteristics and year reported:** DHA sensitivity data using a standard 48 hour assay on parasite lines HL1204, HL1210 and HL1212 were reported in a study done by Schalkwyk *et al.*; drug sensitivity information on HL1402 is being published for the first time in this study.

Parasite Line	Origin	Region	Characteristics	Year Isolated	Reference
<b>3D7</b>	Netherlands	Europe	Lab adapted line	1985	Walliker <i>et al.</i> , 1987
<b>HL1204</b>	Kenya	Africa	Field isolate, culture adapted	2012	Schalkwyk <i>et al.</i> , 2013
<b>HL1210</b>	Ghana	Africa	Field isolate, culture adapted	2012	Schalkwyk <i>et al.</i> , 2013
<b>HL1212</b>	Nigeria	Africa	Field isolate, culture adapted	2012	Schalkwyk <i>et al.</i> , 2013
<b>HL1402</b>	Kenya	Africa	Field isolate, culture	2014	unpublished

### 4.3 Quality assurance of a solution of dihydroartemisinin in dimethyl sulfoxide

To verify that freeze-thaw of DHA aliquots did not change drug concentration, a 100ul aliquot of frozen stock drug in DMSO was tested to confirm it contained 2.84 mg/ml of the active pharmaceutical ingredient DHA. This was done to ensure that the DHA drug concentration remained the same when freeze-thawed each time for new sets of experiment.

HPLC separation of a solution of DHA (2.84 mg/ml) in DMSO

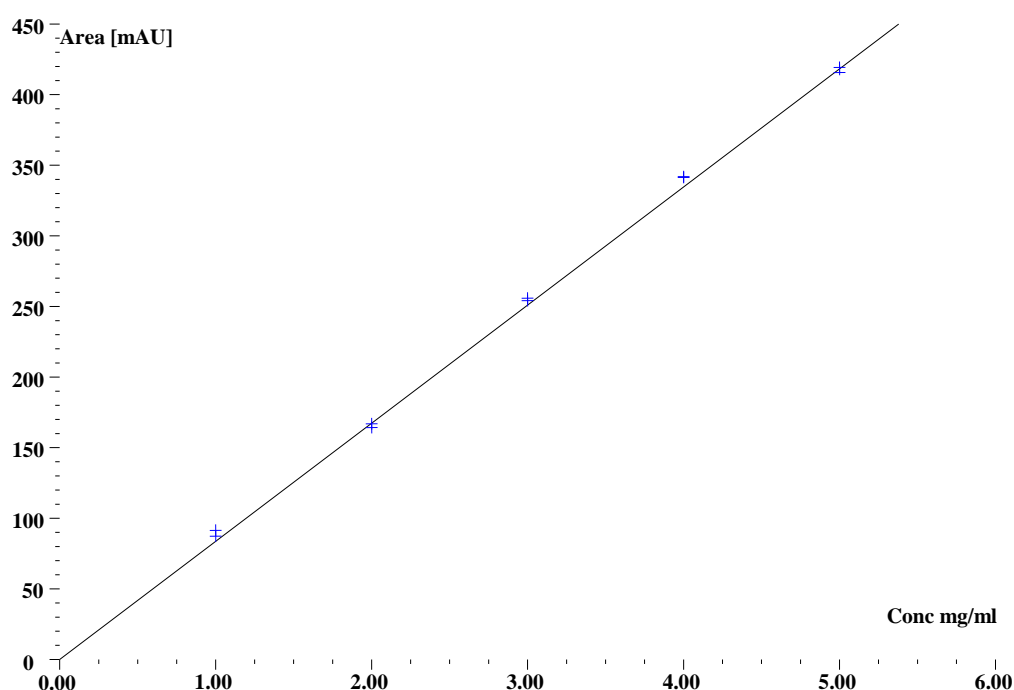


**Figure 4.1:** HPLC separation curve showing the concentration of the analysed sample. The solution had an initial DHA concentration of 2.84mg/ml in DMSO. This sample was tested in the pharmacology lab of the London School of Hygiene and Tropical Medicine and by Dr Harpakash Kaur.

The test solution was subjected to high performance liquid chromatography (HPLC) analysis and confirmed to contain 2.75 mg/ml DHA. The solution contained the DHA in the acceptable range. Prior to analysis, the solution was sitting on the bench for a day because the machine had technical issues. As a result, the measured amount was not exactly 2.85 mg/ml but is within the acceptable 10% range by HPLC.

This result is very useful as DHA aliquots were thawed each time an experiment had to be carried out. Also, DHA aliquots in eppendorf tubes sometimes are placed in the level 3 safety cabinet for at least an hour before being used for an experiment. This result shows that in the event that this has happened, the DHA drug concentration did not deviate from the acceptable HPLC range.

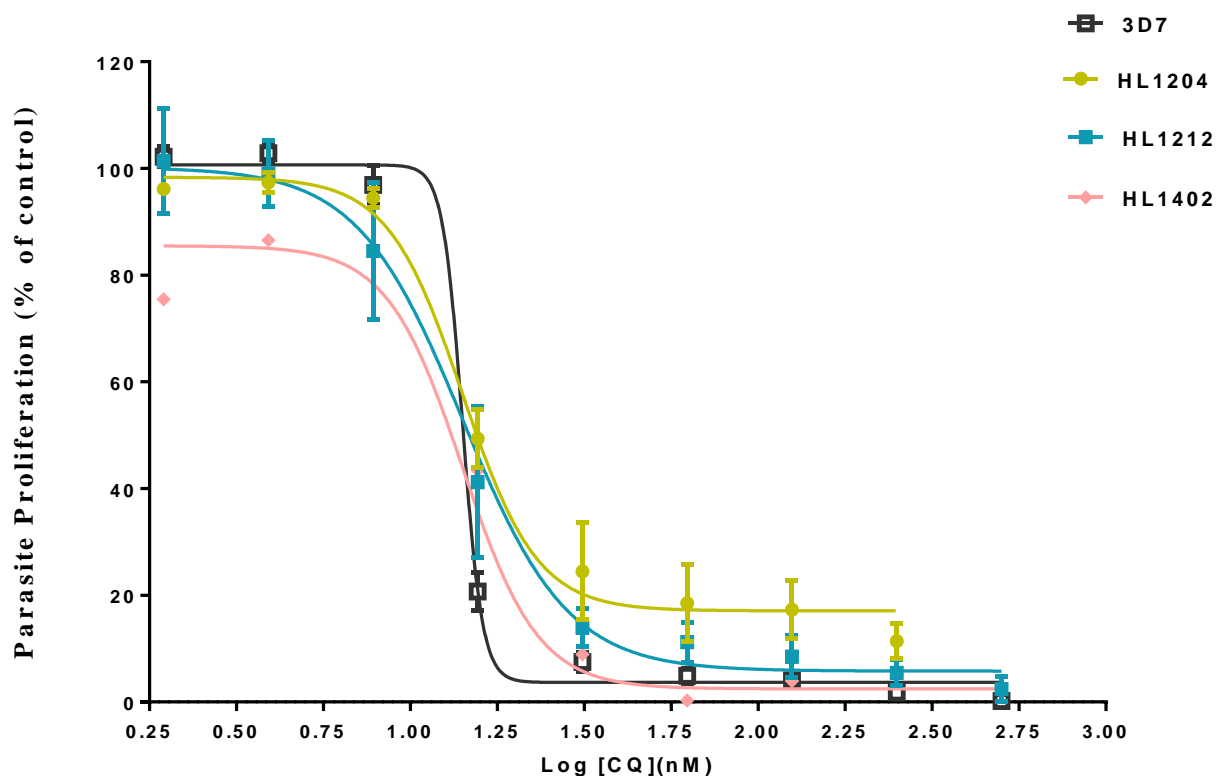
Calibration curve of solutions of DHA [0.0 -5.0 mg/ml] used to determine the concentration of the submitted solution.



**Figure 4.2:** HPLC calibration curve showing the concentration of the analysed sample. This sample was tested in the pharmacology lab of the London School of Hygiene and Tropical Medicine and by Dr Harpakash Kaur.

#### 4.4 Standard 48-hour ( $EC_{50}^{48}$ ) sensitivity to chloroquine *in-vitro*

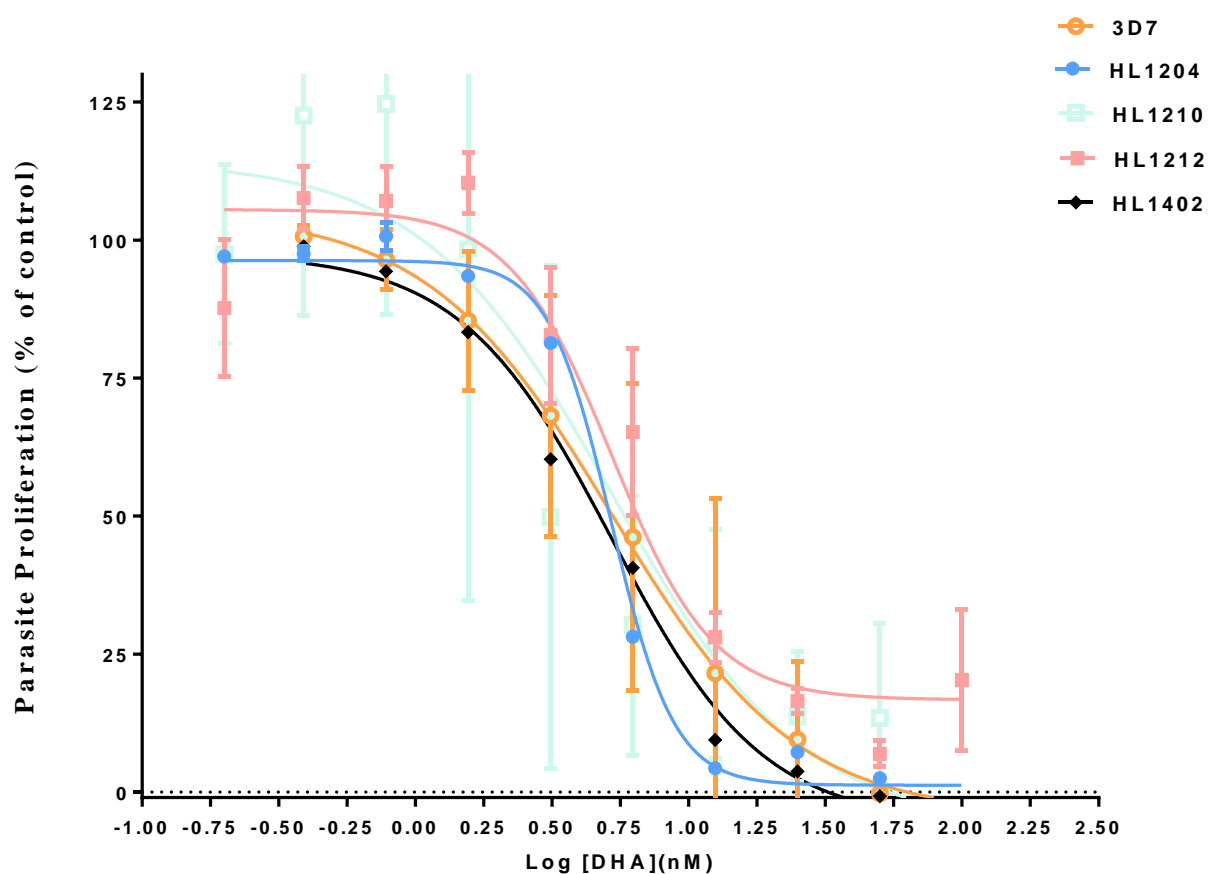
To determine the *in-vitro* responses of all *P. falciparum* lines used in this study, the chloroquine sensitivity of all lines had to be established using the standard 48-hour assay as described in section 3.4. The dose response curve shows chloroquine drug sensitivity assay for isolates HL1204, HL1212, HL1402, HL1210 and 3D7. Isolates with starting parasitaemia of 0.5% exposed to 1000nM of chloroquine showed a mean  $EC_{50}^{48}$  value of 12.9nM, 10.0nM, 15.7nM, 238nM and 14.5nM respectively. All the isolates except HL1210 were sensitive to chloroquine and showed no significant difference in  $EC_{50}$  values for chloroquine drug sensitivity. The HL1210 isolate was chloroquine resistant.



**Figure 4.3** A standard 48-hour drug response curve for African isolates: Chloroquine drug sensitivity assays for parasite lines 3D7, HL1204 and HL1212. The standard 48 hour assay does not successfully discriminate between parasite lines as indicated by the  $EC_{50}$  values ( $EC_{50}^{48}$ ) for both drugs.

#### 4.5 Standard 48-hour ( $EC_{50}^{48}$ ) sensitivity to dihydro-artemisinin *in-vitro*

In order to establish the DHA sensitivity of all *P.falciparum* lines used in this study, the *in vitro* response of all lines to DHA were determined using the standard 48-hour assay as described in section 3.4. Sensitivity to DHA *in vitro* for African isolates HL1204, HL1212, HL1402, HL1210 were analysed with 3D7 as control. Isolates with starting parasitaemia of 0.5% exposed to 100nM of DHA showed a mean  $EC_{50}^{48}$  value of 4.7nM, 6.7nM, 6.8nM, 2.8nM and 4.0nM respectively. All the isolates were sensitive to DHA and showed no significant difference in  $EC_{50}$  values for drug sensitivity.

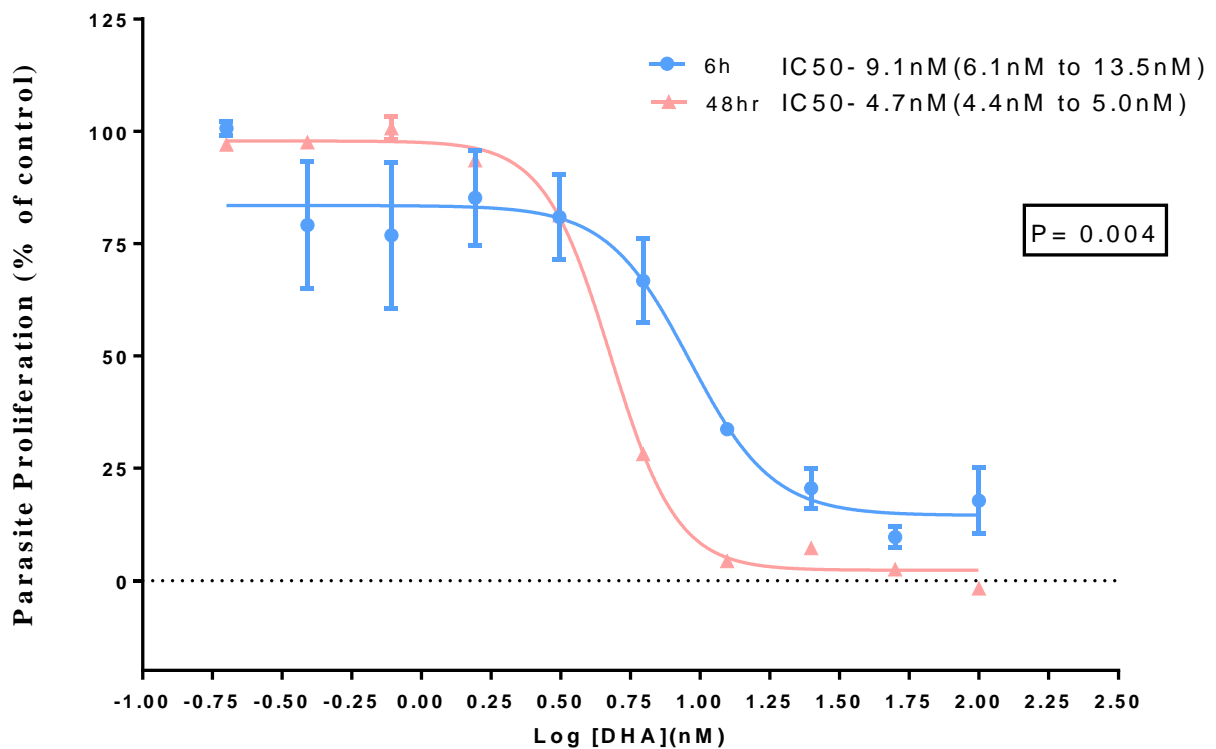


**Figure 4.4** Comparison of standard 48-hour drug response curve for African isolates: DHA drug sensitivity assays for parasite lines HL1204 and HL1402, HL1210 and HL1212 with 3D7 as a control lab adapted line. The standard 48 hour assay does not successfully discriminate between parasite lines as indicated by the  $EC_{50}^{48}$  values ( $EC_{50}^{48}$ ) for DHA.

#### 4.6 Sensitivity to dihydro-artemisinin drugs *in-vitro* for African isolates

The standard 48-hour assay could not successfully discriminate between the DHA  $EC_{50}$  values of the 4 African isolates (Fig 4.4). In order to improve ability to discriminate, a novel 6-hour pulse assay, where synchronised isolates (at 0.5% parasitaemia) were exposed to a 6hr DHA pulse at varying drug concentrations was designed (see Chapter 3.5). DHA drug exposure at the ring stage of the parasites life cycle was carried out and a dose response curve of DHA drug concentration and percentage parasite viability was plotted for all isolates.

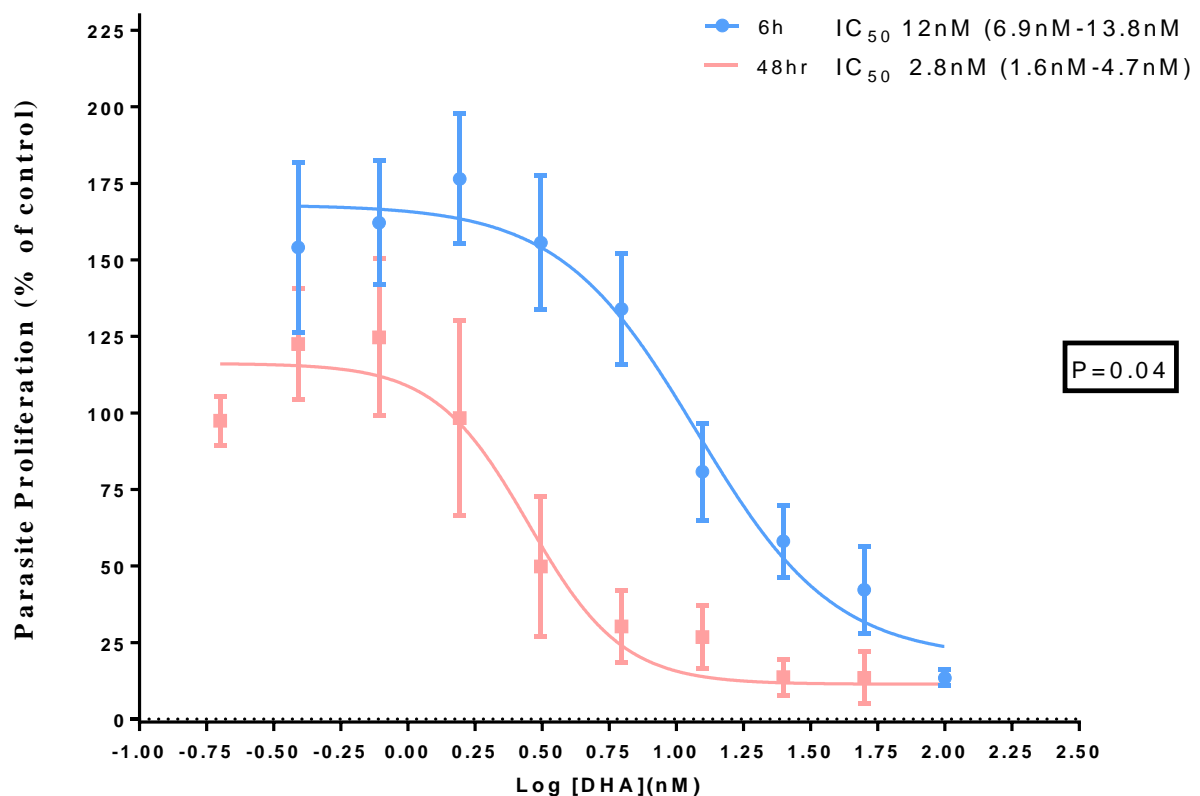
##### 4.6.1 Sensitivity to dihydro-artemisinin drugs *in vitro* for HL1204 isolate from Kenya



**Figure 4.5** 6hr pulse and 48hr exposure of HL1204 isolate to dihydro- artemisinin *in-vitro*: Mean DHA ( $EC_{50}^{6hr}$ ) drug sensitivity assay for parasite line HL1204 shows a higher value when compared to the standard ( $EC_{50}^{48hr}$ ) assay for DHA. Experiments were carried out in quadruplets and repeated 4 times.

The mean  $EC_{50}$  value for the parasite line HL1204 using the standard 48-hour assay was 4.5nM while the mean  $EC_{50}$  using the 6-hour pulse assay was 9.1nM. This is shown by a shift to the right in  $EC_{50}$  values of the dose response curve. The DHA ( $EC_{50}^{6hr}$ ) assay was able to successfully show differences in  $EC_{50}$  values that exist for DHA ( $\Delta EC_{50} = 4.4nM$ ) using the same isolate HL1204, with a P value = 0.004.

#### 4.6.2 Sensitivity to dihydro-artemisinin drugs *in vitro* for HL1210 isolate from Ghana

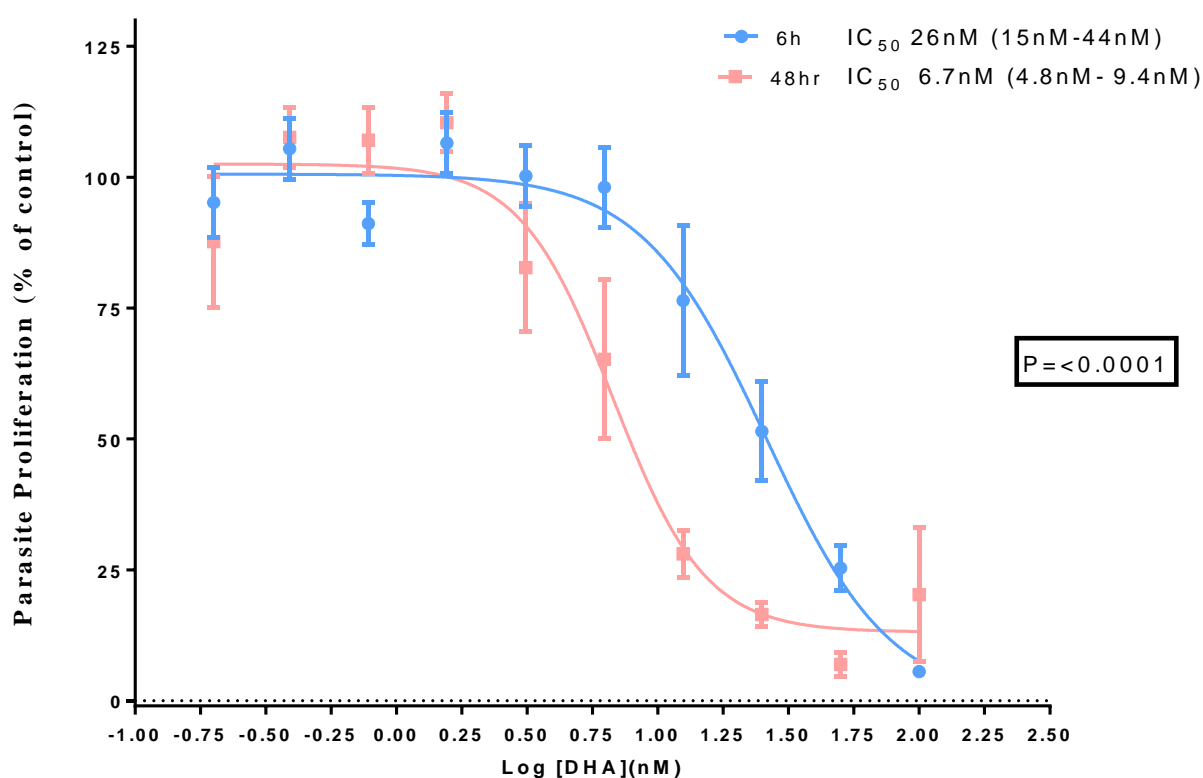


**Figure 4.6 Sensitivity of HL1210 isolate to DHA *in vitro* with a 6 hr pulse and a 48 hr assay:** DHA ( $EC_{50}^{6hr}$ ) drug sensitivity assay for parasite line HL1204 shows a higher value when compared to the standard ( $EC_{50}^{48hr}$ ) assay for DHA with an  $EC_{50}$  of 12nM for the pulse assay and 2.8nM for the standard assay. The DHA ( $EC_{50}^{6hr}$ ) assay was able to successfully show differences in  $EC_{50}$  values for DHA with a P value = 0.04 (Two tailed t-test).



The parasite isolate from Ghana, HL1210, had a mean  $EC_{50}$  value of 2.8nM for the standard 48-hour assay and a mean  $EC_{50}$  value of 12nM for the 6-hour pulse assay. Again, the same trend of a shift to the right in the dose response curve was observed. The pulse assay was able to successfully show differences in  $EC_{50}$  values that exist for DHA ( $\Delta EC_{50} = 9.2nM$ ) using the same isolate HL1210, with a P value of 0.04 (two tailed t-test) which was statistically significant.

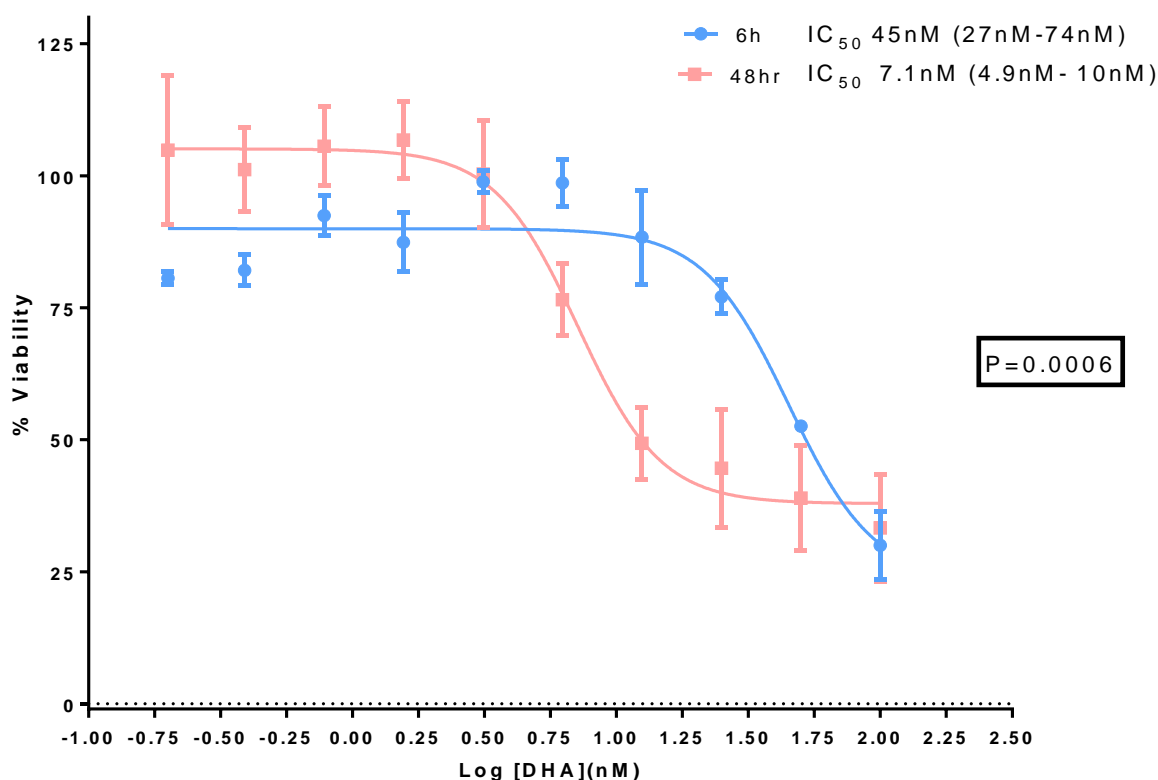
#### 4.6.3 Sensitivity to dihydro-artemisinin drugs *in vitro* for HL1212 isolate from Nigeria



**Figure 4.7** 6 hr pulse and 48 hr exposure of HL1212 isolate to dihydro-artemisinin *in vitro*: DHA ( $EC_{50}^{6hr}$ ) drug sensitivity assay for parasite line HL1212 shows a shift to the right when compared to the standard ( $EC_{50}^{48hr}$ ) assay for DHA with an  $EC_{50}$  of 26nM for the pulse assay and 6.7nM for the standard assay. The DHA ( $EC_{50}^{6hr}$ ) assay was able to successfully show differences in  $EC_{50}$  values for DHA with a P value = 0.0001 (two tailed t-test).

For isolate HL1212 from Nigeria, the mean  $EC_{50}$  value for the parasite line using the standard 48-hour assay was 6.7nM while the mean  $EC_{50}$  value using the 6-hour pulse assay was 26nM. This is shown by a shift to the right in  $EC_{50}$  values of the dose response curve, similar to other isolates. The DHA ( $EC_{50}^{6hr}$ ) assay was able to successfully show differences in  $EC_{50}$  values that exist for DHA using the same isolate HL1212. The difference in mean  $EC_{50}$  values between both assay ( $\Delta EC_{50} = 19.3nM$ ) was statistically significant with a P value of  $<0.0001$ .

#### 4.6.4 Sensitivity to dihydro-artemisinin drugs *in vitro* for HL1402 isolate from Kenya



**Figure 4.8 Sensitivity of HL1402 isolate to DHA *in vitro* with a 6 hour pulse and a 48 hour assay:** DHA ( $EC_{50}^{6hr}$ ) drug sensitivity assay shows a shift to the right when compared to the standard ( $EC_{50}^{48hr}$ ) assay for DHA with an  $EC_{50}$  of 45nM for the pulse assay and 7.1nM for the standard assay. The DHA ( $EC_{50}^{6hr}$ ) assay was able to successfully show differences in  $EC_{50}$  values for DHA with a P value = 0.0006 (Two tailed T-test).

The HL1402 isolate had a mean  $EC_{50}$  value of 7.1nM using the standard 48-hour assays while the mean  $EC_{50}$  value using the 6-hour pulse assay was 4.9nM. This is shown by a shift to the right in  $EC_{50}$  values of the dose response curve, similar to other isolates. The DHA ( $EC_{50}^{6hr}$ ) assay was able to successfully show differences in  $EC_{50}$  values that exist for DHA using the same isolate. The difference in mean  $EC_{50}$  values between both assay ( $\Delta EC_{50} = 37.9nM$ ) was statistically significant with a P value of <0.0006. It is worth noting that the difference between HL1402 and HL1204 (both from Kenya) is that they both have different genotypes. HL1402 comprises of novel mutations (see Chapter 6). The former was collected in 2014 while the latter was collected in 2012.

#### 4.7 Multiplicity of Infection in clinical Isolates

The numbers of parasite types present in the isolates, which are uncloned, were estimated by nested PCR amplification of the *P. falciparum* merozoite surface protein 1 (*Pfmsp1*) and 2 (*Pfmsp2*). Clonal multiplicity for isolates HL1204, HL1210 and HL1212 which were successfully adapted to culture were estimated in a previous study (Schalkwyk et al, 2013). Before experiments were carried out for this project, *Pfmsp1* and *Pfmsp2* genotyping was repeated to confirm the population of clones present in each isolate. This experiment was repeated after a few weeks of the parasites being in culture in order to examine if the population of clones in each isolate had changed with time. For isolate HL1402, PCR amplification of the *P. falciparum* *msp1* and *msp2* were estimated for the first time (Table 4.2).

**TABLE 4.2: *Pfmsp1* and *Pfmsp2* genotype of isolate HL1204. HL1210 and HL1212** (Schalkwyk et al, 2013)

Isolate	Sample	pfmsp1			pfmsp2		Minimum number of clones*	
		K1	MAD20	R033	FC27	IC	Per sample	Per isolate
HL1204 Kenya	Day 0 pre-treatment	0	1	2	0	1	3	4
	Day 1 post-treatment	0	2	2	0	1	4	
	culture-adapted	0	1	1	0	1	2	
HL1210 Ghana	Day 0 pre-treatment	2	2	0	0	1	4	4
	Day 1 post-treatment	2	2	0	0	1	4	
	culture-adapted	2	2	0	0	1	4	
HL1212 Nigeria	Day 0 pre-treatment	3	2	1	2	1	6	6
	culture-adapted	2	1	0	3	1	4	

In the previous study by Schalkwyk et al, HL1204, HL1210 and HL1212 were estimated to have 4, 4 and 6 clones respectively. When this experiment was repeated, HL1204 comprised of 2 clones present while HL1212 comprised of 3 clones. Further *pfmsp1* and *pfmsp2* genotyping was carried out after parasites had been in continuous culture for about 3 weeks and the results show that HL1204 clonal population reduced to one clone while HL1212 comprised of two clones. HL1402 isolate comprised of one clone after culture adaptation.

It is worth noting that drug sensitivity assays were carried out on each isolate a few days after being freeze thawed. This was done each time for every experiment because the results show that the relative abundance changes in continuous culture and it was important to keep the experiment conditions as consistent as possible, and to represent the complexity of natural infections.

**Table 4.3 *Pfmsp1* and *Pfmsp2* genotype of isolate HL1204, HL1210 and HL1212:** The clone population present are shown after parasites had been freeze thawed and were adapted to culture. A no treatment control and 3D7 were used as controls.

Isolate	Pfmsp1			Pfmsp2		Interpretation
	K1	Mad20	R033	IC (3D7)	FC	
3D7 1:50 dilution	1	-	-	-	-	positive control
HL1402_K	1	-	-	-	1	1 clone
HL1204_K	-	-	1	1	-	2 clones
HL1212_N	1	-	-	1	2	3 clones

**Table 4.4: TABLE 4.3 *Pfmsp1* and *Pfmsp2* genotype of isolate HL1204, HL1210 and HL1212:** The clone population present are shown after parasites had been freeze thawed and were adapted to culture. The parasites had been in culture for about 3 weeks. A no treatment control and 3D7 were used as controls. The table shows the drop in clone population with increased culture time.(K =Kenya, N= Nigeria).

Isolate	<i>Pfmsp1</i>			<i>Pfmsp2</i>		Interpretation
	K1	Mad20	R033	IC (3D7)	FC	
3D7 1:50 dilution	1	-	-	-	-	positive control
HL1402_K	1	-	-	-	1	1 clone
HL1204_K	-	-	-	1	-	1 clones
HL1212_N	1	-	-	-	1	2 clones

**Table 4.5 Chloroquine and DHA sensitivity assay:** A standard 48 hour assay was carried out using chloroquine and DHA drugs. Assays were carried out using parasite lines 3D7, HL1204, HL1210, HL1212 and HL1402. Data shows the number of experiments carried out and the number of replicates per experiment with corresponding EC<sub>50</sub> values.

PARASITE	CQ Assay : EC <sub>50</sub> <sup>48h</sup>			DHA Assay 1: EC <sub>50</sub> <sup>48h</sup>		
	# Experiments	# replicates per experiment	mean EC <sub>50</sub> (SD)	# Experiments	# replicates per experiment	mean EC <sub>50</sub> (SD)
3D7	6	8	14.5 ± 3.2	4	4	4.0 ± 0.6
HL1204_K	3	8	12.9 ± 1.1	4	4	4.7 ± 0.2
HL1212_N	4	8	10 ± 4.5	6	4	6.7 ± 3.9
HL1210_G	3	8	238 ± 26.3	4	4	2.8 ± 1.4
HL1402_K	3	8	15.7 ± 0.3	3	4	6.1 ± 1.0

The abbreviations for isolates are K, G, N for Kenya, Ghana and Nigeria respectively. The EC<sub>50</sub> values are averaged for at least three independent experiments with some repeated with up to six occasions. All data are represented as mean ± S.D

**Table 4.6 DHA standard 48hr and 6hr pulse assay:** The standard 48 hour assay was carried out using DHA. Data shows mean EC50 values for the 6 hour pulse assay and the standard 48 hour assay with corresponding fold difference and results for statistical significance

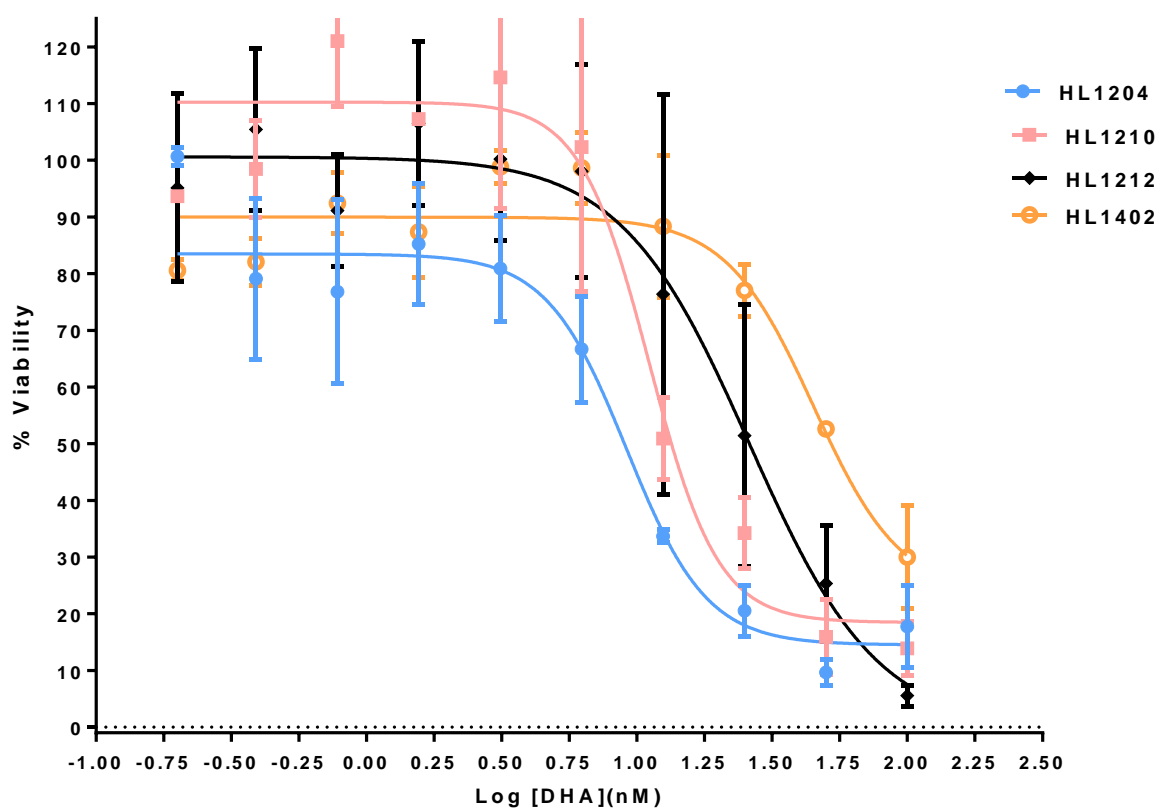
PARASITE		DHA_Assay 2: EC <sub>50</sub> <sup>6h</sup>		DHA_Assay 1: EC <sub>50</sub> <sup>48h</sup>				
	# Experiments	# Replicates	mean EC50 (SD)	mean EC50 (SD)	# Experiments	# Replicates	fold difference	P value (Ttest)
3D7	3	4	16.9 ± 4.0	4.0 ± 0.6	4	4	4.2	0.13
HL1204_K	3	4	9.1 ± 0.8	4.7 ± 0.2	4	4	1.9	0.004
HL1212_N	3	4	26 ± 13.0	6.7 ± 3.9	6	4	3.8	<0.0001
HL1402_K	3	4	44.5 ± 5.0	6.1 ± 1.0	4	4	7.2	0.0006
HL1210_G	4	4	12 ± 0.8	2.8 ± 1.4	3	4	4.2	0.04

The abbreviations for isolates are K, G, N for Kenya, Ghana and Nigeria respectively. The EC50 values are averaged for at least three independent experiments with some repeated with up to six occasions. All data are represented as mean ± S.E.M. A two-tailed test was used to test significance at P >0.05



#### 4.8 DHA ( $EC_{50}^{6hr}$ ) sensitivity profiles for HL1204, HL1210 and HL1212 isolates

When all the data for the 6 hr pulse assays were superimposed, it showed that HL1212 isolate had the highest DHA  $EC_{50}$  value of 26nM. This was followed by HL1210 from Ghana (12nM) and lastly, HL1204 from Kenya (9.1nM) showed a higher sensitivity to dihydro-artemisinin.



**Figure 4.9 Comparison of DHA ( $EC_{50}^{6hr}$ ) drug sensitivity profiles of African isolates:** The graph shows the mean DHA ( $EC_{50}^{6hr}$ ) for isolates HL1204, HL1210 and HL1212 all superimposed. Isolate HL1204 has the lowest  $EC_{50}$  value while HL1212 has the highest  $EC_{50}^{6hr}$  value. These differences are statistically significant.

**Table 4.7 Mean Difference in DHA EC<sub>50</sub> (nM) between African isolates, by the standard 48hr assay and 6hr pulse assay:** Mean difference in DHA sensitivity of the 6 hour pulse assay and standard 48 hour assay. Comparisons were made between each African isolate and the P value of this difference is shown. (P < 0.05)

<b>Region</b>	<b>6hr Pulse assay: Mean difference nM (P value)</b>	<b>48hr Standard assay: Mean difference nM (P value)</b>
<b>3D7 vs. HL1204</b>	7.8 (0.14)	0.8 (0.12)
<b>3D7 vs. HL1210</b>	4.9 (0.01)	1.1 (0.20)
<b>HL1212 vs. 3D7</b>	9.1 (0.09)	2.8 (0.43)
<b>HL1402 vs. 3D7</b>	27.6 (0.07)	2.9 (0.43)
<b>HL1212 vs. HL1204</b>	16.9 (0.02)	2.0 (0.11)
<b>HL1402 vs. HL1212</b>	18.5 (0.02)	2.1 (0.78)

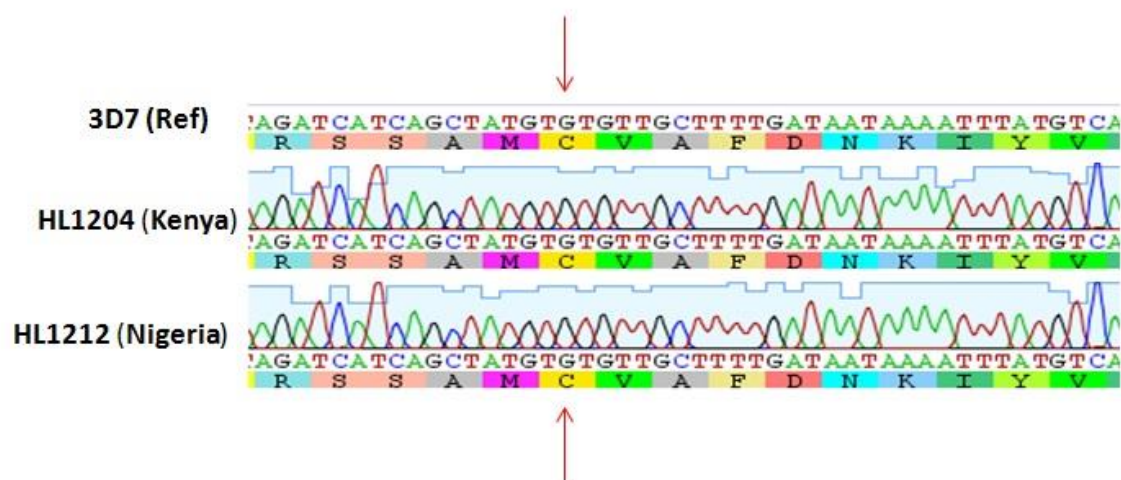
When all the isolates were compared with each other, it was clear that the DHA EC<sub>50</sub><sup>48hr</sup> assay was not successful in discriminating the differences in DHA sensitivity between parasites from the three regions of Africa. The mean differences were very little and had no statistical significance. However, when the isolates were compared using the DHA EC<sub>50</sub><sup>6hr</sup> assay, the mean difference between each comparison was significantly different at P >0.05 (Table 4.7).

DHA EC<sub>50</sub> figures were compared between isolates to ascertain if the assay could tease out small differences in DHA sensitivity that may exist. Isolate HL1212 and HL1402 showed a mean difference of 16.9nM, with a statistically significant difference and P value of 0.005 (appendix).

When HL1204 was compared to HL1402, the difference in DHA EC<sub>50</sub><sup>6hr</sup> was statistically significant (mean difference 35.4, P=0.04). It can be seen that the two isolates from Kenya are different.

#### 4.9 PfK13 Genotyping of HL1204 (Kenya) and HL1212 (Nigeria)

The isolates which showed the biggest differences in DHA  $EC_{50}^{6hr}$  were genotyped in the *P. falciparum* propeller region (*Pfk13*) to investigate if this region had any described mutations associated with artemisinin resistance. The sequence was compared to a lab adapted control isolate, 3D7.



**Figure 4.10 *Pfk13* Genotyping of artemisinin resistant locus:** HL1204 and HL1212 isolates are wild-type at position C580.

Results show that both parasites do not have the C580Y mutation associated with artemisinin resistance. Other positions were also investigated in this study but both isolates were wild type at all positions associated with artemisinin resistance.

#### 4.10: Susceptibility of H1204 and HL1212 to Artemisinin partner drugs

To investigate the susceptibility of *Plasmodium falciparum* isolates, HL1204 and HL1212 to partner drugs in vitro, isolates were exposed to piperazine and lumefantrine for 48 hours. Failure to partner drugs like lumefantrine has been reported in Southeast Asia (Amaratunga *et al.*, 2016; Duru *et al.*, 2015; Leang *et al.*, 2015 Spring *et al.*, 2015), where artemisinin resistance has occurred. As a result, it is important to ascertain the susceptibility of these drugs in the African isolates.

**TABLE 4.8 Susceptibility of African isolates to partner drugs:** Standard 48-hour assay of partner drugs to HL1204 (Kenya) and HL1212 (Nigeria). The data shows EC<sub>50</sub> and EC<sub>90</sub> values with corresponding standard deviation figures. CQ- Chloroquine, DHA- Dihydroartemisinin, PIP- piperazine, LUM- lumefantrine.

	CQ (nM)		DHA(nM)		PIP (nM)		LUM (nM)	
	EC <sub>50</sub>	EC <sub>90</sub>	EC <sub>50</sub>	EC <sub>90</sub>	EC <sub>50</sub>	EC <sub>90</sub>	EC <sub>50</sub>	EC <sub>90</sub>
<b>3D7</b>	15 ± 2	23 ± 3	5.5 ± 1.1	13 ± 2	15 ± 1	21 ± 2	86 ± 27	224 ± 70
<b>HL1204</b>	14 ± 2	21 ± 2	2.7 ± 0.4	6.3 ± 1.1	26 ± 4	38 ± 6	115 ± 17	232 ± 38
<b>HL1212</b>	9.3 ± 0.6	15 ± 1	3.8 ± 0.9	9.7 ± 1.9	21 ± 4	34 ± 7	88 ± 22	213 ± 58

The data shows that for HL1204, piperazine EC<sub>50</sub> value is 26nM while that of HL1212 is 21nm. For lumefantrine, the EC<sub>50</sub> values are 115nM and 88nM respectively. Both HL1204 and HL1212 are sensitive to the partner drugs tested based on this data.

## 4.10 Discussion

This chapter aims to explore appropriate methodology to discriminate between parasite genotypes with small differences in artemisinin susceptibility. It aims to investigate which method best discriminates between DHA sensitive and tolerant isolates by using two assays, the standard 48-hour drug assay and a 6 hour pulse assay. The 6 hour pulse assay ensures antimalarial drugs are washed off the parasite after 6 hrs, to simulate short half-life in vivo conditions and to observe any differences that may exist in DHA EC<sub>50</sub> values.

The chloroquine standard assay on all isolates tested showed that all were sensitive to chloroquine apart from isolate HL1210 (Ghana). When comparisons were carried out to see if the sensitivity profiles of these isolates from three different African countries differed, no significant difference in mean EC<sub>50</sub> values were observed. Chloroquine mean EC<sub>50</sub><sup>48hr</sup> values were 12.9nM, 10.0nM, 15.7nM and 14.5nM for HL1204, HL1212, HL1402 and 3D7 respectively (Fig.4.3). These findings are consistent with another study which tested chloroquine sensitivity profiles of different African isolates. The study found the chloroquine EC<sub>50</sub><sup>48hr</sup> values of the same strains, HL1204 from Kenya and HL1212 from Nigeria for example, to be within the range of 13nM ± 2 and 12nM ± 3 and 3D7 being 15nM ± 2 (Schalkwyk et al, 2013).

The DHA standard 48-hour assay on all isolates tested showed that all were sensitive to DHA. The mean DHA EC<sub>50</sub><sup>48hr</sup> values for HL1204, HL1212, HL1402, HL1210 and 3D7 were 4.7nM, 6.1nM, 6.8nM, 2.8nM and 4.0nM respectively (Table 4.5). Again, these values are consistent with other published studies ((Schalkwyk et al, 2013). When comparisons were made between isolates to see if the sensitivity profiles differed, there was no significant difference in mean EC<sub>50</sub><sup>48hr</sup> values were observed (Table 4.3). This means that the standard DHA EC<sub>50</sub><sup>48hr</sup> assay could not successfully discriminate between the different African isolates, including the lab adapted strain 3D7. A study carried out by Witkowski et al, where culture-adapted parasites from 13 fast- and 13 slow-clearing infections collected in Pursat, Cambodia, in 2010 were exposed to DHA in a standard 48-hour assay showed similar findings. The isotope-based sensitivity assay (ISA) that monitored replication of parasites exposed to drug for 48 h, showed that the fast- and slow-clearing parasites did not differ significantly in EC<sub>50</sub> values for DHA (Witkowski et al, 2013).

A potential reason why the standard  $EC_{50}^{48hr}$  assay has failed to tease out the differences in DHA  $EC_{50}$  between different isolates is that parasites are exposed for 48 hr while parasites *in vivo* are exposed to concentrations of DHA for only 1-2 hr. Artemisinin resistance in *P. falciparum* has been associated with decreased susceptibility of ring-stage parasites (Saralamba *et al.*, 2011; Klonis *et al.*, 2013). As a result, the standard assay does not focus on this stage, rather it extends to the whole life cycle and so might miss out on the important stage of the parasite life cycle. Moreso, another study showed that a consistent decrease in sensitivity using standard *in-vitro* assays was not exhibited in parasites that were isolated from patients that showed delayed clearance *in-vivo* (Dondorp *et al.*, 2009). This means the standard assay is not sensitive enough to discriminate between different artemisinin sensitivity profiles. As a result, the 6 hr pulse assay was used to find a more sensitive method of assaying artemisinin and to validate *in vitro* assays on African parasite lines.

The effect of the 6 hr pulse format is a marked shift to the right in the dose response curve graph which was observed for all the isolates tested with both CQ and DHA. This simply reflects the fact that overall, the mean DHA  $EC_{50}$  values for the 6 hour pulse assays were higher than the mean  $EC_{50}$  values for the standard 48 hr assay. This result shows that there is a difference in  $EC_{50}$  values when both assay methods are compared. The results also show that this difference is larger in isolates HL1210 and HL1212 compared to HL1204. A two tailed T-test carried out showed that the differences in DHA  $EC_{50}$  for both assays are statistically significant (Table 4.6). For the laboratory clone 3D7, the  $EC_{50}$  value for DHA for the 6 hr assay was approximately 3-fold higher than that of the 48 hr assay.

The same trend of a shift to the right was observed in all isolates for the chloroquine drug sensitivity assay when the 6-hour pulse was compared to the standard 48-hour assay (appendix 1). The 48hr assay using chloroquine was also included as a control due to the fact that there is extensive wealth of knowledge regarding chloroquine  $EC_{50}$  value and drug action.

HL1210 from Ghana was chloroquine resistant hence the high mean  $EC_{50}^{48hr}$  value for chloroquine (238 nM  $\pm$  26) while HL1204, HL1212 and HL1402 were all sensitive to chloroquine. These findings are consistent with published studies which reported chloroquine resistance (150nM  $\pm$  7) in the same HL1210 strain and chloroquine sensitivity in the HL1204 and HL1212 strain. (Schalkwyk *et al.*, 2013). Isolates that are resistant to chloroquine have been found to be sensitive to artemisinin (Henriques *et al.*, 2013). A very early study done by

Brasco and Bras (1993) demonstrated this and showed that chloroquine-resistant isolates were significantly more susceptible to artemisinin with  $EC_{50}$  values of (7.67 nM), arteether ( $EC_{50}$  3.88 nM), artemether ( $EC_{50}$  3.71 nM), and artelinate ( $EC_{50}$  3.46 nM). In this study, the HL1210 chloroquine resistant isolate was found to be the most sensitive isolate to DHA ( $EC_{50}^{48hr}$  2.8nM $\pm$ 1.4).

The mean DHA  $EC_{50}$  and *in vivo* values for the standard 48 hr assays which ranged from 2.8nm -6.7nm for all isolates are not far from other known  $EC_{50}$  values in published studies which gives confidence in the data from this study. In a study that tested the *in vitro* susceptibility of ACTs in travellers returning with *Plasmodium*, mean  $EC_{50}$  for DHA was 3.62nM (Pillai *et al.*, 2012). Our laboratory reported a range of 1.7nM to 5.5nM among 7 isolates from travellers returning into the UK (Schalkwyk *et al.*, 2013).

When the African parasite lines were compared to each other (Table 4.3) using the standard 48-hour assay, there was no significant difference in DHA  $EC_{50}^{48h}$  values for all isolates. For example, comparison of HL1212 (Nigeria) and HL1402 (Kenya) were not significantly different (mean difference = 2.0nM,  $P = 0.15$ ) which means the standard assay was not successful at teasing DHA drug sensitivity differences apart. However, when the isolates were compared using the 6-hour pulse assay, the mean difference in  $EC_{50}$  value was statistically significant (mean difference = 16.9nM,  $P = 0.005$ ).

*In-vitro* studies have shown that artemisinin effects vary across the different intra-erythrocytic parasite stages, as mature trophozoites and schizonts are susceptible to artemisinin irrespective of *in-vivo* phenotype, in contrast to ring-stage, trophozoites (Kuile *et al.*, 1993; Witkowski *et al.*, 2010). Studies have shown however, that ring-stage parasites aged 0–3 hr possess differential ability to survive pulses of artemisinin *in vitro* among parasites that are genetically distinct (Witkowski *et al.*, 2013; Dogovski *et al.*, 2015). Klonis *et al.* (2013) showed that tightly synchronized parasites exposed to short drug pulses show large stage-dependent differences in their response to artemisinin and this correlates with haemoglobin digestion throughout most of the asexual cycle. As a result, ring-stage parasites can exhibit >100-fold lower sensitivity to short drug pulses than trophozoite stage.

The pulse assay designed in this study also focused on the tightly synchronized ring stage and the application of a 6 hour pulse was introduced to mimic *in vivo* conditions and as a result, higher estimates of  $EC_{50}$  to drug pulses were observed when compared to the standard assay which had the asexual stages that digest haemoglobin. Crucially, DHA  $EC_{50}^{6h}$  estimates

showed greater variability between isolates. The findings in this chapter are consistent with published work that show that laboratory strains show little differences in drug sensitivity in standard in vitro assays but when exposed to short drug pulses, exhibit substantial (>95-fold) difference in sensitivity (Klonis et al, 2013).

It is worth mentioning that pulse assays were carried out in the same 96 well plate as standard assays so parasites in both assays were from the same vial and were under the same conditions. Also, when 48 hr assays were done independently with all isolates, the Mean EC<sub>50</sub> values shown were in close range to EC<sub>50</sub> values of a standard 48 hr assay done in the same 96 well plate as the pulse assay.

Variations in EC<sub>50</sub> values were seen between each experiment in a single isolate. This may have been due to the polyclonal nature of isolates used in this study. The HL1212 isolate however had the most variation within experiments (6hr pulse and 48hr assay) compared to the other isolates and had a higher population of clones (6 clones). Another possible reason could be linked to a study carried out in Nigeria about a possible artemisinin-based combination therapy resistance (Ajayi et al, 2013).

Variations in DHA EC<sub>50</sub> values between each isolate could be explained by the different drug policies that exist in these countries. For example, Ghana and Nigeria which have a higher mean DHA EC<sub>50</sub> value in the pulse assay are neighbouring West African countries which have a different drug use history compared to Kenya which is in East Africa.

What all African countries however have in common, is that the use of Artemether-Lumefantrine (AL), has been the front-line anti-malarial treatment recommended by the WHO compared to the greater Mekong region, this treatment (ACT) still has high cure rates in Africa (Sawa *et al.*, 2013). There are efficacy studies from a few African countries such as Uganda (Yeka *et al.*, 2016), Nigeria (Ajayi *et al.*, 2013) and Angola (Kiako *et al.*, 2015; Plucinski *et al.*, 2015) that show a decrease in cure rates of patients given AL compared to baseline studies performed a decade ago when ACTs were first introduced. Moreso, treatment failure was documented in some case reports in non-immune travellers who got infected with malaria and were treated with AL in European hospitals (Farnet *et al.*, 2012; Repetto *et al.*, 2011). Another study carried out in Mbita, Kenya in 2009 found that 20.7% of AL-treated children showed recurrent parasitaemia within 42 days after commencement using a combination of PCR and microscopy end-points (Henriques *et al.*, 2014). In the study by Schalkwyk *et al.*, (2013), isolates HL1212 and HL1204 had lumefantrine EC<sub>50</sub> estimate of



67nM and 119nM while HL1210 was sensitive to lumefantrine with an estimate of 24nM. Sensitivity to lumefantrine was assessed for HL1402 in this study and the  $EC_{50}^{48}$  was found to be 50nM. To date, there is no validated  $EC_{50}$  cut off below which lumefantrine susceptibility is considered adequate. One possible explanation could be that there has been no correlation of *in-vitro* estimation of lumefantrine to *in-vivo* studies or treatment response.

Studies from Southeast Asia show that reduction in susceptibility to artemisinins is associated with single point mutations in the propeller region of the *P. falciparum* *pfk13* locus (Ashley *et al.*, 2014; Ariey *et al.*, 2014; Witkowski *et al.*, 2013). However, no point mutation was found in the propeller region of the K13 gene in African isolates in this study. Although another study has shown that reduction in AL is associated with carriage of alleles of a few resistance-associated genes (Henriques *et al.*, 2014), *Pfk13* was not included in the resistant genes (Muwanguzi *et al.*, 2016).

The 6 hr pulse assay can serve as a useful tool in assaying artemisinin resistance on the African continent and can be used to test the DHA susceptibility of artemisinin resistant parasite lines from Cambodia for validation.

## 4.11 Conclusion

In this chapter, a difference in DHA EC<sub>50</sub> values in all three field isolates tested for the 6 hr pulse assay compared to the standard 48 hr assay was found and the differences were statistically significant. The assay was able to distinguish differences in drug sensitivities that exist between parasites from different regions. HL1212 isolate from Nigeria had the highest EC<sub>50</sub><sup>6h</sup> value compared while the HL1204 isolate from Kenya had the lowest EC<sub>50</sub><sup>6h</sup> value. Further investigation into the *Pfk13* region of the propeller domain found no point mutation associated with artemisinin resistance.

Although a recent study found a correlation between *in-vitro* and *ex-vivo* assay (Witkowski *et al.*, 2013) and another study found a molecular marker for artemisinin resistance (Ariey *et al.*, 2013) both studies used isolates from Cambodia and so are not representative of the isolates found in the field in Africa where majority of deaths from malaria does occur. If a significant difference in DHA EC<sub>50</sub> values exists between the 6 hr pulse and the 48 hr assay within and between isolates, then we may be able to identify a new phenotype which could form the basis to which artemisinin resistance could be further explored.

The next chapter would involve including Cambodian isolates with known artemisinin resistance and see how they compare with the African isolates used in this chapter. This would involve using 700nM DHA exposure to mimic the high drug exposure that occur *in vivo* for more pharmacological relevance and to validate already published data on DHA sensitivity. This is done to compare assays in the African isolates and the Cambodian isolates in the hope to find a more sensitive approach of drug sensitivity testing.

### **6.1 Strengths of study**

- Parasite lines used in this study are representative of the current drug pressure experienced in the field in Africa
- There is no known study that has carried out a 6 hour pulse assay using field isolates from Africa and compared with the standard assay used in drug susceptibility tests.

### **6.2 Study limitation**

- There was no use of a known slow clearing parasite as control (e.g. from Cambodia) in this study so this caused limitations in direct comparison of DHA EC<sub>50</sub> values between isolates in this study and other studies.
- Parasites in this experiment were exposed to low concentration of DHA for both 6 hrs and 48hrs even though parasites in-vivo are exposed to much higher drug concentrations of DHA for only 1-2 hours.

**CHAPTER 5** *In-vitro* studies of Cambodian *Pfalciparum* isolates with known *Pfk13* C580Y/R359T polymorphisms: new approaches to the *in-vitro* phenotype

## 5.1 Background

In the previous chapter, the novel 6-hour pulse assay was successful at discriminating differences in DHA sensitivity that exists among 4 *P. falciparum* isolates from Africa. A limitation of the chapter was not including an isolate with known slow clearance as this would have been an artemisinin resistant control and perhaps, validated the study. Slow clearing isolates from Cambodia, were obtained from Rick Fairhurst and David Fidock and the DHA sensitivity of this isolate panel according to the novel 6hour pulse assay was ascertained.

Clinical efficacy studies designed to study artemisinin resistance showed that parasite clearance rates were twice as slow in Battambang, a western Cambodia province (Noedl *et al.*, 2010), Pursat (Amaratunga *et al.*, 2012) and Pailin (Dondorp *et al.*, 2009) compared to the Thai-Myanmar border, despite adequate drug levels. Although other studies indicated slow parasite clearance (Phyo *et al.*, 2012). It has since been reported that slow clearing infections are common across mainland Southeast Asia (Kway *et al.*, 2013; Ashley *et al.*, 2014).

Despite the deterioration of artemisinin clinical response *in vivo*, slow parasite clearance could not be demonstrated *in-vitro* using the standard 48-hour assay (50% growth inhibition in a standard 48 hr exposure). After the studies carried out by Dondorp *et al.*, (2013) showed that artemisinin resistance affected predominantly ring-stage parasites, Witkowski *et al.*, (2013) described a ring-stage survival assay which confirmed that ring stages of *P. falciparum* isolates from western Cambodia were highly resistant to DHA, independently of host variables (Witkowski *et al.*, 2013). This assay deploys a 700nM DHA exposure for 72 hours and an endpoint microscopic readout and has some flaws; therefore our 6 hr pulse assay may be a better method for phenotyping Cambodian parasites.

To ascertain a phenotype for DHA drug resistance, it is important to develop a sensitive *in-vitro* assay as the current one being used (standard 48hr) has not been successful in discriminating between differences in DHA IC<sub>50</sub> values in parasite lines (Chapter 4.5). The RSA has proved a very useful research tool but has three serious flaws that limit its

usefulness as already mentioned in chapter 4. The novel 6-hour pulse assay which was successful in discriminating small differences in DHA EC<sub>50</sub> that exists between parasite lines from Africa will be used to test the DHA susceptibility of KH2 and Cam 3.11 artemisinin resistant lines from Cambodia.

This chapter aims to validate a bespoke 6h pulse assay method deployed on artemisinin resistant Cambodian lines, with full dose-response capacity in 96-well format, using the fluorescent DNA reporter Pico-green to measure growth inhibition by DHA.

Towards this effort, we obtained a KH2 strain from Cambodia (Miotto et al, 2014) and added this to be tested along with our African lines from HTD which are samples that have origin in regions where artemisinin combination therapies (ACTs) are routinely used for treatment of malaria. We also obtained another artemisinin resistant line Cam 3.11 (Straimer et al, 2014), which was retrieved as a result of the unsuccessful separation of the contaminated KH2 artemisinin resistant strain. Further assay work was carried out using this isolate.

During lab experiments, the KH2 strain contaminated with another strain and so efforts were focused on trying to separate mixed *Plasmodium falciparum* cultures through phenotypic and genetic characterisation. This chapter also focuses on the work carried out to isolate the KH2 clone from a mixed culture.

## 5.2 Aims

The specific aims of this chapter are:

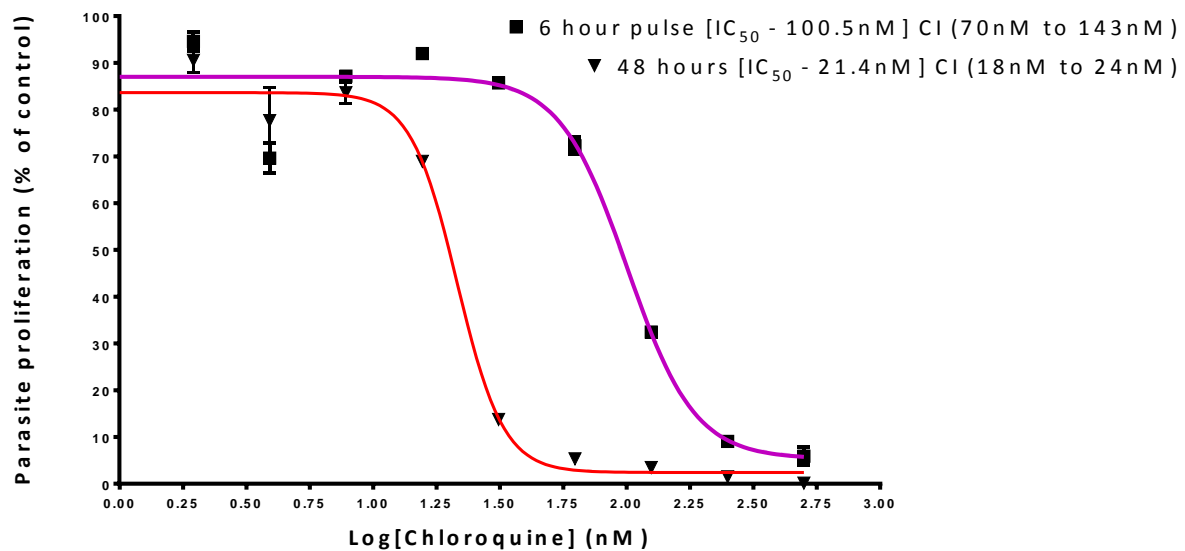
1. To genetically characterise all Cambodian lines.
2. To determine DHA in vitro responses of all Cambodian lines using the standard 48-hour assay and the novel 6hr pulse assay.
3. To compare DHA susceptibility data of the Cambodian lines to the HL1204, HL1210, HL1212 and HL1402 parasite lines to see if the 6-hour pulse assay can tease out any differences in DHA EC<sub>50</sub> values.
4. To identify a scalable assay to discriminate between the artemisinin resistant lines Cam 3.11<sup>C580Y</sup>, and Cam 3.11<sup>R539T</sup>, and sensitive line Cam 3.11<sup>WT</sup> (Straimer et al, 2014).

5. To test if the assay can discriminate between the isolates from Cambodia and the African lines in Chapter 4.
6. To identify phenotypes that could be relevant to the genetic exploration of artemisinin resistance in Cambodia and in Africa.

## 5.3 Results

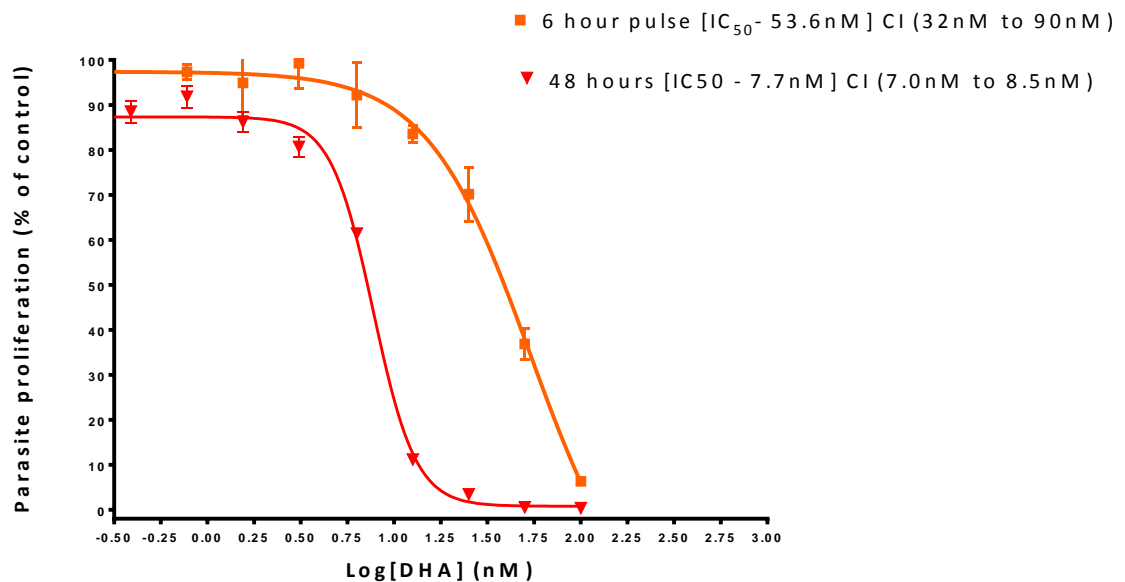
### 5.3.1 DHA and chloroquine sensitivity of KH2 *in-vitro*

To determine the *in-vitro* response of KH2 to DHA and chloroquine, parasites were exposed to drugs using the standard 48 hour assay and a 6 hour pulse assay as described in Chapter 3.5 and 3.6 with starting parasitaemia of 0.5%. The dose response curve shows the percentage viability and the subsequent EC<sub>50</sub> values for each drug exposure.



**Fig 5.1 Chloroquine EC<sub>50</sub><sup>6hr</sup> pulse assay and 48hr assay:** Graph showing EC<sub>50</sub> values of KH2 isolate after treatment with chloroquine in the 6 hour pulse assay and 48 hour assay.





**Fig 5.2 DHA EC<sub>50</sub><sup>6hr</sup> pulse assay and 48hr assay:** Graph showing EC<sub>50</sub> values of KH2 isolate with treatment with DHA in the 6 hour pulse assay and 48 hour assay.

When KH2 was exposed to chloroquine and DHA, there was an elevated EC<sub>50</sub> value in the dose response curve. This is the same trend that was observed in chapter 4 using the 6 hour pulse assay on all the African isolates. From the 48 hour assay result, it was noticed that the mean EC<sub>50</sub> value for chloroquine was 21.9nM. KH2 comes from a chloroquine resistant background (Imwong et al, 2010) and as a result, the EC<sub>50</sub> value was expected to be a lot higher. As a result, it was important to characterise the KH2 isolate genetically and to also characterise the phenotype before further experiments on drug sensitivity using the pulse assay was carried out.

## 5.4 *PfK13* Genotyping of isolate KH2

Since KH2 is supposed to be an artemisinin resistant isolate harbouring mutation in *PfK13*, genotyping of the kelch propeller region was carried out using methods described in chapter 3.7. DNA was extracted from parasites maintained in continuous culture, immediately after drug assay experiments were carried out. The positive control used was 3D7.

**Table 5.1: Genetic information of *Pfk13* region of KH2**

Isolate	Origin	Condition	<i>Pfk13</i>
3D7	Netherlands	Culture adapted	Wild-type
KH2* (Passage # 2)	Cambodia	Culture adapted	Wild-type

\*Since receipt at The London School of Hygiene and Tropical Medicine

The results showed that after KH2 was placed in continuous culture and drug assay experiments were carried out, the *Pfk13* mutation could not be detected. Further experiments were carried out to ascertain the genetic characteristics of the KH2 before further drug assays were carried out.

## 5.5 *Msp 2* Genotyping of KH2 clone after 700nm of DHA drug exposure

The KH2 isolate was adapted to culture after a freeze thaw. Genotyping experiments were carried out and it was confirmed that the KH2 culture also contained an additional parasite with a genotype similar to 3D7. Since the KH2 isolate is artemisinin resistant and contains a Kelch mutation, we decided to select for the KH2 isolate by exposing it to 700nm DHA for 72 hours. This was done in the hope that the high concentration of DHA will kill off any 3D7 in culture (artemisinin sensitive). After this, the surviving parasites were placed in culture and

then cloned through limiting dilution method (Chapter 3). DNA was extracted from successfully cloned parasites and msp genotyping was carried out.

**Table 5.2: Msp Genotyping of KH2 clones derived after limiting dilution method**

<b>samples loaded for msp2 reaction</b>	<b>Band seen in msp2 IC family (3D7)</b>	<b>Band seen in msp2 FC family</b>	<b>Interpretation</b>
<b>3D7</b>	1	0	positive control confirmed
<b>KH2 clone D4</b>	1	0	3D7
<b>kH2 clone H6</b>	1	0	3D7
<b>KH2 clone F12</b>	1	0	3D7
<b>KH2 clone A6</b>	0	0	3D7
<b>KH2 clone C2</b>	1	0	3D7
<b>KH2 clone C5</b>	1	0	3D7
<b>KH2 clone C8</b>	1	0	3D7
<b>KH2 clone A11</b>	1	0	3D7
<b>Culture DNA (cryo vial)</b>	1	1	Culture contains two clones

After the clonal dilution of KH2, 8 clones were derived. The letter and the number of each clone signify the well row and position on a 96 well plate.

The table shows that all the clones are 3D7 and not KH2. The culture DNA sample which was put into culture from a cryopreserved vial that was stored right after the initial KH2 clone was adapted to culture showed that two clones are present. One of the clones present was 3D7 and the other might have been KH2 but further experiments were done to confirm this. There was no signal for sample KH2 A6 and so an msp1 genotyping experiment was carried out to confirm this result. The msp1 result showed the sample was indistinguishable from 3D7.

## 5.6 Msp 2 Genotyping of cryopreserved KH2

Following the result of the sample which was put into culture from a cryopreserved vial (Table 5.2), DNA was directly extracted from a cryopreserved vial (was not put in culture) to confirm if the KH2 clone was present in the original sample stock and if it was already contaminated with an additional 3D7 like parasite. Extracted DNA sample was used to carry out further Msp 2 genotyping experiment as this gene alone can discriminate KH2 from our putative contaminant. The result is shown in table 5.3.

**Table 5.3: Msp 2 Genotyping of cryopreserved KH2**

samples loaded for msp2 reaction	Band seen in msp2 IC family (3D7)	Band seen in msp2 FC family	Interpretation
3D7	Yes	No	Expected 3D7 band seen
KH2 from cryo vial	Yes	Yes	Expected KH2 band seen + band indistinguishable from 3D7

The results confirm the presence of at least two clones, one of which is indistinguishable from 3D7 (IC family). It was likely that the other clone was the derived KH2 (FC family). Thus an alternative way to isolate a pure KH2 culture from this mixture was sought.

## 5.7 Chloroquine drug pressure of KH2

The DHA drug pressure was unsuccessful in selecting for the KH2 DHA resistant strain. Since the KH2 strain has a chloroquine resistant background, a chloroquine drug pressure was carried out in order to kill any chloroquine sensitive 3D7 present and at the same time, select for the chloroquine resistant KH2 strain.

**TABLE 5.4 *Pfmsp1* and *Pfmsp2* genotype of isolate KH2 before and after CQ drug pressure:** The clone population present are shown after parasites had been freeze thawed and were adapted to culture. A no treatment control and 3D7 were used as controls.

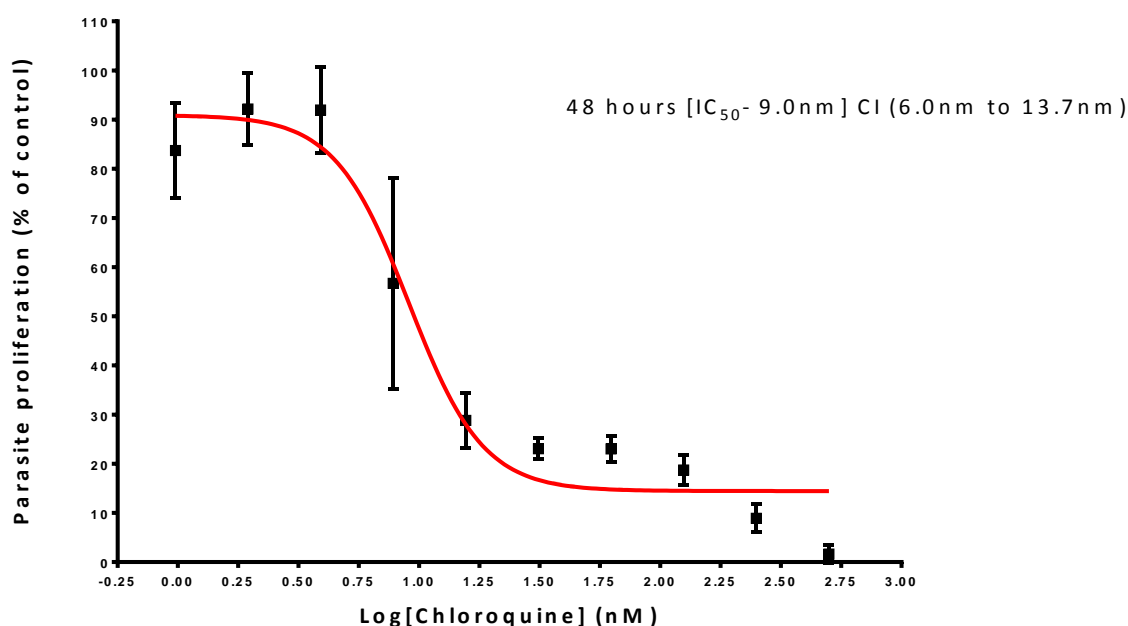
Isolate	K1	<i>Pfmsp1</i> Mad20	R033	<i>Pfmsp2</i> IC (3D7)	FC	Interpretation
3D7	1	-	-	-	-	positive control
KH2 from cryo vial	1	1	-	1	1	2 clones
KH2 clone A6 (no drug)	1	-	-	1	-	2 clones
KH2 clone I5 CQ 100nm	1	1	-	1	1	2 clones
KH2 clone D3 CQ 50nm	1	1	-	1	1	2 clones

KH2 clone A6 was culture adapted and cloned through limiting dilution method. KH2 from cryo vial came straight from the cryopreservation tank and DNA extraction followed (no continuous culture). KH2 clone I5 was exposed to 100nm of chloroquine drug pressure once while KH2 D3 was exposed to 50nm of chloroquine drug pressure twice. All drug pressure was carried out for 72 hours at 37°C.

Before Chloroquine drug pressure of KH2 (100nM), two clones, 3D7 and KH2 were present in the culture. After 100nm of chloroquine drug pressure, two clones still appeared on the agarose gel except this time; the KH2 presumed band had a stronger signal while the presumed contaminant band had a weaker/faint signal. There was no difference in the number of bands before chloroquine pressure and after chloroquine drug pressure. The chloroquine drug pressure was unsuccessful in killing off the contaminant retaining the KH2 isolate in culture.

### 5.7.1 Standard 48-hour Chloroquine drug assay on KH2

To further investigate the presence of the KH2 isolates and its composition in culture, a standard 48 hour drug assay was carried out on one of the chloroquine drug pressured KH2 clone. This was done to ascertain the  $EC_{50}$  value of the isolates in culture and compare with known  $EC_{50}$  values already established in previous experiments and other studies. If the isolate can be identified by its drug susceptibility and therefore its  $EC_{50}$  value, its identity could possibly be deduced.



**Fig 5.3 DHA  $IC_{50}^{48hr}$  assay:** Graph showing  $EC_{50}$  value of KH2 isolate (Clone I5) after a 48 hour exposure to chloroquine.

The dose-response curve shows that the  $EC_{50}$  value for the chloroquine drug pressured KH2 was 9nm. This value is within the  $EC_{50}$  range for the standard 48hr value for 3D7 (9nM-15nM) and not in the range of the  $EC_{50}$  of a chloroquine resistant isolate, which KH2 is expected to be. This dose response result confirms the presence of a fast-growing CQ sensitive parasite in the continuous culture mix with KH2.

## 5.8 *Pfcr*t Genotyping of KH2

Previous results have shown the presence of both KH2 and a contaminant in culture. Since the chloroquine resistant phenotype was not picked up in the drug assay experiment, a *Pfcr*t genotyping experiment was carried out in order to confirm if the KH2 isolate has a chloroquine resistant mutation. The table below shows the results.

**Table 5.5: *Pfcr*t haplotype of KH2 isolate and derived clones**

Parasite Line	Condition	Haplotype <i>Pfcr</i> t 72-76
<b>3D7</b>	Lab adapted line	CVMNK
<b>DD2</b>	Lab adapted line	CVIET
<b>7G8</b>	Lab adapted line	SVMNT
<b>KH2 cryo</b>	From cryopreserved sample	CVMNK
<b>KH2</b>	Culture adapted line	CVMNK
<b>KH2 I5</b>	100nm drug pressure	CVIET
<b>KH2 D3</b>	Two rounds of 500nM drug pressure	CVMNK

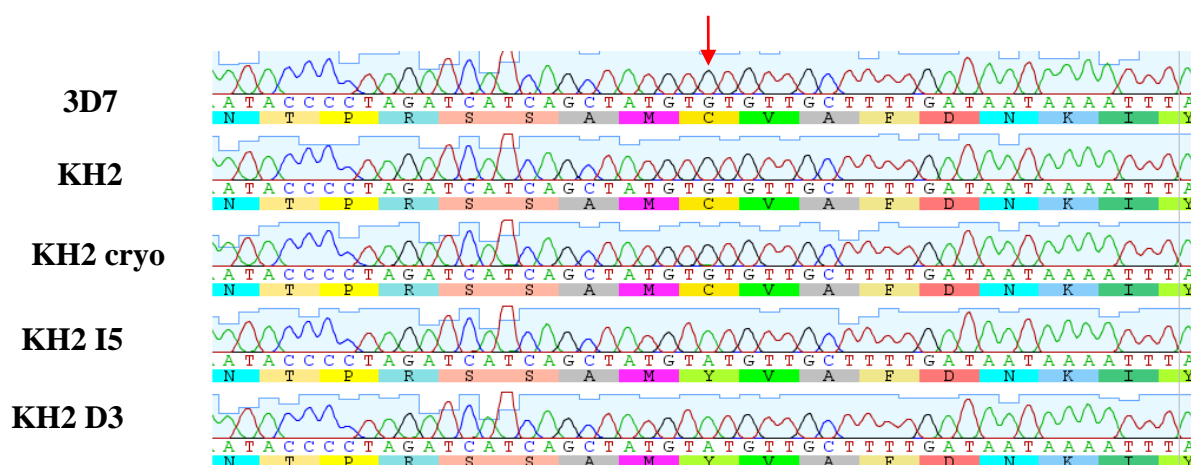
The KH2/contaminant culture has both isolate mixes which has been very difficult to separate (Table 5.2). The 100nM drug pressure killed off majority of the parasites which are chloroquine sensitive. Since KH2 is chloroquine resistant (fig 5.3), it was able to grow in culture and recover from the drug pressure. Immediate DNA extraction after drug pressure and further analysis by *Pfcr*t genotyping showed that after drug pressure, mostly KH2 isolate remains as shown in Table 5.4 (KH2-I5) where the CVIET genotype can be observed. The KH2 isolate before drug pressure shows a strain with genotype CVMNK (Table 5.4: KH2, KH2-cryo). This data shows that the contaminant is the dominant clone in *in-vitro* culture, and enjoys a fitness advantage over KH2 *in-vitro* in the absence of drug pressure.



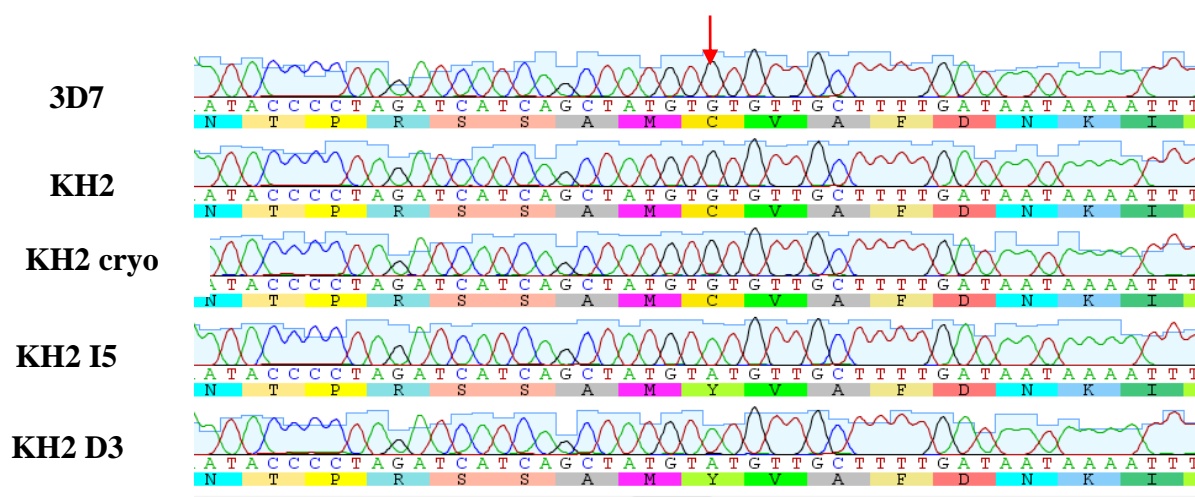
After chloroquine drug pressure where the majority of the non-KH2 parasites were killed off, the *Pfcrt* genotype expressed was CVIET, which is a chloroquine resistant mutation known to be fixed among artemisinin resistant Cambodian parasites.

## 5.9 Pfk13 Genotyping of Chloroquine drug pressured KH2

Seeing the chloroquine pressured KH2 isolates had the CVIET genotype (Table 5.5), it was important to confirm the *Pfk13* genotype on these cloned isolates as they were expected to also harbour the artemisinin resistant genotype.



**Fig 5.4a *Pfk13*forward alignment:** The arrows show the C580Y mutation in the chloroquine pressured KH2 clones



**Fig 5.4b *Pfk13*Reverse alignment:** The arrows show the C580Y mutation in the chloroquine pressured KH2 clones

The drug pressured cloned KH2 isolates comprised of artemisinin resistant mutation at position C580Y. This result was expected. A limiting dilution experiment was carried out on the isolates to separate the resistant isolates from the mixed culture. *Msp* genotyping was again carried out to confirm the clonal mix. The experiments were unsuccessful in separating KH2 from the contaminating genotype or genotypes.

### 5.10 Acquisition of artemisinin resistant Cam3.11 lines.

The previous experiments described in the first part of this chapter highlight the difficulty involved in separating a mixed *Plasmodium falciparum* culture. The experiments to separate KH2 from 3D7 isolate were discontinued. This was because artemisinin resistant isolates were provided by Jill Davies at the Liverpool School of Tropical Medicine and originally developed by David Fidock & Judith Straimer (Straimer *et al.*, 2014). Once expanded in our laboratory, the 6 hr pulse assay and the standard 48 hr assay were carried out with the new artemisinin resistant lines. Three isolates were retrieved and their information can be seen in table 5.6 below.

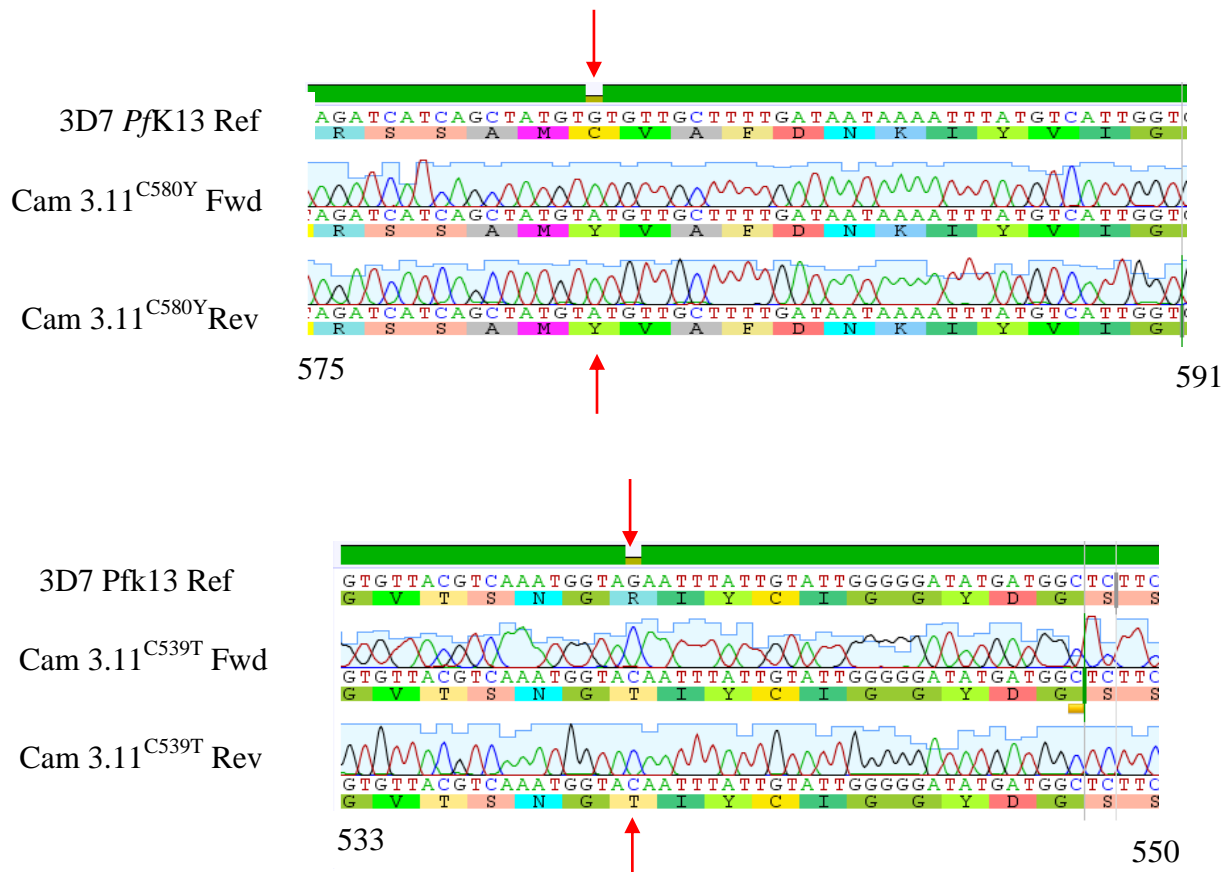
**Table 5.6 List of *Plasmodium falciparum* lines, origins, characteristics and year isolated:** The isolates are comprised of artemisinin sensitive and resistant lines.

Parasite Line	Origin	Region	Characteristics	Year reported	Reference
Cam 3.11 WT	Cambodia	SE Asia	Genetically modified	2014	Straimer et al, 2015
Cam 3.11 C580Y	Cambodia	SE Asia	Genetically modified	2014	Straimer et al, 2015
Cam 3.11 R539T	Cambodia	SE Asia	Parental	2014	Straimer et al, 2015

Cam 3.11<sup>WT</sup> isolate is an artemisinin sensitive (wild type) strain. Cam 3.11<sup>C580Y</sup> and R539T are artemisinin resistant strains with mutations in the C580 and R539 positions of the *Pfkelch* 13 gene respectively.

## 5.11 *Pfk13* Genotyping of Cam 3.11 lines

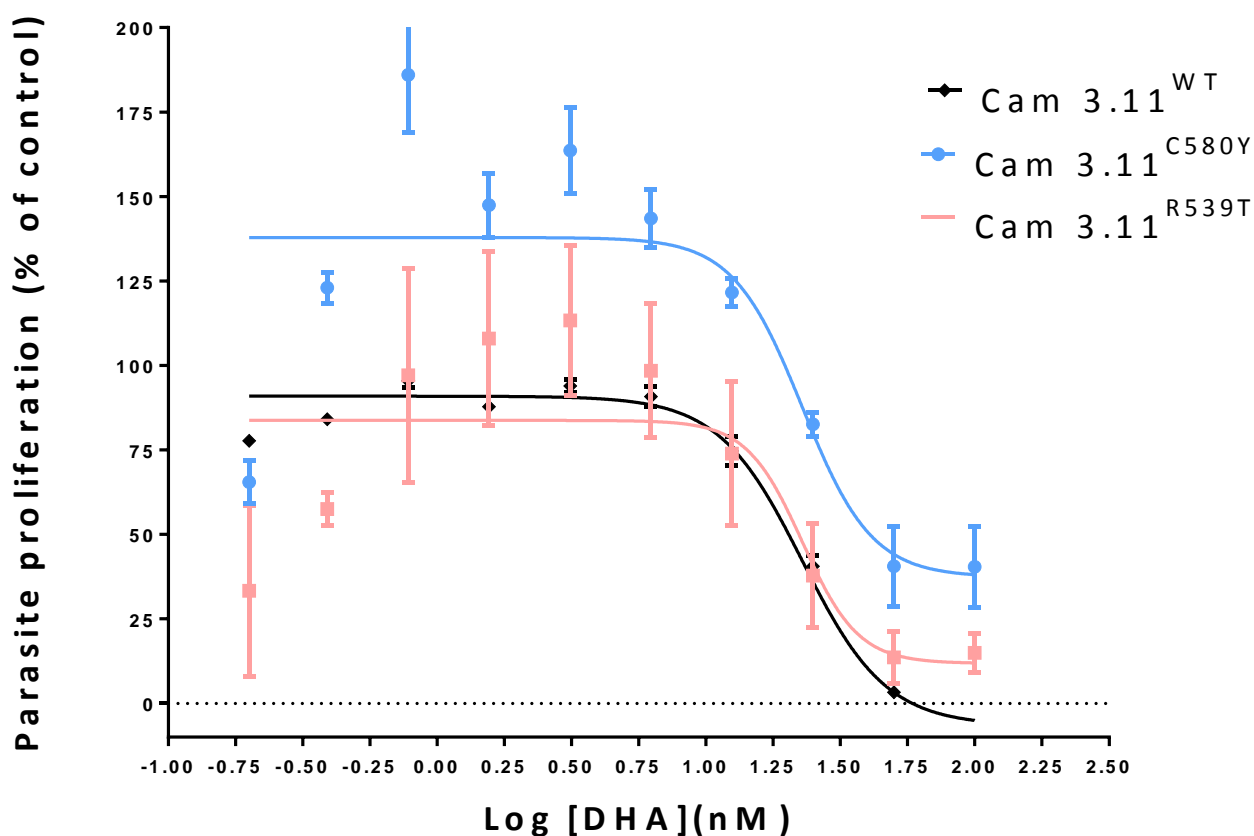
Before any assays were carried out on the new artemisinin resistant Cam 3.11 lines, it was important to ascertain the genotypic information of all the isolates first. *Pfk13* genotyping was done on both resistant lines Cam 3.11 lines (C580Y, R539T) as described in Chapter 3.11.3.



**Fig 5.5 Direct sequencing of *pfk13* gene in Cam 3.11 lines:** Both parasites were confirmed to have the kelch mutation at the positions expected, C580Y (figure above) and R539T (figure below). The numbers correspond to the amino acid number on the *Pfk13* gene.

## 5.12 Sensitivity to dihydroartemisinin *in-vitro* for Cam 3.11 lines

All 3 isolates (Table 5.6) from Cambodia were exposed to DHA in a 6 hour pulse and a standard 48-hour pulse in order to ascertain if the 6-hour pulse can discriminate differences in DHA  $EC_{50}^{6hr}$ . Parasites were synchronised at ring stage (0.5% parasitaemia) and exposed to a 6hr drug pulse at varying drug concentrations (Chapter 3.5). A dose response curve of DHA drug concentration and percentage parasite viability was plotted for all Cambodian isolates.



**Fig 5.6** Mean DHA  $IC_{50}^{6hr}$  pulse assay of *P. falciparum* isolates Cam 3.11: 6 hour pulse assay on Cam 3.11<sup>WT</sup> (artemisinin sensitive), Cam 3.11<sup>C580Y</sup> and Cam 3.1<sup>R539T</sup> lines (artemisinin resistant). See table 5.7 for mean  $EC_{50}$  values.

When all the isolates from Cambodia were exposed to DHA for 48 hours, there was no difference in the EC<sub>50</sub> values observed. This trend was also observed when all African isolates were exposed to DHA for 48 hours (Chapter 4, Table 4.5).

When the Cambodian isolates were exposed to a 6-hour pulse of DHA, differences were observed when compared to the 48-hour assay. Cam 3.11WT had a 4.8-fold difference in DHA EC<sub>50</sub> between the pulse assay and the standard assay with a P value of 0.005. Cam 3.11 C580Y had a 4-fold difference in DHA EC<sub>50</sub> between the pulse assay and the standard assay (P = 0.01), while Cam 3.11 R539T had a 3.5-fold difference in DHA EC<sub>50</sub> between the pulse assay and the standard assay with a P value of 0.01.

However, when all the isolates were compared to each other using the 6 hour pulse assay, this novel assay could not discriminate between DHA EC<sub>50</sub> values i.e the assay could not tease out differences that exist between the Cam 3.11WT (artemisinin sensitive line) and Cam 3.11 C580Y, R539T which are the artemisinin resistant lines (Table 5.8).

**TABLE 5.7 DHA standard 48hr and 6hr pulse assay:** The standard 48-hour assay was carried out using DHA. Data shows mean EC<sub>50</sub> values for the 6-hour pulse assay and the standard 48hour assay with corresponding fold difference and results for statistical significance between 2 assays.

PARASITE		DHA_Assay : EC <sub>50</sub> <sup>6h</sup>		DHA_Assay : EC <sub>50</sub> <sup>48h</sup>				
	# Experiments	# Replicates	mean EC <sub>50</sub> (SD)	mean EC <sub>50</sub> (SD)	# Experiments	# Replicates	fold difference	P value (Ttest)
Cam 3.11 <sup>WT</sup>	3	4	24.0 ± 3.2	6.0 ± 0.6	3	4	4.8	0.005
Cam 3.11 <sup>C580Y</sup>	3	4	20.0 ± 1.05	6.0 ± 0.2	3	4	4.0	0.01
Cam 3.11 <sup>R539T</sup>	3	4	23.0 ± 4.5	6.0 ± 3.9	3	4	3.5	0.01

The abbreviations for isolates are Cam (Cambodia), WT (wild type), and C580Y and R539T are the mutation positions. The EC<sub>50</sub> values are averaged for at least three independent experiments. All data are represented as mean ± S.E.M. A two-tailed test was used to test significance at P > 0.05



**TABLE 5.8 Mean difference in DHA EC<sub>50</sub> (nM) between the Cambodian isolates, by the standard 48hr assay and 6hr pulse assay:** Mean difference in DHA sensitivity of the 6 hour pulse assay and standard 48 hour assay. Comparisons were made between each African isolate and the P value of this difference is shown. (P < 0.05)

Isolates	6hr Pulse assay: Mean difference nM (P value)	48hr Standard assay: Mean difference nM (P value)
Cam R539T vs. Cam C580Y	0.1 (0.98)	0.3 (0.85)
Cam 967WT vs. Cam C580Y	3.4 (0.21)	0.3 (0.71)
Cam 967WT vs. Cam R539T	3.3 (0.17)	0.6 (0.71)

Further comparison of all Cambodian *P. falciparum* isolates showed the same trend. The mean difference in EC<sub>50</sub> values between Cam 3.11<sup>R539T</sup> and Cam 3.11<sup>580Y</sup> was 0.1 (P value = 0.98). This means that the pulse assay was unsuccessful in discriminating between the differences in drug sensitivity that may exist between the two isolates. Comparisons between the wild type Cambodian isolate and each artemisinin resistant line also gave a similar result. The mean difference in EC<sub>50</sub> between Cam 3.11WT and Cam 3.11 C580Y was 3.4nM. This differences was not statistically significant (P= 0.21). More so, comparisons in EC<sub>50</sub> values between Cam 3.11WT and Cam3.11 R539T (3.3nM) also was not statistically significant (P = 0.17) (Table 5.8). Comparisons of the mean difference in EC<sub>50</sub> values between the artemisinin sensitive and the artemisinin resistant lines using the standard 48 hour assay also showed no significant differences in DHA sensitivity between these isolates (Table 5.8)

This means that like the standard 48 hr assay, the 6 hr pulse assay was not successful in teasing apart differences in DHA sensitivity profiles that may exist between the artemisinin sensitive line and the artemisinin resistant lines derived from the same Cambodian parent isolate.

**TABLE 5.9 Mean difference in DHA EC<sub>50</sub> (nM) between isolates from Cambodia and African isolates, by the standard 48hr assay and 6hr pulse assay:** Mean difference in DHA sensitivity of the 6-hour pulse assay and standard 48-hour assay. Comparisons were made between each isolate and the P value of this difference is shown. ( $P < 0.05$ )

Region	6hr Pulse assay: Mean difference nM (P value)	48hr Standard assay: Mean difference nM (P value)
Cam C580Y vs. HL1204	11.6 (0.01)	1.0 (0.17)
Cam R539T vs. HL1204	11.7 (0.02)	1.3 (0.51)
HL1402 vs Cam C580Y	23.8 (0.04)	1.1 (0.34)
HL1402 vs. Cam R539T	23.7 (0.07)	0.8 (0.67)
Cam C580Y vs. HL1210	8.7 (0.02)	2.9 (0.38)
Cam R539T vs. HL1210	8.8 (0.04)	3.2 (0.19)
HL1212 vs. Cam C580Y	5.3 (0.91)	1.0 (0.86)
HL1212 vs. Cam R539T	5.3 (0.91)	0.7 (0.97)

To see if the pulse assay could discriminate between the DHA sensitivity profiles of the isolates from Cambodia and those from Africa, a comparison was made using the mean differences. The pulse assay this time was very successful in teasing apart the differences that exist between the isolates from Cambodia (C580Y, 539T) versus those from Africa (HL1204, HL1210). However, the assay could not differentiate between the DHA sensitivity profile of both C580Y and R539T when compared with the HL1212 isolate (Nigeria: Mean difference: 5.3nM,  $P = 0.91$ ; 5.3nM,  $P = 0.91$ )

The pulse assay could discriminate different DHA-susceptibility phenotypes between the Cam 3.11 C580Y, Cam 3.11 R539T when compared with HL1204 and HL1210 but not HL1212. The mean EC<sub>50</sub> value for HL1212 is 26nM while the mean EC<sub>50</sub> value for both Cam 3.11 C580Y and R539T are 23nM. The standard 48 hour assay could not discriminate the differences in EC<sub>50</sub> in any pairwise comparison (Table 5.9) and this is the same observation made in the previous chapter and in other published studies. In the 6 hour assay, the 2 Cambodian lines with K13 mutation were less susceptible to DHA compared to the African lines HL1204, HL1210. However, the least susceptible to DHA HL1212, was indistinguishable from the Cambodian lines in this assay.

### 5.12 DHA EC<sub>50</sub><sup>6h T0-T4</sup> assay on Cambodia and Africa lines

Since it was important that the pulse assay should produce consistent results (it did not achieve discrimination in the Cambodian lines like it did the Africans). A variation of the 6-hour pulse assay was designed, which incorporates a tightly synchronised ring stage of the parasite with comparison to a later stage, to observe any differences that may exist between stages experiencing a short pulse.

According to the literature, the ring stage is the parasite stage that has been directly linked to artemisinin resistance so it was important to make sure this stage was captured and compared with a later parasite stage to see if a more consistent phenotype could be achieved. As a result, a novel assay format was used where a 6 hr DHA pulse at time 0hr was compared to 6 hr DHA pulse at time 4hr. This means that the synchronised parasites were exposed to DHA at time 0hr and time 4hr and then the drug was washed off six hours later, at times 6hr and 10hr respectively, and plated in a 96 well plate for another 43 hours (a total of 48 hours incubation time). The difference between the two time point exposures (at time 0 hr and time 4 hr) gives a measurement designated as the EC<sub>50</sub><sup>6h T0-T4</sup>. Isolates were stratified according to the EC<sub>50</sub> from the differences in both time points. Isolates with a low  $\Delta T_0 - T_4$  EC<sub>50</sub><sup>6hr</sup> value are on the left of Figure 5.7 while isolates with a high EC<sub>50</sub><sup>6h T0-T4</sup> are on the right.

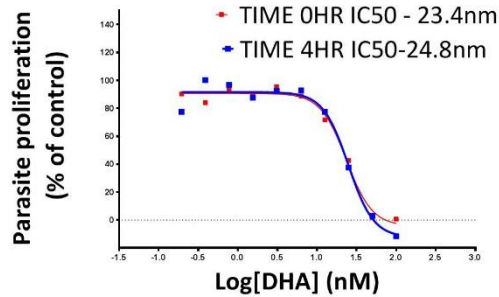
Across the board it can be seen that isolates that are artemisinin sensitive (Cam3.11WT, HL1204, HL1210) all show little differences in DHA EC<sub>50</sub> values at exposure time points 0 hour and 4 hours, thereby displaying a low EC<sub>50</sub><sup>6h T0-T4</sup> value. Whereas isolates that have been shown to be resistant to artemisinin and have the K13 mutation show much higher differences in DHA EC<sub>50</sub> values at exposure time points 0 hour and 4 hours, thereby showing higher differences in their EC<sub>50</sub><sup>6h T0-T4</sup> value. The HL1212 isolate however, show a different trend with higher differences in DHA EC<sub>50</sub> values (compared to HL1204 and HL1210) at exposure time points 0 hr and 4 hrs with an absence of mutations in the propeller domain of the *Pfk13* gene (chapter 4, figure 4.9).

## $\Delta T_0$ -T4 - DHA-SUSCEPTIBLE

## $\Delta T_0$ -T4 - DHA-TOLERANT

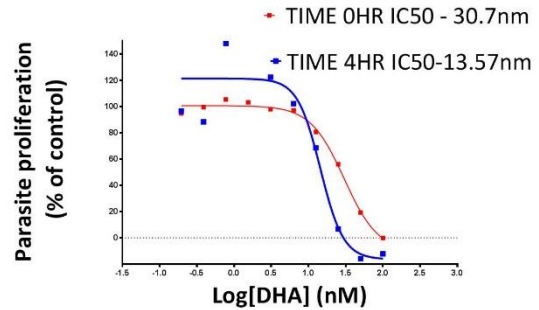
**A**

$IC_{50}^{6hr}$  value of Cam 3.11<sup>WT</sup> at time 0hr and 4hr



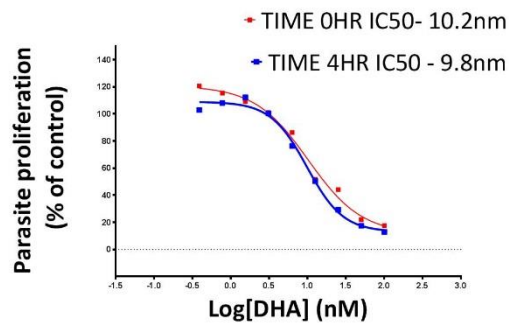
**D**

$IC_{50}^{6hr}$  value of Cam 3.11 C580Y at time 0hr and 4hr



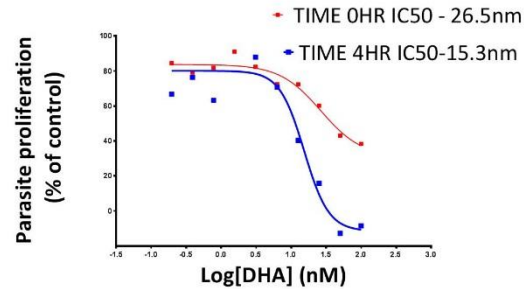
**B**

$IC_{50}^{6hr}$  value of HL1204 at time 0hr and 4hr



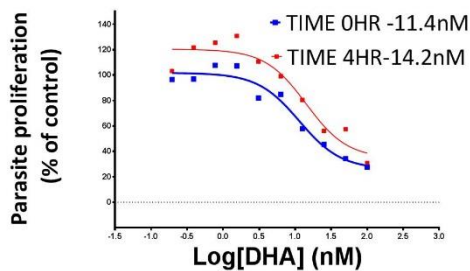
**E**

$IC_{50}^{6hr}$  value of Cam 3.11 R539T at time 0hr and 4hr



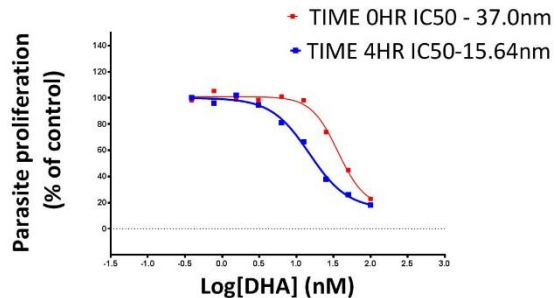
**C**

$IC_{50}^{6hr}$  value of HL1210 at time 0hr and 4hr



**F**

$IC_{50}^{6hr}$  value of HL1212 at time 0hr and 4hr



**Fig 5.7 DHA  $EC_{50}$   $\Delta T_0$ -T4 assay of *P. falciparum* isolates from SE Asia and Africa:** 6 hour pulse assay on Cam 3.11<sup>WT</sup> (artemisinin sensitive), Cam 3.11<sup>C580Y</sup> and Cam 3.1<sup>R539T</sup> lines (artemisinin resistant) and HL1204, HL1210 and HL1212. Isolates with a low  $EC_{50}^{6hr T_0-T_4}$  value are on the left (A, B, C) while isolates with a high  $EC_{50}^{6hr T_0-T_4}$  are on the right (D, E, F). The dose-response curve show an elevated  $EC_{50}^{6hr T_0-T_4}$  value in the less susceptible lines and are superimposed in the wild-type lines.

### 5.12.1 DHA $EC_{50}^{6h\ T0-T4}$ assay for Cam 3.11 lines

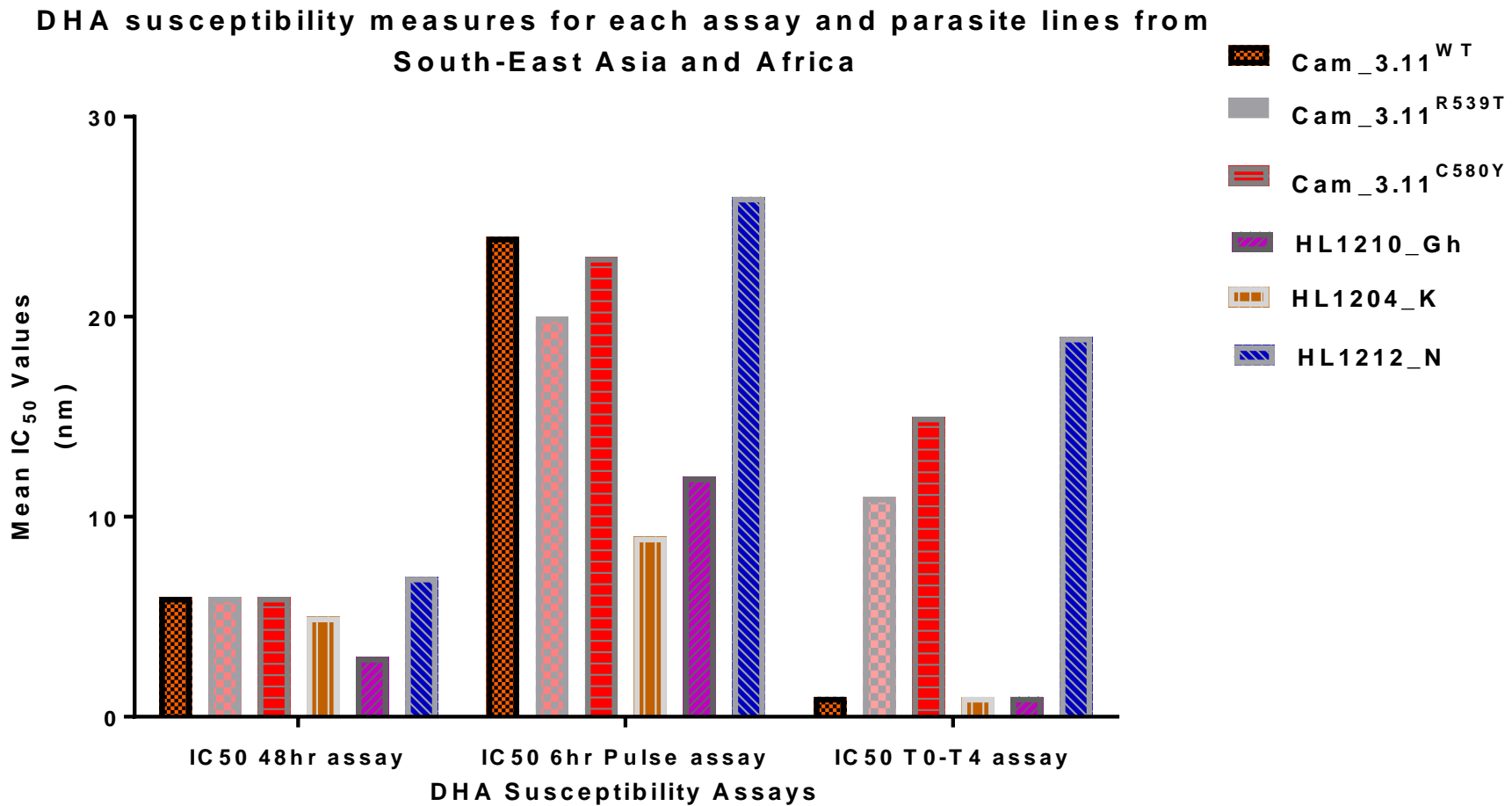
After DHA drug exposure at time T0-T4, wild type *P. falciparum* isolates Cam 3.11<sup>WT</sup> had a  $\Delta EC_{50}^{6h\ T0-T4}$  value of 1.4 with a P value of 0.07. This result shows that the changes in  $EC_{50}$  between the two time point exposures are not significant (Mean  $EC_{50}$  T0 -23.3nM, T4-24.8nM). In comparison, both artemisinin resistant lines Cam 3.11 C580Y (Mean  $EC_{50}$  at T0-30.6nM, T4 -14.7nM) and R539T (Mean  $EC_{50}$  at T0 -36.5nM, T4 -15.3nM) had a  $\Delta T_0 - T_4$  value of 15.9 and 11.2 with a P value of 0.04 and 0.0007 respectively, which shows a statistically significant difference in both exposure time points (Table 5.10).

### 5.12.2 DHA $EC_{50}^{6h\ T0-T4}$ assay for the African lines

The DHA pulse assay carried out on isolates HL1204, HL1210 and HL1212 showed very interesting results. The HL1204 isolate (artemisinin sensitive) had a  $\Delta EC_{50}^{6h\ T0-T4}$  value of 1.2 with a P value of 0.68 while the HL1210 isolate, also artemisinin sensitive, had a  $EC_{50}^{6h\ T0-T4}$  value of 1.6 with a P value of 0.16. The  $EC_{50}$  differences in both exposure time points were not statistically significant. However, the HL1212 isolate which does not have a K13 mutation, had a  $\Delta EC_{50}^{6h\ T0-T4}$  value of 19.2 with a P value of 0.008. The  $EC_{50}$  differences in both exposure time points for HL1212 were statistically significant. All the data for DHA  $EC_{50}^{6h\ T0-T4}$  assay (assay 3) are summarised in Table 5.10 below.

**TABLE 5.10 Mean difference in DHA EC<sub>50</sub> (nM) between isolates from Cambodia and African isolates, by the standard 48hr assay and 6hr pulse assay:** Mean difference in DHA sensitivity of the 6-hour pulse assay and standard 48-hour assay. Comparisons were made between each isolate and the P value of this difference is shown. (P < 0.05)

PARASITE	DHA_Assay 3: IC <sub>50</sub> <sup>6h T0-T4</sup>					P value (Ttest)
	# Expts	# replicates per xperiment	mean IC <sub>50</sub> <sup>T0</sup> (SD)	mean IC <sub>50</sub> <sup>T4</sup> (SD)	Δmean IC <sub>50</sub> <sup>T0-T4</sup>	
3D7	2	4	14.0 ± 3.1	5.6 ± 0.8	8.4	0.11
Cam_3.11 <sup>WT</sup>	2	4	23.4 ± 0.5	24.8 ± 0.2	1.4	0.07
Cam_3.11 <sup>R539T</sup>	2	4	26.5 ± 0.3	15.3 ± 0.4	11.2	0.0007
Cam_3.11 <sup>C580Y</sup>	2	4	30.6 ± 0.1	14.7 ± 1.5	15.9	0.04
HL1204_K	2	4	10.8 ± 0.8	12.0 ± 3.1	1.2	0.68
HL1210_Gh	2	4	12.0 ± 0.7	13.6 ± 0.7	1.6	0.16
HL1212_N	2	4	38.2 ± 1.7	18.3 ± 3.7	19.2	0.008



**Fig 5.8 Comparison of all 3 assays tested on *P.falciparum* isolates from SE Asia and Africa:** The graph shows data from the standard 48 hour assay, the 6 hour pulse assay and EC<sub>50</sub><sup>6h T0-T4</sup> assay carried out on isolates Cam 3.11<sup>WT</sup> (artemisinin sensitive), Cam 3.11<sup>C580Y</sup> and Cam 3.1<sup>R539T</sup> (artemisinin resistant) and HL1204, HL1210 and HL1212 (artemisinin sensitive). The graph shows the assay which best discriminates between the differences in DHA sensitivity.

### 5.13 Comparison of the 3 assays tested on *P. falciparum* isolates from Southeast Asia and Africa

The general aim of this Chapter is to ascertain the best assay that can discriminate between differences in DHA susceptibility that may exist between isolates from different regions. From the data analysis (Figure 5.9), the standard 48 hour assay ( $EC_{50}^{48hr}$ ), which is the assay that has been used in the literature for years (developed initially for chloroquine), was unsuccessful in discriminating between DHA  $EC_{50}$  differences that exists between the parasite lines.

The novel hour pulse assay ( $EC_{50}^{6hr}$ ) was successful in discriminating the differences that exist between the DHA sensitivity profiles of HL1204, HL1210 and HL1212. This assay however was unsuccessful in discriminating between DHA  $EC_{50}$  differences that exists between the Cam 3.11 parasite lines from Cambodia (Figure 5.9).

The novel 6 hour pulse assay at Time 0 hours and 4 hours ( $EC_{50}^{6h\ T0-T4}$ ) was successful at discriminating the differences in DHA  $EC_{50}$  that exists between the artemisinin resistant Cambodian line and the artemisinin sensitive Cambodian and African line (Figure 5.9). This assay was also able to show a different phenotype for HL1212 ( $\Delta T_0 - T_4 = 19.2$ ; P value 0.008) even though genetically, it does not have the K13 mutation.



#### 5.14 Ring stage survival assay (RSA<sup>0-3hr</sup>) on *P. falciparum* isolates from SE Asia and Africa

According to the literature, the ring stage survival assay has been shown to produce a phenotype for artemisinin resistance *in-vitro*, based on evidence that artemisinin resistance affects predominantly the ring-stage of the parasite lifecycle (Witkowski et al, 2013). We therefore set out to compare relative DHA susceptibility by ring stage survival assay compared to our other assays on the *P.falciparum* lines from Cambodia and Africa. This was done to validate the RSA<sup>0-3hr</sup> assay and to also investigate if the assay could produce a phenotype *in-vitro* for the African lines. Parasites were exposed to 700nM DHA concentration at the tightly synchronised early ring stage of the parasite. The drug was washed off after a 6 hour exposure and the cells incubated at 37°C for 72 hours. The RSA method has been described extensively in Chapter 3.

**TABLE 5.10 Mean difference in DHA EC<sub>50</sub> (nM) between isolates from Cambodia and African isolates, by the standard 48hr assay and 6hr pulse assay:** Mean difference in DHA sensitivity of the 6-hour pulse assay and standard 48-hour assay. Comparisons were made between each isolate and the P value of this difference is shown. (P < 0.05)

PARASITE	DHA_Assay 4: RSA		
	# Expts	# replicates per experiment	Survival (%)
Cam_3.11 <sup>WT</sup>	1	1	0
Cam_3.11 <sup>R539T</sup>	1	1	26.5
Cam_3.11 <sup>C580Y</sup>	1	1	14
HL1204_K	2	1	0
HL1210_Gh	2	1	0
HL1212_N	2	1	0

The results from the RSA experiments showed that Cam3.11 WT had a 0% survival. This was the same trend in the African lines HL1204, HL1210 and HL1212 which all do not have a PK13 mutation. However, Cam3.11<sup>R539T</sup> had a 26.5% survival while Cam\_3.11<sup>C580Y</sup> had a 14% survival. Both isolates are artemisinin resistant with mutation in *PfK13* region of the propeller domain.



## 5.15 Discussion

This Chapter aimed to determine *in vitro* responses of Cam 3.11 artemisinin resistant and artemisinin sensitive isolates in comparison to the African lines and to find new approaches to the *in-vitro* phenotype. Three assays were used to determine DHA sensitivity profiles between African and Asian lines. Some assay formats were able to successfully discriminate the differences that exist between artemisinin resistant/tolerant and artemisinin sensitive lines while others were unsuccessful. One format which derived DHA sensitivity differences is the  $EC_{50}^{6h\ T0-T4}$  assay and this approach could now be developed further with lines of interest.

Parasite lines from Cambodia (Cam 3.11<sup>WT</sup>, Cam 3.11<sup>C580Y</sup> and Cam 3.11<sup>R539T</sup>) showed no significant difference in  $EC_{50}$  values when exposed to DHA for 48 hrs. The comparisons of the mean difference in DHA showed that the 48 hr assay was unsuccessful in discriminating between artemisinin resistant and the artemisinin sensitive isolates. The corresponding P values for the comparisons show that the results were not statistically significant (Table 5.8). This result is consistent with published data which show that the standard 48 hr assay was unsuccessful in teasing out differences in DHA sensitivity between fast clearing and slow clearing parasites (Dondorp *et al.*, 2009; Witkowski *et al.*, 2013).

When the novel 6 hour pulse assay was compared to the standard 48 hour assay for the Cambodian lines, the mean difference in  $EC_{50}$  for all 3 isolates were statistically significant and showed a higher mean DHA  $EC_{50}$  value. The pulse assay showed a shift to the right in the dose response curve which a similar trend observed with DHA pulse exposure in the African lines (Chapter 4), and this shift in  $EC_{50}$  was statistically significant in all Cam 3.11 lines (Table 5.7). However, when the mean differences between the artemisinin sensitive and the resistant parasites were compared using the 6 hour pulse assay, the result showed that the assay could not tease out differences in  $EC_{50}$  that exists between the 3 Cambodian parasite lines (Table 5.8). This suggests that the 6 hour pulse assay, while very successful in discriminating the differences in DHA sensitivity that exists between African parasites, could not tease apart differences in DHA sensitivity of the Cambodian parasites in the current test format.

A further comparison of the 6 hr pulse assay between the Cambodian lines and the African lines showed that the assay could in fact discriminate the differences in DHA sensitivity between these isolates with one exception, the HL1212 isolate which showed that the mean difference was not statistically significant (Table 5.9,  $P = 0.91$ )

Since the 6 hr pulse assay was not discriminating among fast and slow clearing Cambodian lines, an altered assay ( $EC_{50}^{6h\ T_0-T_4}$ ) was designed to take into account the early ring stage of the parasite lifecycle, which had been shown to be directly associated with artemisinin resistance (Witkowski *et al.*, 2013; Dogovski *et al.*, 2015). This assay involved exposing parasites to DHA between the 0-6 hr stages of its lifecycle, the stage associated with artemisinin resistance, and comparing the exposure with an exposure, 4-10 hrs into the parasite lifecycle. By altering the assay, a phenotype was derived using the  $\Delta T_0 - T_4$  measurement. The artemisinin sensitive Cam 3.11<sup>WT</sup> line showed a different phenotype to the artemisinin resistant Cam 3.11<sup>C580Y</sup> and Cam 3.11<sup>R539T</sup> lines. The  $\Delta T_0 - T_4$  for the sensitive line was 1.4 while the resistant lines were 15.9nM and 11.2 nM respectively, a difference that was statistically significant. This suggests that the  $\Delta T_0 - T_4$  assay was capturing the important stage of the parasite life cycle where artemisinin resistance occurs. Although the 6 hour pulse assay is also based on a tightly synchronised ring stage, the 6 hour pulse assay may have missed out on the very important early ring stage (0-3hr). The 6 hour pulse assay, not based on the early ring stage, contains a mixture of ring stages (early, mid, late ring) and as a result, this may have affected the ability for the assay to tease out the differences in DHA sensitivity of the Cambodian lines like it successfully did with the African lines.

The  $EC_{50}^{6h\ T_0-T_4}$  assay was also successful in discriminating between African lines with different DHA susceptibility. While HL1204 (artemisinin sensitive) and HL1210 (artemisinin sensitive, chloroquine resistant) had a  $\Delta T_0 - T_4$  of 1.2 and 1.6nM respectively, HL1212 had a  $\Delta T_0 - T_4$  value of 19.2nM, an observation similar to the artemisinin resistant isolates from Cambodia, except that HL1212 does not have any K13 propeller domain mutation (Chapter 4). The  $\Delta T_0 - T_4$  assay, taking the early ring stage of the HL1212 parasite lifecycle into account shows that this parasite has lower susceptibility to artemisinin when compared to the other African lines and as such, this must be due to a *Pfk13*-independent event which is not clearly understood. The ring stage assay was successful as it validates studies carried out by Witkowski *et al.*,

(2014) where parasites with the R539T mutation had a higher percent survival compared to parasites with the C580Y mutation. The RSA also showed that the African lines are artemisinin sensitive according to this assay. The HL1212 line had 0% parasite survival using the RSA. This suggests that while the RSA is successful at identifying a phenotype associated with artemisinin resistance in the Cambodian lines, it may not be very useful in elucidating small differences in DHA susceptibility among African lines. The RSA is an absolute measure which works perfectly when artemisinin resistance has already occurred in Cambodia, however, resistance takes some time to arise and as a result, a useful assay for this purpose would be able to measure subtle DHA sensitivity changes over time. As a result, the 6 hr pulse assay and/or the  $\Delta T_0 - T_4$  assay can be a useful assay for tracking down artemisinin resistance on the African continent.

Comparison of all assays tested show that the  $EC_{50}^{6h T_0-T_4}$  assay could potentially provide new approaches to the *in-vitro* phenotype as it was successful in discriminating the differences in DHA sensitivity that may exist between parasites from different regions of the world.

The assay format was a drawback in this study. Experiments were carried out in 1.5ml microcentrifuge tubes which meant that the parasites at the bottom of the tube may not have been exposed to DHA like the parasites at the top of the microcentrifuge tubes. A solution around this would be to carry out the experiments in a 96 well plate where the surface area is big enough for all the parasites to get exposed to DHA. Another drawback of the study was that the HL1212 isolate had a high standard deviation. This may be due to the polyclonal nature of this isolate.

ASSAY	ADVANTAGE	DISADVANTAGE
EC <sub>50</sub> <sup>48h</sup>	<ul style="list-style-type: none"> <li>- less laborious</li> <li>- cheap</li> </ul>	Does not discriminate between parasite lines
EC <sub>50</sub> <sup>6hr</sup>	<ul style="list-style-type: none"> <li>- Discriminates between African lines</li> <li>- scalable</li> <li>- cheap</li> </ul>	<ul style="list-style-type: none"> <li>- Does not discriminate between Cambodian lines</li> <li>- prone to contamination</li> </ul>
EC <sub>50</sub> <sup>6hrT0-T4</sup>	<ul style="list-style-type: none"> <li>-Discriminates between both African &amp; Cambodian lines</li> <li>- scalable</li> <li>- cheap</li> </ul>	Quite laborious, prone to contamination Takes time
RSA	Discriminates between Cambodian isolates	<ul style="list-style-type: none"> <li>- absolutely laborious,</li> <li>- long prep time before assay,</li> <li>- expensive compared to the other assays</li> <li>- gives absolute measure for African lines,</li> <li>- not very scalable, not useful for African lines with <i>Pfk13</i> independent mutation</li> </ul>

**TABLE 5.11: Advantages and disadvantages of the EC<sub>50</sub><sup>48h</sup> EC<sub>50</sub><sup>6hr</sup> EC<sub>50</sub><sup>6hrT0-T4</sup> and the Ring stage survival assay.**

## 5.16 Conclusion

With rising artemisinin resistance, it is crucial to develop an in vitro assay that can successfully track down resistance before it spreads. While the ring stage assay successfully discriminates between DHA resistant and DHA sensitive Cambodian parasites, it is laborious, expensive and time consuming. The 6 hour pulse assay, while not successful in discriminating  $EC_{50}$  differences between isolates from Cambodia, it successfully does so with the African lines and can be used as a surveillance tool on the African continent to prevent the spread of artemisinin failure. The  $EC_{50}^{6h\ T0-T4}$  assay which has been successful in teasing out the differences in DHA sensitivity that exists between all isolates used in this project, can be also be used as a new approach to the in vitro phenotype. It can also be used as a surveillance tool to contain artemisinin resistance.

**CHAPTER 6:** Genetic and genomic analysis of multi-parasite  
lines; genotype-phenotype associations



## 6.1 Background

Emergence of drug resistance has continuously occurred after new drugs for the treatment of malaria have been introduced. Drug resistance emerged for chloroquine, sulfadoxine/pyrimethamine and mefloquine. Resistance also emerged for atovaquone, which occurred the same year the drug was introduced (Looareesuwan *et al.*, 1996). Malaria became very difficult to treat after *P. falciparum* became resistant to these derivatives are usually combined with a partner drug, typically from a chemical family such as the 4- aminoquinolones or the aryl alcohols, to make up artemisinin-based combination therapies (ACTs). Artemisinins are usually given in combination because they will delay the appearance of drug resistance occurring since with ACTs, parasites would need to acquire resistance to two drugs instead of one (artemisinins have a short half-life, so combination with a drug with a longer half-life is a strategy to prevent resistance). ACTs are currently the most effective treatments for *P. falciparum* malaria, however, clinical trials from Southeast Asia have shown that parasites have acquired artemisinin resistance when given as monotherapy and some ACTs appear to be losing effectiveness (Saunders *et al.*, 2014). If there continues to be a decline in ACT efficacy, this could result in malaria becoming a very difficult disease to treat.

There are ongoing clinical trials on novel classes of antimalarials compounds, but none will be available for licensing within the next two years. As a result, scientists have been trying to identify genetic markers that would predict artemisinin drug resistance in the parasite. These markers will be used to track the spread of resistance and hopefully, be used for containment which would in turn prevent early stage treatment failures and possible deaths from occurring. A genetic marker or set of markers could also be used as surveillance to identify the widespread presence of alleles associated with resistance in any geographic region. In early studies that looked at genes involved in artemisinin resistance, it was hypothesised that transporter-encoding genes contribute to resistance. These include *pfmdr1* (Wilson *et al.*, 1989) which encodes the *P. falciparum* multidrug resistant protein 1 (*pfmdr1*), and *pfcr1* (Sidhu *et al.*, 2002), encoding the *P. falciparum* chloroquine resistance transporter (*pfcr1*). Studies have shown that artemisinin sensitivity increases when the

copy number of genes encoding *Pfmdr1* decreases (Sidhu et al, 2006). Mutations in *pfmdr1* have also been shown to modulate IC50 values for artemisinin *in vitro* (Sidhu et al, 2005). Furthermore, after treatment with parasite causing recurrent infections using artemether-lumefantrine, field studies in Africa show an overrepresentation of the *pfmdr1* I1876V mutation (Dahlstrom et al., 2009).

Prevalent mutant forms of *pfcr* that confer chloroquine resistance have been shown to increase parasite susceptibility to artemisinin, and epidemiological studies have shown selection for wild-type *pfcr* occurs in endemic settings where ACTs are used (Sisowath et al., 2009; Henqirues et al., 2014). There have been reports of associations between candidate genes, *pfatp6*, *pfap2mu* and *pfubp1* to resistance in artemisinins (Eckstein-Ludwig et al., 2003; Krishna et al., 2004; Henriques et al., 2013). Despite this, in Cambodia where artemisinin resistance occurs and where monotherapy has been used, it was found that these candidate genes are not strongly correlated with resistance (Imwong et al., 2010). Although there is a lack of disease association with these genes in Asia, it does not mean they do give a different resistance phenotype or contribute to drug resistance.

In order to investigate *in-vivo* artemisinin susceptibility, researchers genetically exposed *Plasmodium chabaudi* rodent parasites with sub-lethal concentrations of artemisinin and artesunate to create resistant parasites. The artemisinin resistant line was then crossed with an artemisinin sensitive line of parasites that were different from the original at many genetic positions (Hunt et al., 2005). Selection in bulk, was applied to the recombinant progeny lines and the relative proportion of alleles for each of the two parent lines was determined by pyrosequencing, using a method known as linkage group selection (Martinelli et al., 2005). The region enriched in resistant parasites was sequenced and two different mutations were identified in a gene encoding a deubiquitinating enzyme, *pcubp-1*, which suggested this gene could have alleles that confer resistance (Hunt et al., 2007). Another study showed that variant alleles of the *pfubp-1* gene in *P.falciparum* (E1528D) were significantly more prevalent (P<0.001) in Kenyan children post-treatment, with reduced responsiveness to ACTs than in those who had a better ACT response (Henriques et al., 2014).

In terms of sensitivity and cost, whole genome sequencing and analysis methods are becoming more tractable. These methods have allowed researchers genes that are involved in drug resistance through analyzing whole genome sequence of multiple resistant clones from *in vitro* experiments. Tucker and colleagues created several artemisinin and artelinic resistant lines (Tucker et al, 2012) and found changes in candidate genes, including *pfmdr1* copy number variations. Whole genome sequence analysis showed that there were nonsynonymous mutations, including in one protein which was uncharacterised and present on chromosome 13, *pf13\_0238*. In the reference genome, this locus is designated *PF3D7\_1343700* and is now known as “*kelch13*” (Ariey et al, 2014). In order to identify mutations associated with drug resistance, another study by Cui and colleagues used gene expression microarrays to parental parasites that were 25 times more resistant to DHA and had amplifications of a locus containing *pfmdr1* (Cui et al, 2012).

Genome sequencing has also been an important tool to identify artemisinin drug resistance markers within a population-based study setting. The design of parasite population genetic studies that could be used to map genes involved in resistance was spurred after it was shown that parasites with heritable resistance exists (Anderson et al, 2010). Researchers sought to identify genomic regions under selection using large collections of parasites that already existed, when there was absence of patient phenotype data. For many years, it had been known that linkage disequilibrium exists around genes which are involved with either pyrimethamine resistance (Roper et al, 2004) or chloroquine resistance (Wooton et al, 2002). It was hypothesized that genomic regions in disequilibrium which correlates with artemisinin sensitivity might exist.

In a study, 61 parasite lines were screened against 2,816 compounds, which were provided by the NIH Chemical and Genomic Centre Pharmaceutical collection. Genotyping of the lines facilitated an examination of the association of differential drug sensitivity to endoperoxides (Yuan et al, 2011).

The genes that were found to be associated with response to artemisinin included *pfa0655w* (SURFIN), *mal13p1.268* (a *Plasmodium* conserved protein), *pfe0565w* (a conserved *Plasmodium* protein), *pf08\_0130* (a ribosomal-RNA-processing WD-repeat protein), *pf11\_0188* (a heat shock protein 90), and *pfi0355c* (an adenosine triphosphate-dependent heat shock protein) (Yuan et al, 2011).

Subsequently, 189 culture-adapted parasites collected from diverse locations were genotyped including 146 from Asia (Mu et al, 2010). In the study, a genome wide scan for loci associated with dihydroartemisinin (DHA) response showed novel loci on chromosome 1,3 and 8, using only Asian parasites (Mu et al, 2010). Another study which used 45 culture adapted *P. falciparum* parasites from different geographical regions ( Park *et al.*, 2012) revealed some chromosomal regions found to be associated with an increase in DHA and artemisinin sensitivity, notably on chromosome 4, but none of the associations were significant (Park *et al.*, 2012). There was strong evidence of selection around known resistance genes such as *pfdhfr*, *pfcr* and *pfmdr1* in both of these studies (Park *et al.*, 2012).

Later studies utilized both clinical phenotype data and parasite samples to demonstrate resistance. These studies recruited patients and measured the amount of time needed for parasites to clear after artemisinin monotherapy was administered. Parasite material for genome analysis was obtained from areas like Cambodia where there was confirmed genetically determined resistance and also from control areas (Phyo *et al.*, 2012). In 2012, the first study which analyzed 91 parasites samples from Thailand, Laos and Cambodia which was phenotyped for parasite clearance time was published (Cheeseman *et al.*, 2012). The researchers demonstrated that a clinical slow parasite clearance rate was strongly associated with a selective sweep on chromosome 13, although artemisinin resistance probably did not occur as a result of a single originating event either temporally or geographically i.e. not a “hard” sweep. A 35kb region on chromosome 13(bases 1,759,466 to 1,794,766, PlasmoDB 11.1) was proposed as a possible drug resistance marker (Cheeseman *et al.*, 2012).

Three hundred and thirty one parasites that had been phenotyped for parasite clearance time, after artesunate monotherapy were genotyped from patients with clinical infections from Cambodia, Pailin, Wang Pha, Bangladesh and Thailand

(Takala-Harrison et al, 2013). Takala-Harrison and colleagues used an Affymetrix SNP array to analyze parasite genotypes at 8,079 positions. Two SNPs were calculated to be ‘located within a top-ranked signature of recent positive selection’ (Takala-Harrison et al, 2013). Both SNPs were found on chromosome 13 at locations MAL13-1718319 and MAL13-1719976, which were described to be within 2,000 bp of each other. One SNP was within *pf3d7\_1343400* (formerly *mal13p1.216*, located between bases 1,714,443 to 1,719,255, PlasmoDB 11.1). The specific alleles causing resistance were not identified in this study as it was not designed to do so, however, the authors further highlighted the importance of the 100-kb region on chromosome 13 (Takala-Harrison et al, 2013).

Miotto and colleagues genotyped 825 *P. falciparum* infections from 10 locations in West Africa and Southeast Asia in order to identify possible causal SNPs in the locus under selection. The phenotype for identifying resistant infections was lengthened parasite clearance time after treatment with artesunate monotherapy in Southeast Asia, and genotyping was by short-read high throughput sequencing on an Illumina platform (Miotto *et al.*, 2013). The Cambodian parasites exhibited distinct population structure with 5 sub-population identified. One resistant subpopulation of parasites from Southeast Asia (KH2) was shown to have a single haplotype which extended across half of chromosome 13, from 1.4 Mb to 3.4 Mb, indicating a recent selective sweep with strong evidence. This group highlighted the importance of this region and its association to artemisinin drug resistance, but failed to identify a discrete marker.

In a report published in 2014, it was shown that a candidate artemisinin-resistance gene was identified with high confidence (Ariey *et al.*, 2014). In this study, a combination of population genetics studies and next generation whole genome sequence of an artemisinin resistant line that was selected through continuous exposure for five years *in-vitro* were used. The group compared the genomic sequence from their laboratory evolved artemisinin-resistance isolate with that of an isogenic parent. After ruling out synonymous mutations, variants in multigene families and alleles with mixed reads, eight non-synonymous candidate mutations in seven genes, each of which had emerged during resistance selection, were identified (Ariey *et al.*, 2014). The time of mutations arising was examined retrospectively, and

it was established that the M476I coding change in *PF3D7\_1343700*, a Kelch propeller domain- containing protein (K13), emerged concurrently with the appearance of strong artemisinin resistance in their *in vitro* population. The study asserted that it was most likely causative (Ariey et al, 2014).

The regions around the eight candidate SNPs were sequenced in 49 culture-adapted isolates from Southeast Asia which were associated with artemisinin sensitivity data *in vivo*. Ariey and colleagues showed that only mutations in *PF3D7\_1343700*, which included a C580Y change, had strong associations with long parasite clearance half-life in patients and survival in the ring-stage assay (RSA) *in-vitro*. It is worth noting that the *in vitro* derived M476I mutation was not found in their sample (Ariey et al, 2014). The frequency of mutations in this gene was then analysed in parasite samples from regions with and without resistance and further associations between resistance and this gene were discovered. Follow-up work has now looked at the frequency of kelch13 mutations globally (Miotto *et al.*, 2015; Kamau *et al.*, 2015; Menard *et al.*, 2016).

Another detailed study which characterised parasite clearance half-life after patients were given artesunate monotherapy at ten sites, found long parasite clearance times in Indochina with no significant resistance in any patient from India or Africa (Ashley *et al.*, 2014). A strong association between mutations in *pfkelch13* and the artemisinin-resistance phenotype was found.

Genetic engineering studies further showed that mutations in the propeller-domain region in *pfkelch13* were only strongly associated with resistance and were not causal. A method known as the CRISP-Cas9 system, a genome editing method created for use in other eukaryotes (Jinek et al, 2012) and has been adapted in *P. falciparum* (Ghorbal *et al.*, 2014), was used to show conclusively that one of the alleles caused resistance. The change in position C580Y was engineered into the Kelch propeller domain of *pfkelch13* in the drug resistant NF54 background which is of unknown origin. *In-vitro* assays carried out on two clones which were modified showed evidence of an increase in ring-stage resistance (Ghorbal *et al.*, 2014). This provided additional support that the certain mutations in *pfkelch13* gene plays a role in drug resistance, and these were sufficient on their own to generate a measurable resistant phenotype *in-vitro*.

## Rationale

The majority of malaria infections occur in sub-Saharan Africa where ACTs still appear to clear parasites quickly and where mutations in *pfkelch13* have not yet appeared at elevated rates (Ashley *et al.*, 2014). Although Southeast Asian alleles seem to be absent in Africa (Torrentino-Madamet *et al.*, 2014), it is worth noting that parasites in Africa have other mutations in *pfkelch13* (Taylor *et al.*, 2015). The common African mutation, A578S, was not associated with clinical or *in vitro* resistance to artemisinin in one other study (Menard *et al.*, 2016). In another study where dihydroartemisinin-piperaquine was administered, one patient harboured a non-synonymous mutation at position A578S on day 0; however, this allele was replaced by the wild-type form on day 3, showing no association with any phenotype (Muwanguzi *et al.*, 2016). These studies highlight that events in Africa are very different from those in other regions of Southeast Asia and thus, might be independent of the *pfkelch13*. There may be other important genes which may also play a role or might be involved in artemisinin drug resistance.

It was shown that residual sub-microscopic parasites were present in Kenyan children on day 3 after treatment with ACT and were associated with subsequent recrudescence and transmission (Beshir *et al.*, 2013). Another study showed that parasites which harboured certain mutations in *pfmdr1*, *pfcr1*, *pfubp1*, and *pfap2mu* genes showed a higher survival rate at a sub-microscopic level after treatment with ACTs (Henriques *et al.*, 2014). It is thus important to identify molecular markers which may be associated with artemisinin drug resistance and are *pfk13* independent. To assist with this, whole genome sequencing of 4 African isolates was carried out and SNPs that exist in 11 putative genes associated with artemisinin drug resistance were described. This Chapter aims to link *in-vitro* phenotype work described in Chapters 4 and 5 to genotype information described here. The *in-vitro* IC<sub>50</sub><sup>6hr</sup> pulse studies will be linked to the genome sequence information for each African isolate although no strong phenotype-genotype correlation can be made. The description of non-synonymous mutations present in 13 genes of interests associated with artemisinin drug resistance could inform future functional studies deploying gene editing. The information provided in this Chapter can be used to monitor the presence of drug resistance markers especially as it relates to artemisinin drug sensitivity in Africa.

### **Specific objectives**

1. To produce full genomic sequences for each polyclonal line HL1204, HL1210 and HL1212 lines, together with an extra added field isolate from a patient from Liberia (Sutherland et al, 2016)
2. To examine genomic data for evidence of polymorphisms at established candidate loci. This could inform future functional studies deploying gene editing.
3. To assess the relative abundance of different allele variants among the clones present within each isolate.



## 6.2 Results

The majority of the work done in this Chapter used methods that have not been described elsewhere. Due to this, some of the modifications to methods are described as results. The first part of the result section describes the experiments performed to achieve whole genome sequencing of isolates HL1204, HL1210 and HL1212 while the second part shows the whole genome sequencing data results. This work was the first in-house *P. falciparum* whole genome sequencing experiments carried out using the Illumina sequencing platform at the London School of Hygiene and Tropical Medicine.

### 6.2.1 DNA extraction and quantification

In order to prepare DNA for library construction and whole genome sequencing, parasite DNA was extracted using the Qiagen kit and an adapted/amended protocol. In particular, the experiment was initially carried out using the methods in malaria protocol where 2% parasitaemia was suggested for parasite DNA extraction. However, this approach did not produce high quality DNA or high yield which is essential for the construction of the sequencing libraries before whole genome sequencing can commence.

The adapted method used in this chapter, used 3% -4% parasitaemia in schizont stage with the presence of other stages (rings and trophozoites) in approximately 2% parasitaemia. The adapted method also added an extra step to the DNA extraction protocol where incubation at 70°C was carried out. This adapted method ensured high product yield was achieved. DNA quantity and yield was measured using a nanodrop spectrophotometer as described in the methods section (3.9).

DNA quality and yield were calculated for isolates 3D7, HL1204, HL1210, HL1212 and HL1402. Refer to Section 3.2.1 for information on the characteristics and origination of the isolates.

**Table 6.1: Showing an improvement in DNA quality and concentration after adaptation of the DNA extraction protocol (see chapter 3.6)**

Isolates	DNA quality A260/A280		DNA Concentration	
	Before adaptation	After adaptation	Before adaptation	After adaptation
3D7	1.75/1.45	2.27/2.08	24.2ng/ul	150.9ng/ul
HL1204	1.90/1.28	2.04/1.87	33.5ng/ul	135.8ng/ul
HL1210	1.63/1.77	2.07/1.96	26.8ng/ul	179.3ng/ul
HL1212	1.69/1.55	2.24/2.31	9.9ng/ul	198.3ng/ul

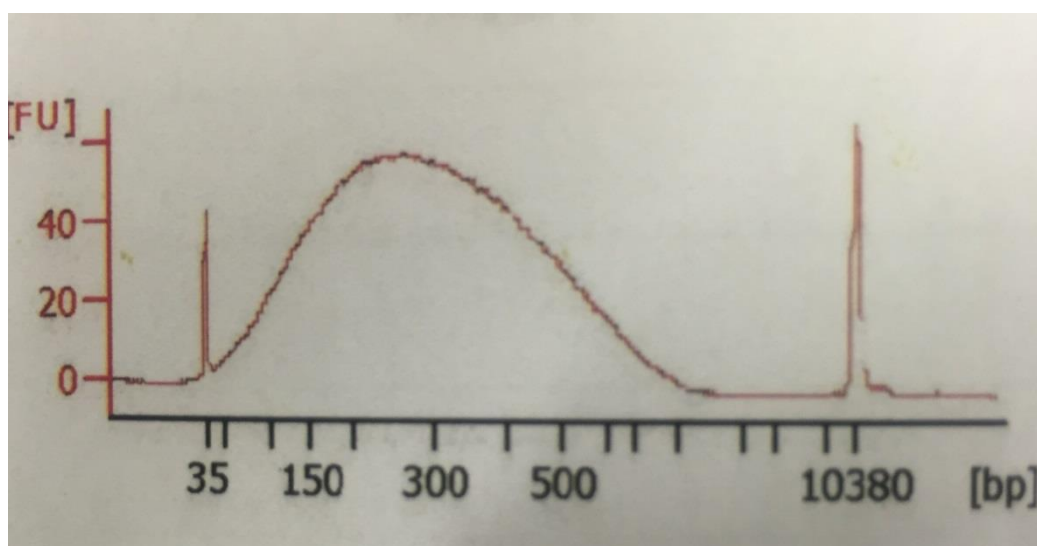
### 6.2.2 DNA shearing

In order to prepare a library for whole genome sequencing, DNA was sheared using an ultrasonicator (described in Chapter 3). This step was very important to obtain band sizes of approximately 200-500bp for library preparations. The protocol for shearing provided by this machine was used initially but after much trial and failure to achieve the required band size, the protocol was adapted to achieve exactly 300bp in this study by varying factors like the peak incident power, duty factor, cycles per burst, treatment time and sample volume. This adapted treatment produced sheared DNA with required size of 300bp as confirmed by gel electrophoresis. Table 6.2 shows the treatment used for DNA shearing, which led to the required band size.

**Table 6.2** Ultrasonicator treatment for extracted DNA from isolates 3D7, HL1204, HL1210 and HL1212.

Target BP (Peak)	200
Peak incident power (W)	50
Duty factor	20%
Cycles per burst	200
Treatment time (s)	240
Temperature (°C)	20
Sample volume (ul)	75

In order to achieve the right sheared DNA size (bp), different treatment times were used with the above condition. These were 25s, 32s, 50s, 65s, 80s, 120s 175s 240s 300s and 375s. The right size was achieved at 240 seconds after sheared DNA with approximately 300bp was seen on an agarose gel.



**Figure 6.1:** The product of ultrasonification using HL1204 shows a band size of 300bp. This DNA size was used for library preparation of all African isolates.

### 6.3 Library preparation

The preparation of the library was initially carried out following methods described by Oyola et al, 2012 (Methods section). However, after many unsuccessful attempts at creating a library, the methods were modified for the purpose of the work in this chapter.

#### 6.3.1 DNA end repair

End repair of fragmented DNA was carried out with less than 100ng of DNA for all isolates initially as instructed by the Illumina NEBNext protocol. Unfortunately, the experiment was unsuccessful as no product was visualised using the bioanalyser. In order to understand the problem, the DNA product was saved at every stage of the

library preparation for troubleshooting after running them on the bioanalyzer. This assisted the understanding of the underlying reasons for unsuccessful steps, and an assessment of the effects of the implement changes in conditions to make the experiments work and improve yield and library integrity.

The end preparation step was modified to use less than 50ng of DNA. The protocol for the end preparatory step, the adaptor ligation step, the clean-up step with Ampure XP beads before and after the PCR amplification steps were carried out following the modified illumina protocol as described in Chapter 3.9.

### **6.3.2 PCR Libraries**

Modification of both the Illumina protocol and the approach suggested by Oyola *et al.*, (2012) were carried out. PCR (50ul) with Kapa HiFi (KAPA Biosystems, South Africa) contained 1x Kapa HiFi buffer (with TMAC), 10mM of dNTP mix, 0.4uM of each primer pair and 1 unit of Kapa HiFi and was amplified with thermocycling conditions in table 6.2. PCR products were purified with 1x Agencourt Ampure XP beads and eluted in EB buffer as described in Section 3.9. PCR amplification products were analysed using Agilent 2100 Bioanalyzer as described in Section 3.9.

It is worth noting that on different experiments in the PCR enrichment step, three different DNA polymerase enzymes were used. These were Phusion, Kappa 2G and Kappa Hifi; all with varying PCR amplification conditions. The conditions that worked best are shown in Table 6.3 and the enzyme that proved most successful was Kapa Hifi, which was also deployed by Oyola et al., (2012).

**Table 6.3:** PCR amplification/enrichment condition: This condition was successfully used throughout the library preparation experiments. The Kapa HiFi Enzyme was the used in this step with success, and was replicated for 3D7, HL1204, HL1212 and HL1402 isolates

Cycle step	Temp	Time	Cycles
Initial denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	12
Annealing	65°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	30 seconds	1
Hold	4°C	∞	

Only successful amplifications were processed for sequencing. Library dilutions at varying concentrations were carried out before whole genome sequencing was done because initial sequencing without library dilution also proved unsuccessful. Below is a summary table of the steps and trials carried out and detail of the condition which worked for sequencing *P. falciparum* isolates in this study.

**Table 6.4a:** The flow of experiments: The experiment numbers do not exactly reflect the number of repeats carried out at each library preparation step before success was achieved. It highlights the flow of experiments and the stages where troubleshooting had to occur before the experiments could carry on. At 7, Kappa 2G and Phusion was swapped for Kappa HiFi. At 8, the PCR amplification/enrichment conditions got changed. At 9, PCR products were diluted (x10 dilution).

Experiment	Library Prep steps						
	DNA extraction yield	DNA shearing	Adaptor ligation	Purification with Ampure beads	PCR amplification	Bioanalyzer	Sequencing
1	x	-	-	-	-	-	-
2	x	x	-	-	-	-	-
3	x	x	-	-	-	-	-
4	✓	x	-	-	-	-	-
5	✓	✓	x	x	x	x	-
6	✓	✓	x	x	x	x	-
7	✓	✓	✓	✓	✓ Kappa HiFi	x	-
8	✓	✓	✓	✓	✓	✓PCR conditions	-
9	✓	✓	✓	✓	✓	✓	✓ Dilution

**Table 6.4b:** Genes sequenced in all African *P. falciparum* isolates. The table shows function of each gene and chromosome location.

Gene	Chromosome	Function
<i>Pfcr1</i> (chloroquine resistant transporter)	7	Transmembrane transporter
<i>Pfkelch13</i>	13	Protein binding
<i>Pfmrp2</i> (multidrug resistance-associated protein 2)	12	Transmembrane transporter
<i>Pfmdr1</i> (multidrug resistance protein 1)	5	Transmembrane transporter
<i>Pfubp1</i> (ubiquitin carboxyl-terminal hydrolase 1)	1	Ubiquitinyl hydrolase activity
<i>Pfap2mu</i> (clathrin vesicle-associated adaptor 2)	12	Vesicle-mediated transport
<i>Pfnhe1</i> (sodium/hydrogen exchanger)	13	Sodium ion transport
<i>Pfatpase6</i> (calcium-transporting ATPase)	1	Calcium ion transport
<i>Pf26s-protsu</i> (26S proteasome regulatory subunit )	14	Endopeptidase activity
<i>Pfatpase4</i> (non-SERCA-type Ca <sup>2+</sup> -transporting P-ATPase )	12	Hydrogen ion transmembrane transporter activity
<i>Pfmrp1</i> (multidrug resistance-associated protein 2)	1	Transmembrane transporter

### 6.3.3 Summary statistic of sequence data

The quality of reads achieved for all African isolates sequenced was analysed for read length, paired end reads and reads mapped against the 3D7 reference. The data summary is shown in Table 6.4b

**Table 6.4c: Summary of sequence data for isolates HL1204, HL1210 and HL1212.**

Isolate	Read length	PE reads	Reads mapped against reference
HL1204:	200	829,157	84.40%
HL1210:	200	5,398,716	84.50%
HL1212:	200	11,334,878	61.10%

PE = paired end, reads were mapped against the 3D7 reference.

### 6.3.4 Isolate from Liberia

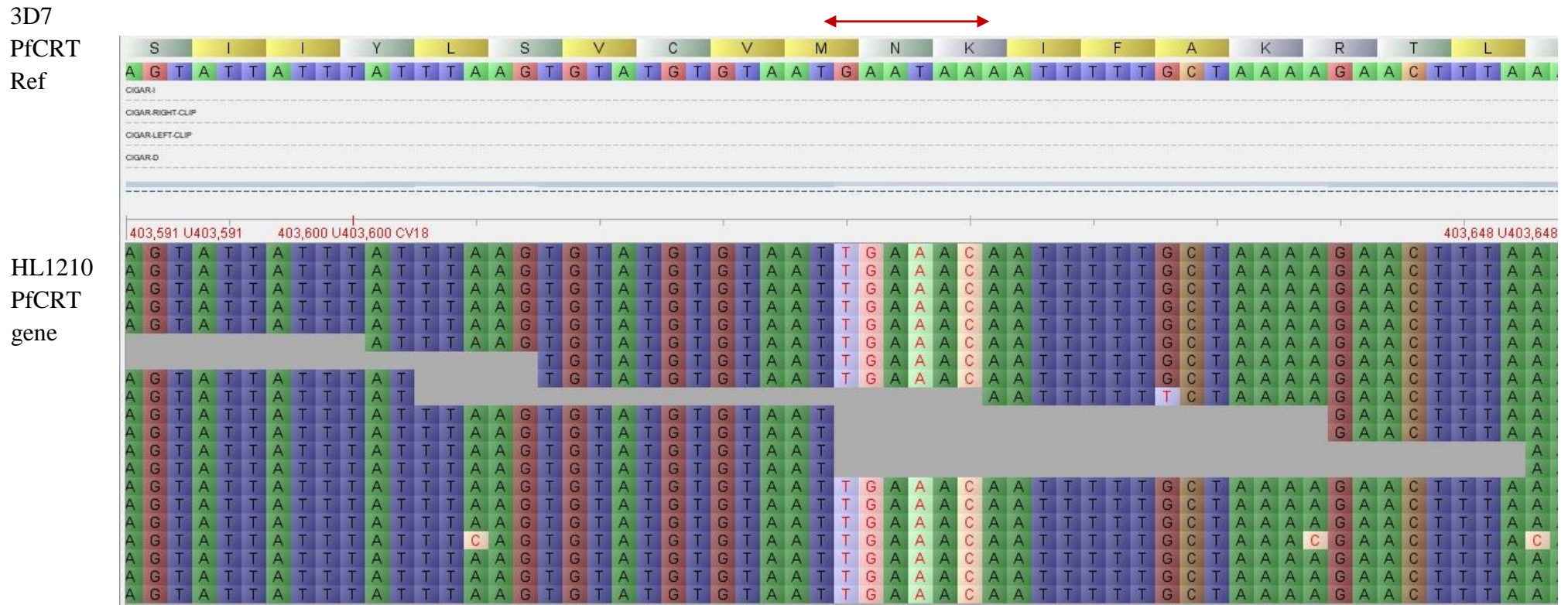
A new isolate from Liberia was added to the analysis carried out in this Chapter. The isolate was from a patient who presented with *P. falciparum* malaria at the Hospital for Tropical Diseases in London after an initial treatment in Liberia with artemether-lumefantrine. The patient was successfully treated with atovaquone-proguanil.

Whole genome sequencing of the isolate before and after treatment with atovaquone-proguanil was carried out by the Sanger Institute while the data analysis for this isolate was carried out as part of this chapter of this thesis. Genome analysis was carried out looking at single nucleotide polymorphisms present in 11 candidate genes of interest, associated with artemisinin resistance. The mutations present before and after treatment were described and presented in the appendix section.



### 6.3.5 Data Analysis

Analysis of whole sequencing data was carried out using both tablet software, which facilitated the visualisation of SNP and Geneious software as described in the Methods section in Chapter 3.10.



**Figure 6.2:** showing a pairwise alignment of the 3D7 *pfcr*t gene and the HL1210 (Ghana) *pfcr*t gene. The arrow shows mutations at position 72-76 which corresponds to change in amino acid from CVMNK to CVIET.

<b>PfCRT</b>	74-76	140	220	239-244	269	297	368
<b>3D7</b>	MNK	S	A	KIDILR	L	S	D
<b>HI1210</b>	IET	S	S	EIFILM	L	S	D
<b>HL1212</b>	MNK	S	A	KIDILR	L	L	D
<b>HL1204</b>	MNK	S	A	KIDILR	L	S	D
<b>Lib</b>	IET	H	A	KIDILR	E	S	I

<b>PfMDR1</b>	86	184
<b>3D7</b>	N	Y
<b>HI1210</b>	Y	F
<b>HL1212</b>	N	F
<b>HL1204</b>	N	F
<b>Lib</b>	N	F

<b>PfMRP2</b>	199	578	583	630	714	796	798	940	1395	1531	1643	1910
<b>3D7</b>	L	N	N	D	K	S	R	R	L	L	A	N
<b>HI1210</b>	L	D	S	D	K	S	A	R	I	L	A	D
<b>HL1212</b>	V	N	N	D	K	A	R	R	L	I	A	N
<b>HL1204</b>	L	N	N	D	K	S	R	S	I	L	A	D
<b>Lib</b>	V	N	N	N	I	S	R	R	L	L	V	N

**Table 6.5:** Whole genome sequencing data from all *P. falciparum* isolates showing single nucleotide polymorphisms (SNPs) present in genes *pfCRT*, *pfmdr1* and *pfmrp2*. The variant frequency shows the percentage of the SNPs present relative to the coverage at that position.

#### 6.4 Polymorphisms in HL1210 isolate

Whole genome sequencing data for HL1210 isolate is shown in Tables 6.5, 6.6 and 6.8. HL1210 has the *pfcr*t mutation CVIET at positions 74-76, which is known to confer resistance to chloroquine. Other observed mutations in the *pfcr*t gene include mutation in position A220S which have been described in studies done in Ghana, Senegal, Mali, Burkina Faso (West Africa), Kenya, Malawi (East Africa), Thailand, Cambodia, Vietnam (Southeast Asia) and Papua New Guinea (Preston *et al.*, 2012). Other mutations found in the *pfcr*t gene of the HL1210 isolate include mutations at positions K239E, D241F, and R244M. These mutations are novel and not been described in any published studies.

Mutations observed in *pfmdr*1 include an amino acid change of asparagine to tyrosine at positions 86, and tyrosine to phenylalanine at position 184. There were 5 non-synonymous mutations found in *pfmrp*2 gene of HL1210 which have not been described in any published work to date. The mutations are N578D, N583S, R798A, L1395I and N1910D (Table 6.5).

Various non-synonymous mutations were found in the *Pfubp*1 gene and none have been described in any published work to date (Appendix III, Table 4 & 5). Similarly, mutations in *Pfnhe*1 for HL1210 were also found at positions L415R, N621F, and D1022P, and none have been described previously.

Non synonymous mutations were found in the *pfap2mu* gene with a mutation at position 336 from asparagine to serine. More so, one mutation was observed in the *pfatpase* 6 gene at position E424Y and another mutation in the *pf26s-protsu* gene at position K529A (Table 6.6, 6.8). These mutations are novel. No mutations in *pfk*13, *Pfmrp*1, *Pfmrp*2 and *Pfatpase*4 were observed in the HL1210 isolate

<b>PfNHE1</b>	415	494	621	950	1022	1362	1366	1380
<b>3D7</b>	L	L	N	V	D	N	K	K
<b>HI1210</b>	R	L	F	V	P	N	K	K
<b>HL1212</b>	L	I	N	V	D	N	K	K
<b>HL1204</b>	L	L	N	V	D	N	K	K
<b>Lib</b>	L	L	N	G	D	H	N	N

<b>AP2MU</b>	336
<b>3D7</b>	N
<b>HI1210</b>	S
<b>HL1212</b>	N
<b>HL1204</b>	N
<b>Lib</b>	N

<b>ATPASE6</b>	424	431
<b>3D7</b>	E	E
<b>HI1210</b>	Y	E
<b>HL1212</b>	E	E
<b>HL1204</b>	E	E
<b>Lib</b>	E	K

**Table 6.6:** Whole genome sequence data from all *P. falciparum* isolates showing SNPs present in genes *pfnhe*, *pfap2mu* and *pfatpase6*. Genes in all African isolates were compared to a 3D7 reference.

## 6.5 Polymorphisms in HL1212 isolate

Whole genome sequencing data for HL1212 isolate are shown in Tables 6.5, 6.6 and 6.8. While HL1212 is wild type at positions 72-76 (CVMNK) in the *Pfcrt* gene, it has a mutation at position 297 with amino acid change from valine to leucine. This non synonymous mutation has not been described in any published work till date.

Mutations in the multi-drug resistant protein 2 of HL1212 show non-synonymous mutations that have been reported in various other studies. These mutations are L199V, S796A and L1531I and were present in the HL1212 isolate with 100% variant frequency. Mutations in the *Pfmdr1* gene of HL1212 also showed a non-synonymous mutation at position 184, with an amino acid change from tyrosine to phenylalanine (Y184F). This mutation gives the NFSND mutation in *Pfmdr1*.

Analysis of the *Pf26-protsu* gene showed a non-synonymous mutation present at position 533 with an amino acid change from tryptophan to alanine (Table 6.8). This mutation has not been described in any published work. One non-synonymous mutation was observed in *Pfnhe* gene at position 494 with an amino acid change from leucine to isoleucine with a 100% variant frequency (Table 6.6).

Analysis of the *pfUbp1* showed the most variation compared to all other genes of interest analysed in this study. All of the identified mutations have not been described in any published work till date (Appendix III, Table 8 & 9). However, the HL1212 *ubp1* gene shares some similar mutations as the HL1210 *pfubp1* mutations shown in the Appendix section.

No SNPs were observed in genes *Pfk13*, *pfmpr1*, *Pfatpase4*, *Pfatpase6* and *Pfap2mu* of the HL1212 isolate. However, there were many nucleotide variations present.

<b>Mitochondria</b>						
Position	Ref	amino acid	Mutation	Change	Gene	SYN/NONSYN
772	TTA	L	CTA	LEUCINE (L)	Cox gene	SYN
<b>Apicoplast</b>						
412	GGT	G-Glycine	AGT	Serine (S)		NON SYN
431	TGT	C-Cysteine	TAT	Tyrosine (Y)		NON SYN
10603	CAA	Q-Glutamine	AAA	Lysine (K)		NON SYN
20427	TTA	L-leucine	TTG	Leucine (L)		SYN

**Table 6.7:** Mutations present in the mitochondrial and Apicoplast genome of HL1212 isolate shows the presence of 3 non-synonymous mutations present at positions 412, 431 and 10603.

## 6.6 Polymorphisms in HL1204 isolate

The HL1204 isolate (Kenya) had wild-type alleles at positions 72-76 for the *Pfcrt* gene (CVMNK). Also this isolate possessed no mutation in the Kelch propeller region of *P. falciparum*. It was also wild-type for SNPs in *Pfap2mu* and *pf26s-protsu* genes. However, HL1204 did have 3 non-synonymous mutations in MRP2 gene (Table 6.5) which have not been described in any published work. For mutations in the *Pfmdr1* gene, HL1204 contains the NFSND mutation with a SNP which changes from tyrosine to phenylalaline at position 184. The *Pfubp1* gene contained low numbers of insertions/deletions, and these have been listed in appendix section.

<b><i>Pfk13</i></b>	112	580	539
<b>3D7</b>	G	C	R
<b>HI1210</b>	G	C	R
<b>HL1212</b>	G	C	R
<b>HL1204</b>	G	C	R
<b>Lib</b>	E	C	R

<b><i>PfATPASE4</i></b>	1045	1081
<b>3D7</b>	N	Q
<b>HI1210</b>	N	Q
<b>HL1212</b>	N	Q
<b>HL1204</b>	N	Q
<b>Lib</b>	K	K

<b><i>Pf26s-PROTSU</i></b>	529	533-534	711
<b>3D7</b>	K	WE	K
<b>HI1210</b>	A	WE	K
<b>HL1212</b>	K	AE	K
<b>HL1204</b>	K	WE	K
<b>Lib</b>	K	WA	R

Table 6.8: Observed non-synonymous mutations in the *pfk13*, *pfatpase4* and *pf26s-protsu* gene in all *P. falciparum* African isolates. The *pfubp1* genes for all isolates sequenced contains SNPs and insertions and deletions and can be found in the appendix section.





	Isolate	Mean DHAEC50(nm)	$\Delta$ T0-T4	pfk13 <sup>a</sup>	pfcr1 <sup>b</sup>	pfmdr1 <sup>c</sup>	pfap2mu <sup>d</sup>	pfubp1	pf26s- protsu	mrp2
1	HL1204	2.4	1.2	wt	CVMNK	NFSND	wt	multiple	wt	multiple
2	HL1210	12	1.6	wt	CVIET	YFSND	N336S	multiple	K529A	multiple
3	HL1212	23	19.2	wt	CVMNK V297L	NFSND	wt	multiple	W533A	multiple

**Table 6.9:** Phenotypic and genetic information of isolates HL1204, HL1210 and HL1212

- Nomenclature used shows wild-type (reference) amino acid encoded at the codon of interest.
- Nomenclature used shows single letter amino acid code for codons 72-76 which is associated with chloroquine resistance
- Mutations in *pfmdr1* locus correspond to changes at codon 86, 184, 1246 (NFD)
- Nomenclature used shows wild-type amino acid encoded at the codon of interest, followed by amino acid encoded by the variant observed
- Multiple: refers to the presence of various mutations in the gene of interest.
- The shaded regions show the isolates which show the biggest difference in both phenotypic and genotypic information.

## Polymorphisms in the isolate from Liberia

Before treatment with atovaquone-proguanil (after initial treatment with artemether-lumefantrine), the isolate from Liberia had the CVIET mutation indicative of resistance to chloroquine. Other mutations present in *Pfcr*t were S140H, L269E and D368I. After treatment with atovaquone-proguanil, all these mutations described were not detected and the isolate harboured the chloroquine wild-type haplotype, CVMNK.

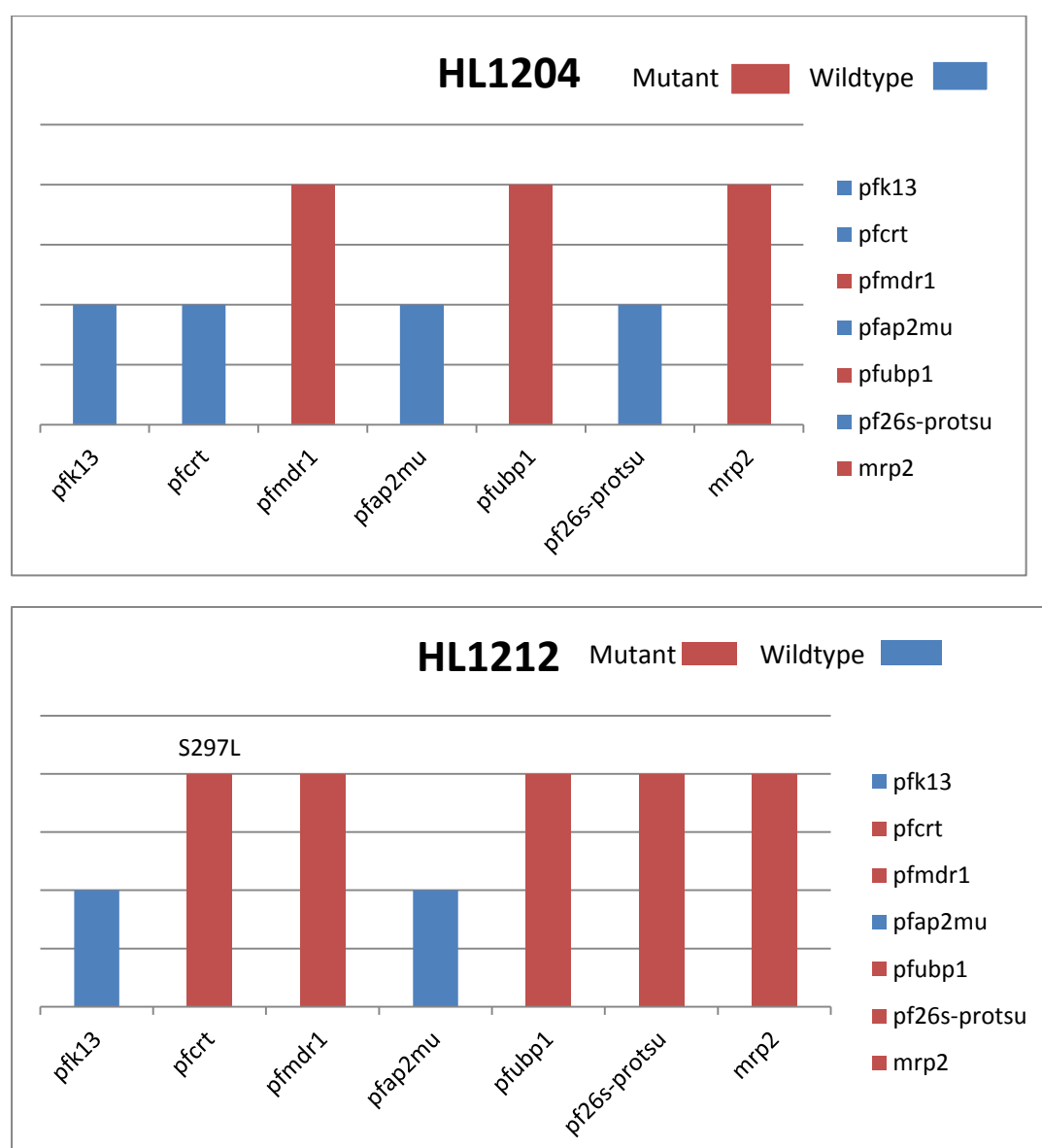
The Liberian isolate harboured a G112E mutation in the *Pfk13* locus which was also present after treatment atovaquone-proguanil. An insertion was observed in the *ap2mu* gene at position 233 (N233k), which was not present after treatment. One SNP in the *Pfatpase6* gene was found before and after treatment (E431K) while 2 SNPs were found in the *atpase4* gene (Q1081K and N1045K). Numerous SNPs were found in the *ubp1* gene (appendix III, Table 11), which were also present after treatment with atovaquone-proguanil. New mutations were also detected in the *ubp1* gene after treatment and these are listed in the appendix.

The *pf26s-protos* gene had 2 non-synonymous mutations which were also detected after treatment with atovaquone-proguanil (E534A, K711R).

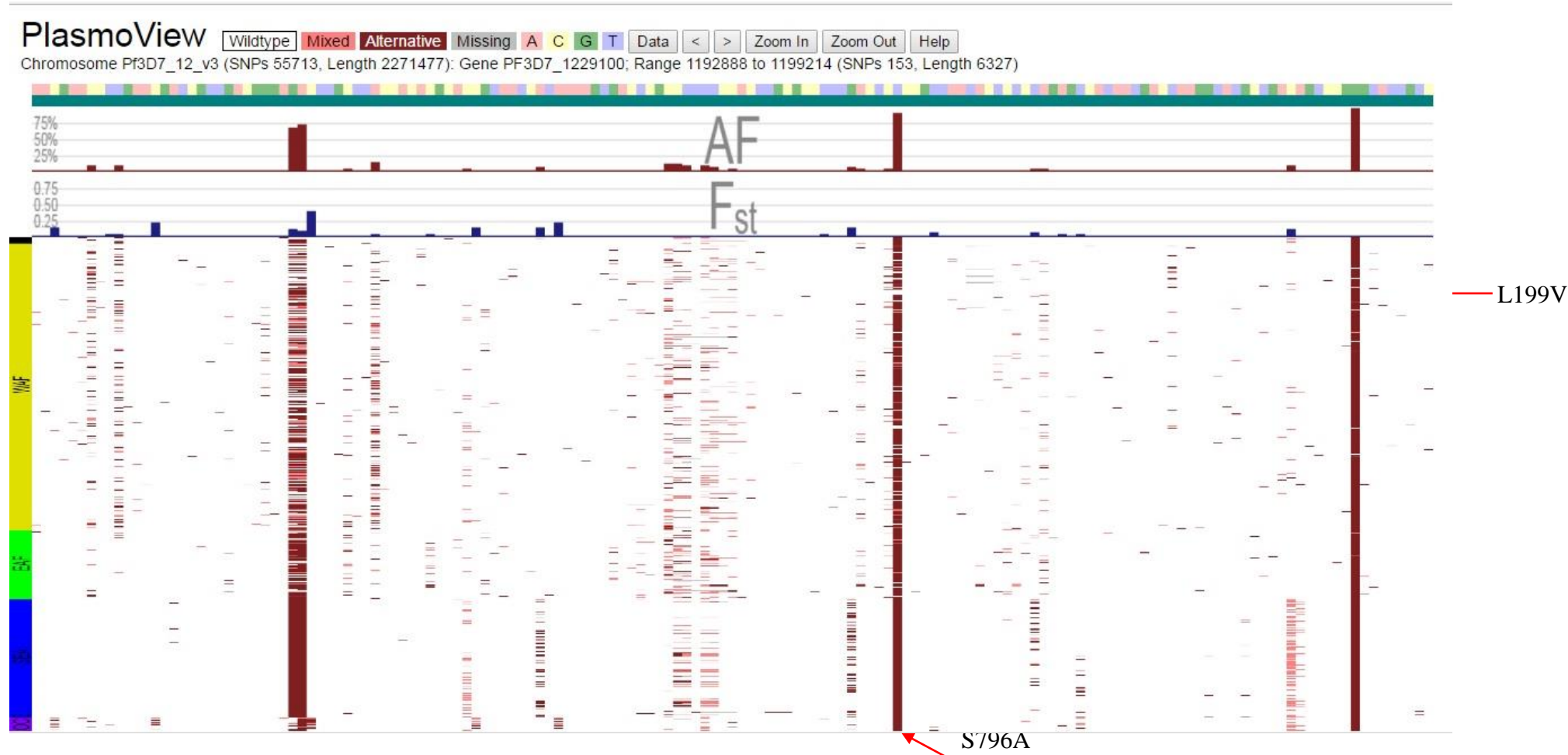
Twelve SNPs were found in the *mrp2* gene, four of which were present after treatment. These are the A1643V, K714I, D630N and L199V mutation. Nine mutations were found in the *Pfnhe1* gene before treatment, four of which were detected after treatment (appendix III, Table 13).

## 6.7 Comparison of genetic information for HL1204 and HL1212

HL1204 and HL1212 isolates showed the biggest difference in mean DHA EC<sub>50</sub> in both Chapters 4 and 5. A comparison of mutations present in selected genes of interest was performed to assess if both isolates differed and to what extent. The graphs show binary data generated for the presence of a mutation (mutant) or the absence of a mutation (wild-type).



**Figure 6.2:** Histogram showing the presence or absence of a mutation in selected genes of interests. Binary data was generated (wild-type =1, mutant=2) to identify the types of mutations present in both HL1204 and HL1212. HL1212 contains CNMKNK and a mutation in S297L. The Y-axis represents binary data that denotes wildtype =1, mutant =2.



**Figure 6.3:** Global sequence diversity in *pfmrp2* gene across genome sequences from *P. falciparum* isolates mapped to 3D7 reference genome (Preston et al, 2014). Coloured bars at any chromosome position (horizontal axis) denote a nonreference substitution at that residue in the relevant isolate (vertical axis). Mutations at codon L199V, S796A and L1531I are highlighted using red arrows. These non-synonymous mutations are present in the HL1212 isolate from Nigeria.

## 6.8 Discussion

This chapter described non-synonymous mutations present in 11 candidate genes associated with artemisinin resistance in the African isolates HL1204, HL1210, HL1212 and the added *P.falciparum* line from Liberia. The HL1204 isolate from Kenya had the least amount of mutations observed in candidate genes when compared to mutations in HL1210 and HL1212 isolates. All isolates HL1204, HL1210 and HL1212 were wild-type at the propeller–encoding domain of the *pfk13* locus, which shows that the phenotypic profiles observed in Chapters 4 and 5, particularly for HL1212 (Mean EC<sub>50</sub> 23nM;  $\Delta T_0$ -T<sub>4</sub> = 19.2) are independent of events in the kelch13 propeller region of the parasite. This result shows that the African isolates do not exhibit reduced artemisinin susceptibility as seen in the Greater Mekong Region (Ashley et al, 2014; Arieu et al, 2014; Menard et al, 2016) and is consistent with other studies which have shown that genetic events in Africa are independent of *pfk13* (Menard et al, 2016).

The isolate from Liberia does have a novel *pfk13* non-propeller region mutation (Gly112Glu) which was present before and after treatment with atovaquone-proguanil. This mutation has not been described elsewhere and would require further study.

Mutations observed in the *Pfcr*t gene coupled with drug sensitivity assay (Chapter 4) show that HL1204 and HL1212 are chloroquine sensitive compared to HL1210 which is resistant to chloroquine. However, HL1212 harboured another mutation S297L which has not been described in published studies. The CVMNK haplotype has been associated with artemether-lumefantrine (AL) in Kenya (Humphreys et al, 2007). The presence of a novel mutation S297L in HL1212 isolate from Nigeria is a potential concern as certain *P.falciparum* genotypes at *pfcr*t have been reported to survive ACT at submicroscopic level and could contribute to onward transmission (Henriques et al, 2013).

Both HL1204 and HL1212 had the NFD mutation at codons 86, 184, and 1246 in the *pfmdr1* locus, which has been shown to be associated with arthemeter-lumefantrine (AL) failure in Kenya and Tanzania (Humphreys et al., 2007; Henriques et al., 2013). None of the isolates contained any mutation at codons 1034, 1042 and 1246 of the *Pfmdr1* genes. The data shown here is consistent with other published research especially for the HL1212 isolate from Nigeria (Oladipo et al, 2015).

Mutations in *pfap2mu* that have been described elsewhere to be associated with artemisinin drug resistance were not found in this study, particularly the S160N mutation. However, another novel mutation at position 336 with amino acid change from asparagine to serine was found in the HL1210 isolate, and a mutation at position N233K (insertion) was found in the isolate from Liberia, before and after treatment with atovaquone-proguanil.

For all African isolates, numerous novel mutations were found in the *Pfubp1* gene. Mutations in the *Pfubp1* gene have also been shown to survive more often after ACT treatment at sub-microscopic level through directional selection (Henriques *et al.*, 2014). A novel mutation was also found in the *Pf26s-protsu* gene of HL1212 which has not been described in any published work to date nor has it been described in the Plasmoview software (Preston *et al.*, 2014). The *Pf26-protsu* gene in the Liberian isolate has two novel mutation E534A and K711R which were present before and after treatment and these have not been published in any study.

Analysis of the *pfmrp2* gene for all isolates showed various non-synonymous mutations. While the HL1204 and HL1210 isolates had 5 non-synonymous mutations each in *Pfmrp2* genes which have not been described in any published work, HL1212 had 3 mutations which have been described in other studies. The first mutation found was L199V, which has been described in West Africa (Gambia, Senegal, Ghana, and Burkina Faso), East Africa (Kenya and Malawi), and Southeast Asia (Thailand, Cambodia and Vietnam) and also Papua New Guinea (PNG) (Preston *et al.*, 2014). The second and third mutations, S796A and L1531I have also been described in countries across West Africa, EastAfrica, Southeast Asia and Papau New Guinea (Preston *et al.*, 2014). The isolate from Liberia also has the L199V mutation found in HL1212 but the other mutations found before and after treatment with atovaquone-proguanil (A1643V, D630N) are novel.

It was shown that *pfmrp2* gene is mostly expressed in the ring stages of *P.falciparum* (Le Roch *et al.*, 2003). Another study has also showed that that drug pressure could induce a subpopulation of ring stages into developmental arrest, which can explain artemisinin tolerance in *P. falciparum* (Witkowski *et al.*, 2010). This may be an explanation as to why the HL1212 isolate showed a high EC<sub>50</sub> value (23nM) and  $\Delta T_0$ -T<sub>4</sub> value (19.2nM) that is comparable to drug sensitivity values for slow-clearing isolates from Cambodia (Chapter 5).

While there were numerous SNPs found in the *pfubp1* gene in HL1210, HL1212 and the Liberian isolate (insertions/deletions in HL1204), none of these have been described in other

studies. All isolates were wild-type at codon 1528, identified by Bormann *et al.*, (2013) as being associated with *in-vitro* parasite responses to artemisinin in Kenya. Other mutations in *pfubp1* found in other studies (Henriques *et al.*, 2014) were also not observed in this study. Analysis of *Pf26s-protsu* in HL1212 from Nigeria showed the presence of a novel gene at codon 533, with a change in amino acid from tryptophan to alanine.

All isolates were polyclonal in nature and exhibited variations at nucleotide positions in the candidate genes of interest analysed (Appendix showing the HL1212 *pfcrt* gene as an example). The role these variations at nucleotide positions play with regards to susceptibility to DHA needs further study.

The HL1204 and HL1212 isolates were prioritised in chapter 5, primarily because they had the largest phenotypic differences measured. Direct comparison of whole genome sequence of both isolates showed a clear difference in the presence of mutations associated with artemisinin drug resistance (Table 6.12 or Figure 6.2). Whilst the HL1204 isolate was shown with the pulse assay to be sensitive to DHA, the H1212 isolate showed a clear reduction in sensitivity to DHA (Chapter 5, Table 6.12), again quite comparable to EC<sub>50</sub> values shown for the slow clearing parasite lines from Cambodia (Cam 3.11<sup>C580Y</sup>, Cam 3.11<sup>R539T</sup>).

## 6.9 Conclusion

Work done in this thesis has shown that small differences in DHA susceptibility do exist and can be measured *in-vitro*. These differences, especially as shown in HL1212, may be mediated by complex multi-locus genotypes as shown in this Chapter, and are definitely not associated with variants of the *pfkelch13* locus seen in the Greater Mekong region. It is therefore important to protect the efficacy of the current front-line ACT by validating potential genetic markers, as those shown in this chapter, for monitoring the African parasite population. The work done in this chapter could inform future functional studies deploring gene editing.



## **CHAPTER 7: General conclusions and future work**

## 7.1 General Conclusion

The central aim of this thesis is to identify a scalable assay able to discriminate between parasite lines with small differences in artemisinin susceptibility and to identify phenotypes relevant to the genetic exploration of artemisinin resistance in African settings. As a result, 4 assays ( $EC_{50}^{48h}$ ,  $EC_{50}^{6h}$ ,  $EC_{50}^{6hT0-T4}$  and  $RSA^{0-3h}$ ) were used to determine DHA susceptibility of African lines and comparisons were made with artemisinin resistant lines from Cambodia to determine which assay best discriminates small differences that exists in DHA sensitivity.

The work done in Chapter 4 showed that the  $EC_{50}^{6h}$  assay was successful in teasing out small differences that exists, in DHA sensitivity of African lines HL1204, HL1210, HL1212 and HL1402. It also showed that the standard assay  $EC_{50}^{48h}$  was not sensitive in discriminating the differences in DHA  $EC_{50}$  that exist in these parasite lines. The pulse assay was done as a development of this project and so the work done in the next chapter tested this methodology and made comparisons using parasite lines from Cambodia (Cam 3.11<sup>wt</sup>, Cam 3.11<sup>C580Y</sup>, Cam 3.11<sup>R539T</sup>). The key findings in this Chapter are that the  $EC_{50}^{6h}$  assay can be used to test artemisinin drug sensitivity in Africa. The assay provides an alternative test that is less laborious, cheaper and developed specifically for African parasites which face the current ACT drug pressure.

In chapter 5, the  $EC_{50}^{6h}$  assay was successful at discriminating small differences in DHA susceptibility that exist between parasite lines from Africa but was unsuccessful in doing the same for slow clearing Cambodian lines with known artemisinin resistance mutation in *pfk13*. As a result, the assay was adapted taking into account evidence that the early ring stage of *P.falciparum* is associated with drug resistance (Teuscher *et al.*, 2010; Witkowski *et al.*, 2010). The adapted assay,  $EC_{50}^{6hT0-T4}$  assay was used to test all parasite lines and this assay was successful at discriminating the differences that exists in DHA sensitivity of both lines from Africa and Cambodia. The HL1212 isolate showed the most interesting result with DHA sensitivity profile similar to the slow clearing Cambodian lines tested even though a *pfk13* mutation was absent. The ring stage survival assay (RSA) was deployed to compare relative DHA susceptibility of African and Cambodian isolates and then see how it compares to our other assays.

The RSA was successful in estimating percentage parasite survival after DHA exposure in the Cambodian lines tested and showed no parasite survival estimates for the African lines.

Key findings in this chapter are that the RSA is successful at identifying artemisinin resistant parasites and also successful at identifying artemisinin sensitive parasites. Since it gives a binary measure, this assay cannot be used to investigate artemisinin resistance over a period of time. As a result, the  $EC_{50}^{6hT0-T4}$  assay is the most sensitive assay that best discriminates the DHA sensitivity that exists between all isolates used in this project. This assay can be used as a new approach to the *in vitro* phenotype and can be used as a surveillance tool to contain artemisinin resistance especially in the African setting. The  $\Delta T_0-T_4$  assay can also be used to check for change in drug sensitivity over a period of time.

The work done in chapter 6 showed single nucleotide polymorphisms present in 11 chosen genes of interest associated with artemisinin resistance. Phenotype data from chapter 4 and 5 were linked to genotype information analysed in chapter 6, although no strong correlation could be made. The presence of novel SNPs in these isolates could be used to inform functional studies deploying gene editing, particularly in HL1212 which showed an elevated mean  $EC_{50}$  estimate in both the  $EC_{50}^{6h}$ , and  $EC_{50}^{6hT0-T4}$  assay. The key findings in this chapter are that the small differences in DHA susceptibility may be mediated by complex multi-locus genotypes, which are not associated with variants of *pfk13* locus seen in the Greater Mekong Region.

## 7.2 Study limitations

The parasite lines used were polyclonal and as a result, variations were observed within and between drug assay experiments for the pulse assay ( $EC_{50}^{6h}$ ). A solution would be to carry out limiting dilution cloning on all African lines to separate the clones and then test all clones using the assay methods and comparing estimates to the parental lines.

The format used to carry out the assays employed 1.5ml microcentrifuge tubes. During experiments, it was observed that not all parasites may have been exposed to the DHA specifically the ones which settle at the bottom of the eppendorf tube. A solution to this would be to use a 24 well plate with bigger surface area to ensure all parasite cells get adequate DHA exposure. This method is currently being investigated in our lab.

### 7.3 Future work

- Limiting dilution of all parental African lines
- Testing the assays ( $EC_{50}^{48h}$ ,  $EC_{50}^{6h}$ ,  $\Delta T_0-T_4$  assay,  $RSA^{0-3h}$ ) on each clone and investigating how they compare to the parental line.
- Carrying out whole genome sequencing of each clone and compare SNPS to parental line
- Using a 24 well plates to optimise the 6 hour pulse assay
- Deploying functional studies testing some of the novel mutations (in *pfmrp2*, *pfubp1*, *pfap2mu*) discovered in chapter 6 and seeing if these allele changes affect the DHA susceptibility of the isolate HL1212. This could be done using zinc-finger nuclease (ZFN) - mediated gene editing or the clustered, regularly interspaced, short palindromic repeat (CRISPR) technology. Transgenic lines can be generated harbouring some of the novel mutation using a laboratory adapted strain. Parasite clones which express the variant alleles can then be tested for their susceptibility to a panel of antimalarial drugs including artemisinin derivatives (DHA) and partner drug

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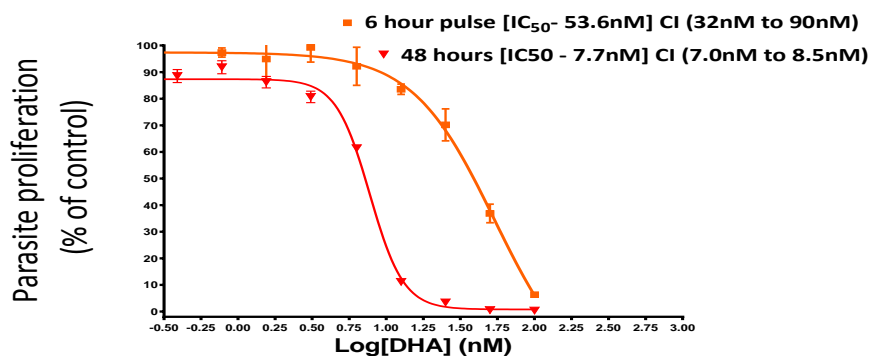
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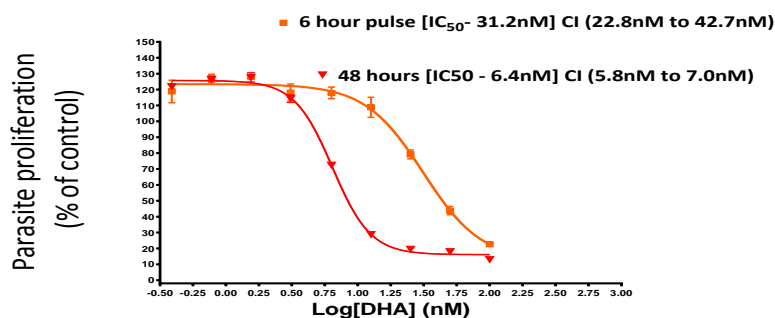


## APPENDIX I -

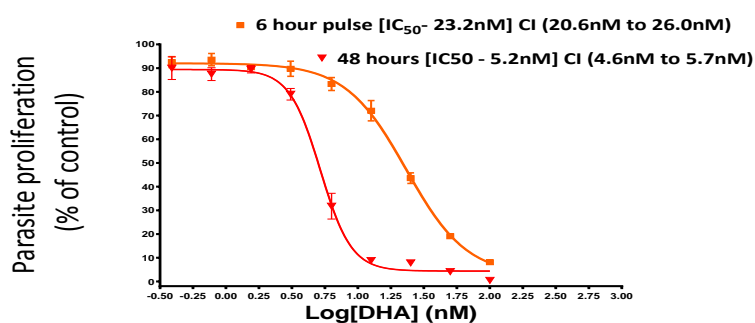
Graph showing IC<sub>50</sub> values of KH2 after a 6 hr and 48hr treatment with DHA



Graph showing IC<sub>50</sub> values of KH2 after a 6 hr and 48hr treatment with DHA

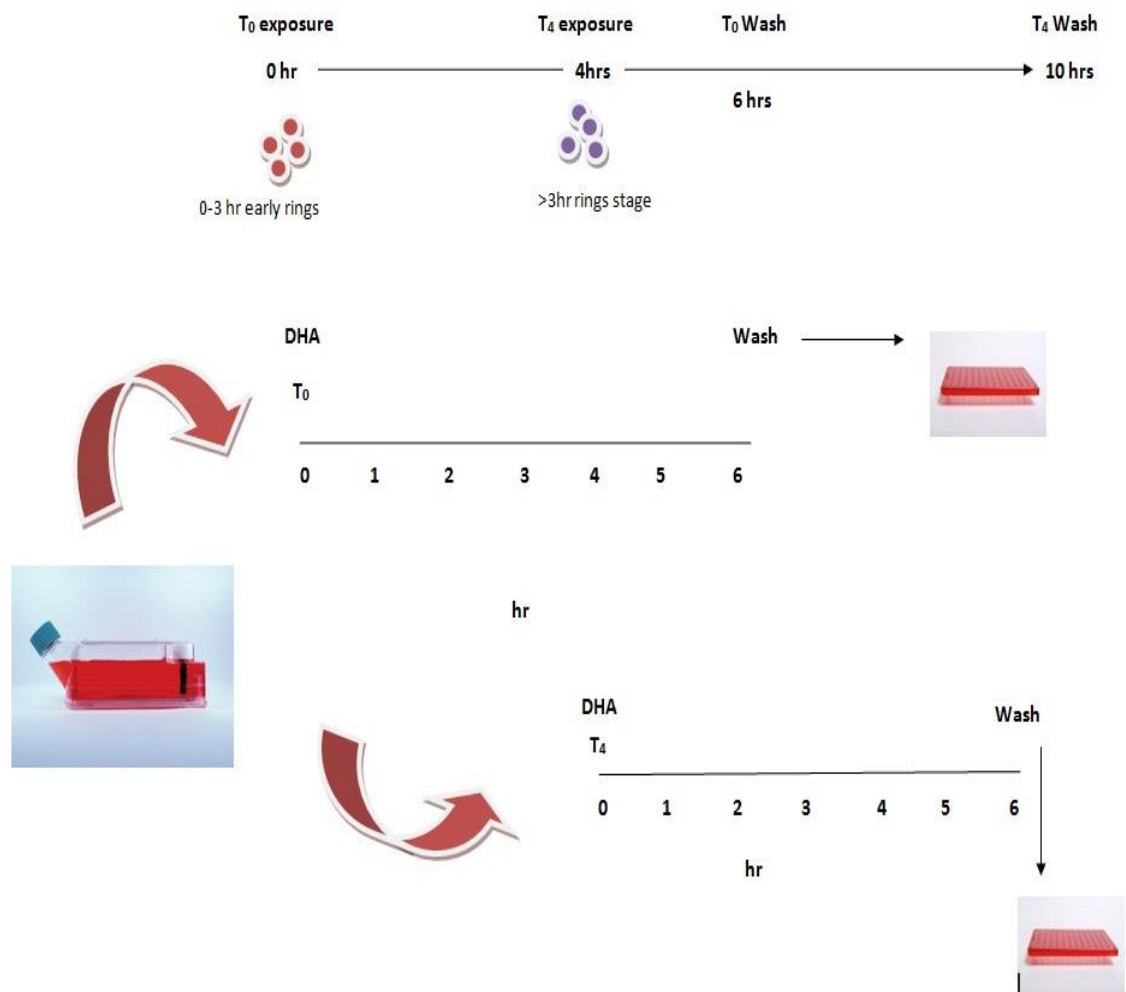


Graph showing IC<sub>50</sub> values of KH2 after a 6 hr and 48hr treatment with DHA



**Figure 1:** Showing the EC<sub>50</sub><sup>6hr</sup> estimate for KH2 isolate changing with each DHA 6-hour pulse assay and 48-hour standard assay. The top graph shows an EC<sub>50</sub><sup>6hr</sup> estimate of 53.6nM, which changed to 31.2nM and finally to 23.2nM (bottom graph). These highlight changes seen in the KH2 isolate signifying a contamination with an unknown isolate (See Chapter 5).

## APPENDIX II -



**Figure 1:** Showing a schematic diagram of the EC<sub>50</sub><sup>6hr T<sub>0</sub>-T<sub>4</sub></sup> assay. The steps are explained in Chapter 3 (Methods Chapter).



### APPENDIX III -

Table 1: Genetic variation present in the HL1212 *pfcr* gene. Variant frequency was set at 5% in order to capture variation present in the gene. HL1212 is a multiclonal isolate with the number of clones described in Chapter 4 of this thesis. Allele variation was also observed in the other African isolates used in this study. This table is used as an example and to highlight this phenomenon is the same for other African isolates.

Change	Min	Max	Change	Coverage	Polymorphism Type	Reference Nucleotide(s)	Variant Frequency	Codon position
A	1,216	1,216	G -> A	34	SNP (transition)	G	8.80%	406
A	1,215	1,215	T -> A	36	SNP (transversion)	T	22.20%	405
G	1,212	1,212	A -> G	46	SNP (transition)	A	8.70%	404
A	1,209	1,209	G -> A	61	SNP (transition)	G	19.70%	403
T	1,206	1,206	A -> T	63	SNP (transversion)	A	6.30%	402
T	1,200	1,200	A -> T	64	SNP (transversion)	A	14.10%	400
G	1,198	1,198	A -> G	62	SNP (transition)	A	25.80%	399
T	1,196	1,196	A -> T	62	SNP (transversion)	A	9.70%	398
T	1,191	1,191	C -> T	44	SNP (transition)	C	18.20%	397
A	1,188	1,188	T -> A	26	SNP (transversion)	T	19.20%	396
A	1,185	1,185	T -> A	23	SNP (transversion)	T	34.80%	395
T	1,184	1,184	A -> T	21	SNP (transversion)	A	19.00%	394
T	1,179	1,179	A -> T	17	SNP (transversion)	A	35.30%	393
A	1,178	1,178	T -> A	17	SNP (transversion)	T	23.50%	392
A	1,173	1,173	C -> A	15	SNP (transversion)	C	26.70%	391
T	1,172	1,172	A -> T	17	SNP (transversion)	A	23.50%	390
A	1,163	1,163	C -> A	13	SNP (transversion)	C	30.80%	387
A	1,161	1,161	T -> A	13	SNP (transversion)	T	30.80%	387
A	1,158	1,158	T -> A	13	SNP (transversion)	T	30.80%	386
T	1,152	1,152	C -> T	15	SNP (transition)	C	20.00%	384
AA	1,146	1,147	TG -> AA	15	Substitution	TG	53.30%	382
G	1,144	1,144	T -> G	15	SNP (transversion)	T	53.30%	381

Table 2: Genetic variation present in the HL1212 *pfert* gene. Variant frequency was set at 5% in order to capture variation present in the gene. HL1212 is a multiclonal isolate with the number of clones described in Chapter 4 of this thesis. Allele variation was also observed in the other African isolates used in this study. This table is used as an example and to highlight this phenomenon is the same for other African isolates.

Change	Minimum	Maximum	Change	Coverage	Polymorphism Type	Reference Nucleotide(s)	Variant Frequency	Codon position
AA	1,141	1,142	TT -> AA	11 -> 13	Substitution	TT	18.2% -> 30.8%	380
A	1,140	1,140	T -> A	11	SNP (transversion)	T	27.30%	380
GTG	1,137	1,139	AAC -> GTG	13 -> 16	Substitution	AAC	30.8% -> 43.8%	379
ATA	1,133	1,135	TCG -> ATA	16	Substitution	TCG	43.80%	377
ATA	1,129	1,131	GAT -> ATA	16	Substitution	GAT	43.80%	376
A	1,112	1,112	G -> A	18	SNP (transition)	G	50.00%	370
TA	1,108	1,109	GT -> TA	18	Substitution	GT	50.00%	369
GAA	1,104	1,106	TGT -> GAA	19	Substitution	TGT	47.40%	368
G	1,103	1,103	A -> G	21	SNP (transition)	A	19.00%	367
T	1,100	1,100	G -> T	21	SNP (transversion)	G	28.60%	366
TAG	1,096	1,098	GCC -> TAG	21	Substitution	GCC	28.60%	365
ATAC	1,038	1,041	TATT -> ATAC	25	Substitution	TATT	16.00%	346
AA	1,025	1,026	CC -> AA	24	Substitution	CC	16.70%	341
			TCGAC ->					
GAATA	1,010	1,014	GAATA	24	Substitution	TCGAC	25.00%	336
G	1,006	1,006	A -> G	28	SNP (transition)	A	21.40%	335
A	1,002	1,002	C -> A	26	SNP (transversion)	C	76.90%	334
A	999	999	C -> A	24	SNP (transversion)	C	16.70%	333
C	991	991	T -> C	24	SNP (transition)	T	33.30%	330
T	989	989	A -> T	20	SNP (transversion)	A	40.00%	329
T	978	978	C -> T	16	SNP (transition)	C	25.00%	326
T	977	977	A -> T	18	SNP (transversion)	A	33.30%	325

Gene	Gene ID	Chromosome	Gene position	Base Change	Coverage	Polymorphism Type	Variant Frequency	Position	Mutation
1	PfCRT	7	403,222 to 406,317	GA -> TG	15	Substitution	100.00%	74	<b>M74I</b>
				T -> A	15	SNP (transversion)	100.00%	75	<b>N75E</b>
				A -> C	15	SNP (transversion)	100.00%	76	<b>K76T</b>
				G -> T	29	SNP (transversion)	100.00%	220	<b>A220S</b>
				A -> T	24	SNP (transversion)	100.00%	239	<b>K239E</b>
				C -> T	24	SNP (transition)	100.00%	241	D241F
				G -> T	26	SNP (transversion)	100.00%	244	R244M
2	PfMDR1	5	957,890 to 962,149	A -> T	45	SNP (transversion)	100.00%	86	N86Y
				A -> T	40	SNP (transversion)	100.00%	184	Y184F
3	PfMRP2	12	1,192,888 to 1,199,214	G-> T	66	SNP (transversion)	100.00%	578	N578D
				A->T	64	SNP (transversion)	100.00%	583	N583S
				A->G	65	SNP (transition)	100.00%	798	R798A
				T-> A	62	SNP (transversion)	100.00%	1395	L1395I
				G-> C	62	SNP (transversion)	100.00%	1910	N1910D

**Table 3:** Whole genome sequencing data from *P. falciparum* isolate HL1210 showing single nucleotide polymorphisms (SNPs) present in genes pfCRT, pfmdr1 and pfmrp2. The variant frequency shows the percentage of the SNPs present relative to the coverage at that position.

	Gene	Gene ID	Chromosome	Gene position	Change	Coverage	Polymorphism Type	Variant Frequency	Position	Mutation
4	PfUBP-1	PF3D7_0104300	1	299,759 to 308,804	C -> T	24	SNP (transition)	100.00%	252	H252Y
					CT -> TC	28	Substitution	100.00%	258	L258S
					G -> A	28	SNP (transition)	100.00%	259	A259T
					G -> A	26	SNP (transition)	100.00%	262	V262I
					G -> T	26	SNP (transversion)	100.00%	263	C263F
					A -> G	22	SNP (transition)	100.00%	2600	K2600E
					G -> A	24	SNP (transition)	100.00%	2608	E2608K
					A -> G	24	SNP (transition)	100.00%	2613	S2613G
					A -> T	24	SNP (transversion)	100.00%	2614	I2614F
					T -> G	32	SNP (transversion)	100.00%	2702	I2702R
					CA -> AG	34	Substitution	100.00%	2704	H2704S
					AC -> GT	42	Substitution	100.00%	2708	T2708V
					T -> A	42	SNP (transversion)	100.00%	2709	S2709T
					CAA -> GTG	42	Substitution	100.00%	2710	S2710C
					C -> T	44	SNP (transition)	100.00%	2712	T2712I
					C -> G	54	SNP (transversion)	100.00%	2721	H2721D
					T -> G	57	SNP (transversion)	100.00%	2723	I2723S
					A -> T	57	SNP (transversion)	100.00%	2724	I2724F
					G -> A	56	SNP (transition)	100.00%	2735	G2735R
					A -> T	63	SNP (transversion)	100.00%	2743	I2743F
					T -> A	58	SNP (transversion)	100.00%	2745	I2745F
					T -> A	59	SNP (transversion)	100.00%	2752	W2752R
					T -> A	54	SNP (transversion)	100.00%	2767	S2767T

**Table 4:** Whole genome sequence data from *P.falciparum* isolate HL1210 showing single nucleotide polymorphisms (SNPs) present in the *pfubp1* gene. The variant frequency shows the percentage of the SNPs present relative to the coverage at that position.

	Gene	Gene ID	Chromosome	Gene position	Change	Coverage	Polymorphism Type	Variant Frequency	Position	Mutation
4	PfUBP-1	PF3D7_0104300	1	299,759 to 308,804	CGCT	->				
					TATA	46 -> 49	Substitution	100.00%	2775	R2775Y
					GA -> TT	51	Substitution	100.00%	2781	R2781I
					A -> G	51	SNP (transition)	100.00%	2779	Q2779R
					G -> A	40	SNP (transition)	100.00%	2794	G2794R
					A -> G	32	SNP (transition)	100.00%	2806	E2806K
					G -> A	32	SNP (transition)	100.00%	2806	E2806K
					C -> T	32	SNP (transition)	100.00%	2805	L2805F
					C -> G	25	SNP (transversion)	100.00%	2827	H2827D
					TG -> GA	29	Substitution	100.00%	2873	C2873D
					CA -> TG	28	Substitution	100.00%	2871	Q2871W
					T -> A	25	SNP (transversion)	100.00%	2870	S2870T
					G -> A	30	SNP (transition)	100.00%	2886	E2886K
					T -> A	31	SNP (transversion)	100.00%	2884	W2884R
					CTCT	->				
					AAAA	36 -> 37	Substitution	100.00%	2893	L2893K
					G -> A	38	SNP (transition)	100.00%	2901	G2901E
					T -> A	37	SNP (transversion)	100.00%	2906	Y2906N
					C -> A	38	SNP (transversion)	100.00%	2911	Q2911K
					T -> C	37	SNP (transition)	100.00%	2925	Y2925H
					T -> C	37	SNP (transition)	100.00%	2924	S2924P
					CCG -> TAA	40	Substitution	100.00%	2915	P- STOP?
					T -> A	24	SNP (transversion)	100.00%	2939	F2939I
					G -> A	26	SNP (transition)	100.00%	2938	R2938H
					T -> A	29	SNP (transversion)	100.00%	2937	S2937T
					T -> C	22	SNP (transition)	100.00%	2944	F2944L

**Table 5:** Whole genome sequence data from *P.falciparum* isolate HL1210 showing single nucleotide polymorphisms (SNPs) present in the *pfub1* gene. The variant frequency shows the percentage of the SNPs present relative to the coverage at that position

	Gene	Gene ID	Chromosome	Gene position	Change	Coverage	Polymorphism Type	Variant Frequency	Position	Mutation
5	pfnhe1	P13_0019	13	169,874 -175789	A->G	66	SNP (transition)	100.00%	415	L415R
					A->T	64	SNP (transversion)	100.00%	621	N621F
					A->C	68	SNP (transversion)	100.00%	1022	D1022P
					T->A	68	SNP (transversion)	100.00%	1773	STOP-R
6	AP2MU		12	717955-719820	G->C	25	SNP (transversion)	100.00%	336	A336S
7	pfatpase6	PFA0310c	1	265,208 to 269,173	A->T	46	SNP (transversion)	100.00%	424	E424Y
					A->C	34	SNP (transversion)	100.00%		STOP-920Y
8	Pf26s- protsu	PF3D7_1466300	14	2,709418-2,713,192	A->C	32	SNP (transversion)	100.00%	529	K529A

**Table 6:** Whole genome sequencing data from *P.falciparum* isolate HL1210 showing SNPs present in genes *pfnhe*, *pfap2mu* and *pfatpase6* and *pf26s-protsu*.

	Gene	Gene ID	Chromosome	Gene Position	Change	Coverage	Polymorphism Type	Variant Frequency	Mutation
1	PfMRP2	PFL1410c	12	1,192,888 to 1,199,214	C -> A	25	SNP (transversion)	100.00%	L1531I
					T -> G	63	SNP (transversion)	100.00%	S796A
					C -> G	80	SNP (transversion)	100.00%	L199V
	Pf26s-								
2	Protsu	PF3D7_1466300	14	2,709,418-2,713,192	A -> C	69	SNP (transversion)	100.00%	W533A
3	PfCRT	MAL7P1.27	7	403,222 to 406,317	C -> T	16	SNP (transition)	100.00%	V297L
4	PfMDR1	PFE1150w	5	957,890 to 962,149	A -> T	68	SNP (transversion)	100.00%	Y184F
5	PfNHE	P13_0019	13	169,874 -175789	G -> A	76	SNP (transition)	100.00%	L494I

**Table 7:** Whole genome sequencing data from *P.falciparum* isolate HL1212 showing single nucleotide polymorphisms (SNPs) present in genes *pfnhe*, *pfap2mu* and *pfatpase6* and *pf26s-protsu*.

Gene	Gene ID	Chromosome	Gene Position	Change	Coverage	Polymorphism Type	Variant Frequency	Mutation
6	PfUBP1	1	299,759 to 308,804	CCG -> TAA	40	Substitution	100.00%	R2915K
				TCA -> ATG	40	Substitution	100.00%	V2912A
				C -> T	38	SNP (transition)	100.00%	D2902N
				A -> T	50	SNP (transversion)	100.00%	Y2790F
				GA -> TT	51	Substitution	100.00%	E2781L
				A -> G	51	SNP (transition)	100.00%	K2779E
				G -> A	43	SNP (transition)	100.00%	G2794R
				G -> A	49	SNP (transition)	100.00%	E2806K
				C -> T	51	SNP (transition)	100.00%	L2805F
				C -> G	31	SNP (transversion)	100.00%	H2827D
				TG -> GA	29	Substitution	100.00%	C2873D
				CA -> TG	39	Substitution	100.00%	Q2871W
				T -> A	39	SNP (transversion)	100.00%	S2870T
				G -> A	30	SNP (transition)	100.00%	E2886K
				T -> A	43	SNP (transversion)	100.00%	W2884R
				CTCT -> AAAA	36 -> 37	Substitution	100.00%	L2893K
				G -> A	49	SNP (transition)	100.00%	G2901E
				T -> A	49	SNP (transversion)	100.00%	Y2906N
				C -> A	53	SNP (transversion)	100.00%	Q2911K
				T -> A	24	SNP (transversion)	100.00%	F2939I
				G -> A	26	SNP (transition)	100.00%	R2938H
				T -> A	29	SNP (transversion)	100.00%	S2937T

**Table 8:** The *pfubp1* gene of isolate HL1212 shows multiple non-synonymous SNPs and 5 substitutions at various codon positions



6	Gene	Gene ID	Chromosome	Gene Position	Change	Coverage	Polymorphism Type	Variant Frequency	Mutation
	PfUBP1	PF3D7_0104300	1	299,759 to 308,804	T -> C	22	SNP (transition)	100.00%	F2944L
					T -> A	53	SNP (transversion)	100.00%	I2745F
					A -> T	63	SNP (transversion)	100.00%	I2743F
					G -> A	57	SNP (transition)	100.00%	G2735R
					A -> T	57	SNP (transversion)	100.00%	I2724F
					T -> G	57	SNP (transversion)	100.00%	I2723S
					C -> G	54	SNP (transversion)	100.00%	H2721D
					C -> T	47	SNP (transition)	100.00%	T2712I
					CAA -> GTG	42	Substitution	100.00%	S2710C
					T -> A	42	SNP (transversion)	100.00%	S2709T
					AC -> GT	42	Substitution	100.00%	T2708V
					CA -> AG	34	Substitution	100.00%	H2704S
					T -> G	38	SNP (transversion)	100.00%	I2702R
					A -> T	34	SNP (transversion)	100.00%	I2614F
					A -> G	34	SNP (transition)	100.00%	S2613G
					G -> A	34	SNP (transition)	100.00%	E2608K
					A -> G	34	SNP (transition)	100.00%	K2600E
					G -> T	26	SNP (transversion)	100.00%	C263F
					G -> A	26	SNP (transition)	100.00%	V262I
					G -> A	28	SNP (transition)	100.00%	A259T
					CT -> TC	28	Substitution	100.00%	L258S
					C -> T	24	SNP (transition)	100.00%	H252Y

**Table 9:** The *pfubp1* gene of isolate HL1212 shows multiple non-synonymous SNPs and 9 substitutions at various codon positions. None of the observed mutations have been described in any published studies

	Gene	Gene ID	Chromosome	Gene Position	Change	Coverage	Polymorphism Type	Variant Frequency	Mutation
1	PfMRP2	PFL1410c	12	1,192,888 to 1,199,214	G-> T	54	SNP (transversion)	100.00%	Q578D
					A-> T	76	SNP (transversion)	100.00%	R582S
					T-> A	69	SNP (transversion)	100.00%	F1395Y
					T-> C	85	SNP (transition)	100.00%	F1745L
					G-> C	65	SNP (transversion)	100.00%	Q1910R
2	PfMDR1	PFE1150w	5	957,890 to 962,149	A-> T	62	SNP (transversion)	100.00%	T184F

	Gene	Gene ID	Chromosome	Gene Position	Change	Coverage	Polymorphism Type	Variant Frequency	Position
3	PfUBP1	PF3D7_0104300	1	299,759 to 308,804	-TG	35	Deletion	100.00%	195
					+T	31	Insertion	100.00%	198
					-G	30	Deletion	100.00%	190
					-TGT	17	Deletion	100.00%	1293
					-T	17	Deletion	100.00%	2279
					(A)2 -> (A)3	16	Insertion (tandem repeat)	100.00%	1072
					-GAGA	16	Deletion	100.00%	2518-2519
					-TGT	17	Deletion	100.00%	1292-1293
					-T	17	Deletion	100.00%	2279
					(A)2 -> (A)3	16	Insertion (tandem repeat)	100.00%	1072
					GAGA	16	Deletion	100.00%	2519-2520

Table 10: Observed non-synonymous mutations in the *pfmrp2* and *pfmd1* gene in the HL1204 isolate from Kenya. The *pfubp1* gene contains insertions and deletions.

	Gene	Gene ID	Chromosome	Gene position	Change	Coverage	Variant Frequency	Position	Before treatment	After treatment
1	Pfcrt	MAL7P1.27	7	403,222 to 406,317	G-> T	18	55.60%	74	M74I	wt
					AT-> GA	17	53.00%	75	N75E	wt
					A-> C	17	58.80%	76	K76T	wt
					G-> C	11	45.50%	140	Q140H	wt
					C-> G	23	43.50%	269	Q269E	wt
					G-> T	30	60.00%	368	R368I	wt
2	PK13	pf3d7_1343700	13	1,724,817-1,726,997	C-> T	49	91.80%	112	G112E	G112E
3	ap2mu		12	717955-719820	T-> A	43	11.60%	233	insertion- N233K	wt
4	pfatpase6		1	265208-269173	A-> T	67	100%	898	I898 (syn)	I898 (syn)
					C-> T	89	100%	431	E431K (syn)	E431K
5	atpase4		12	528999-532792	C-> T	86	50.00%	1128	G1128R	wt
					G-> T	90	67.78	1081	Q1081K	Q1081K
					A-> C	95	53.68	1045	N1045K	N1045K

**Table 11:** Whole genome sequencing data from *P.falciparum* isolate Liberia showing single nucleotide polymorphisms (SNPs) present in genes *Pfcrt*, *Pfk13*, *Pfap2mu*, *Pfatpase6*, and *Pfatpase4*. The variant frequency shows the percentage of the SNPs present relative to the coverage at that position. Before treatment and after treatment refers to treatment with atovaquone- proguanil (section 6.3.4)

6	Gene	Gene ID	Chromosome	Change	Coverage	Variant Frequency	Position	Mutation before treatment	Mutations after treatment	New mutations after treatment
6	pfubp1	PF3D7_0104300		A-> G	93	20.40%	789	N789D	N789D	N754K
				T-> C	90	74.40%	793	D793 (syn)	D793 (syn)	K779N
				G-> A	93	47.30%	795	D795N	D795N	Q841K
				A-> G	85	51.80%	798	N798D	N798D	N1375K
				C-> T	60	45.00%	904	S904 (syn)	S904 (syn)	K1914N
				A-> T	64	40.60%	906	K906N	K906N	E1915K
				G-> C	70	51.40%	1133	R1133S	R1133S	R2238K
				A-> G	60	26.70%	1710	N1710S	wt	
				T-> C	25	48%	2236	L2236 (syn)	L2236 (syn)	
				G-> A	10	50.00%	3121	V3121I	V3121I	
				T-> G	85	64.70%	2817	N2817K	N2817K	
7	pf26- protsu	PF3D7_1466300		C-> T	51	17.60%	427	N427 (syn)	wt	
				T-> A	20	25.00%	789	L783 (syn)	wt	
				A-> C	78	100%	534	E534A	E534A	
				A->G	104	48.08%	711	K711R	K711R	

**Table 12:** Whole genome sequencing data from *P.falciparum* isolate Liberia showing single nucleotide polymorphisms (SNPs) present in genes *Pfubp1* and *Pf26-protsu*. The variant frequency shows the percentage of the SNPs present relative to the coverage at that position. Before treatment and after treatment refers to treatment with atovaquone- proguanil (section 6.3.4) and new mutations listed show mutations that were not present before or after treatment.

Gene	Gene ID	Chromosome	Gene position	Change	Coverage	Variant Frequency	Position	Mutations before treatment	Mutations after treatment
8	Pfmrp2	12	1,192,888 1,199,214	to					
				G-> A	82	46.34%	1643	A1643V	A1643V
				G-> T	63	27.00%	1531	L1531I	wt
				A-> T	64	31.25%	1527	S1527T	wt
				C-> T	36	19.40%	1192	D1192N	wt
				C-> T	40	22.50%	1188	D1188N	wt
				C-> T	89	25.80%	1184	D1184N	wt
				A-> G	73	93.20%	1169	N1169 (syn)	N1169 (syn)
				T-> A	98	75.51%	714	K714I	K714I
				T-> C	32	71.86%	646	N646D	wt
				T-> C	90	27.70%	634	D634 (syn)	D634 (syn)
				C-> T	32	53.13%	630	D630N	Wt
				T-> C	85	18.82%	631	D631G	Wt
				C-> T	84	15.47%	630	D630N	D630N
				G-> C	70	74.30%	199	L199V	L199V

**Table 13:** Whole genome sequencing data from *P.falciparum* isolate Liberia showing single nucleotide polymorphisms (SNPs) present in the *pfmrp2* gene. The variant frequency shows the percentage of the SNPs present relative to the coverage at that position. Before treatment and after treatment refers to treatment with atovaquone- proguanil (section 6.3.4)

Gene	Gene ID	Chromosome	Gene position	Change	Coverage	Variant Frequency	Position	Mutations before treatment	Mutations after treatment
9	Pfnhe1	13	169,874-175,789	C-> A	78	70.50%	1380	K1380N	K1380N
				G-> T	75	44%	1375	H1375N	absent
				C-> G	76	56.60%	1366	K1366N	K1366N
				T-> G	77	26.00%	1362	N1362H	N1362H
				G-> A	76	29.00%	1361	N1361 (syn)	wt
				C-> G	21	47.62%	1168	E1168Q	wt
				C-> T	21	19.05%	1168	E1168K	wt
				T-> C	21	33.33%	1166	K1166E	wt
				C-> T	17	53.00%	1162	R1162K	wt
				C-> T	13	30.77%	1159	K1159 (syn)	wt
				G-> A	63	85.70%	1139	S1139 (syn)	S1139 (syn)
				A-> C	70	100%	950	V950G	V950G

**Table 14:** Whole genome sequencing data from *P.falciparum* isolate Liberia showing single nucleotide polymorphisms (SNPs) present in the *pfnhe1* gene. The variant frequency shows the percentage of the SNPs present relative to the coverage at that position. Before treatment and after treatment refers to treatment with atovaquone- proguanil (section 6.3.4)