Determination of Epigenetic and Genetic Mechanisms involved in Generation of Chloroquine Tolerant Phenotype Clones of *Plasmodium falciparum*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

To my family; especially my parents, for being the cornerstones of my life and providing physical, material and psychological support.

"For we know in part,

- But when that which is perfect is come,

then that which is in part shall be done away."

The Apostle Paul

1 Corinthians 13: 9-10

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ABBREVIATIONS AND ACRONYMS

5-mC	5-methylCytosine
ACT	Artemisinin-based Combination Therapy
bp	Base pair
CE	Capillary Electrophoresis
CQ	Chloroquine
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
GLURP	Glutamate-Rich Protein
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
IC	Inhibitory Concentration
MSP-1	Merozoite Surface Protein-1
MSP-2	Merozoite Surface Protein-2
ng/mL	nanogram/milliliter
PCR	Polymerase Chain Reaction
PfCRT	*Plasmodium falciparum* Chloroquine Resistance Transporter
PfMDR1	*Plasmodium falciparum* multidrug resistance 1
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
SP	Sulphadoxine-Pyrimethamine

ABSTRACT

Development of anti-malarial resistance occurs at a fitness cost to the parasites. It makes sense to assume that *Plasmodium falciparum* would first use alternative mechanisms to survive transient drug pressure and only resort to genetic fixation if the pressure is sustained. In this study, development of chloroquine (CQ) tolerance was assessed by DNA methylation, allelic diversity and genetic changes at *P. falciparum* chloroquine resistance transporter (PfCRT), and *P. falciparum* multidrug resistance 1 (PfMDR1) genes. A chloroquine sensitive 3D7 strain of *P. falciparum* was cloned by limiting dilution and the derived population exposed to increasing CQ concentrations of 4.51 ng/mL, 5.99 ng/mL and 7.15 ng/mL corresponding to 10%, 30% and 50% inhibitory concentrations (IC) of the parental population. Chemo-sensitivity to CQ of the surviving parasite densities at the initial and at each drug level was determined by SYBR Green I fluorassay. Allelic diversity of CQ unexposed and drug pressure surviving parasite strains were assessed by nested PCR that targeted the polymorphic regions of MSP1, MSP2 and GLURP. Global DNA methylation at 5-methylCytosine (5-mC) was assessed by ELISA. Single nucleotide polymorphisms (SNPs) at PfCRT and PfMDR1 genes were assessed by restriction fragment length polymorphism (RFLP), sequencing and probe based quantitative PCR. The chloroquine unexposed population had an IC_{50} of 7.03±1.37 ng/mL, one K1 allele (248 bp), two IC3D7 (482 bp and 596 bp) and one 800 bp GLURP. PfCRT and PfMDR1 were wild type. Global 5-mC DNA methylation was not detectable. Post CQ exposure at 4.51 ng/mL and 5.99 ng/mL, IC_{50} increased to 10.5 ng/mL and 15.05 ng/mL respectively. Parasite growth at 7.15 ng/mL of CQ was minimal and IC_{50} could not be determined. At 5.99 ng/mL of CQ, evidence of clonal selection was marked by allele reduction in parasites carrying the K1 and the 596 bp IC3D7 alleles. At 7.15 ng/mL of CQ, parasites with these two alleles were lost, but the 482 bp IC3D7 and 800 bp GLURP clones survived. Chloroquine tolerant populations remained wild type at PfCRT and PfMDR1. Global 5-mC DNA methylation was not observed in any of the derived parasite populations. These data suggest that, development of CQ tolerance starts by clonal selection. In absence of obvious selection advantages of genetic or epigenetic changes to the surviving clones, further studies are needed to elucidate how CQ induced changes at PfCRT and PfMDR1 genes eventually occur.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Malaria is a major public health problem with a global toll of 219 million clinical cases and nearly 660,000 deaths per year (WHO, 2012). The emergence and spread of anti-malarial resistance is now one of the greatest challenges facing the global efforts to control malaria (Winstanley, 2000). The trigger for emergence of drug resistant strains are not clearly understood, but are thought to involve the continuous use of sub-standard or sub-curative doses of anti-malarials (Hastings, Watkins, & White, 2002). Such resistant strains have single or multiple point nucleotide polymorphisms that affect drug influx/efflux or drug binding affinity, thus allowing natural selection for 'fit' parasites (Wongsrichanalai, Pickard, Wernsdorfer, & Meshnick, 2002). The maintenance of these mutations occur at an evolutionary fitness cost where normal growth and survival is affected (Hayward, Saliba, & Kirk, 2005), raising the possibility of existence of temporary non-genetic changes that would allow the parasites to quickly adapt to transient physiological pressures (Merrick & Duraisingh, 2010), and only result to genetic fixation if the pressures are permanent. Epigenetics is one such mechanism and would allow malaria parasites to temporarily tolerate drug pressure.

Epigenetics is the study of heritable changes in gene loci affecting levels of gene expression without an actual change in the genome sequence (Merrick & Duraisingh, 2010). The epigenome thus refers to the chemically modified state of the gene and its associated proteins at a locus. Epigenetic mechanisms commonly include Histone acetylation, DNA methylation and RNA interference (RNAi), and have profound effects on chromatin remodeling, affecting gene activation and silencing causing variable phenotypic effects. The arrangement of eukaryotic DNA into chromosomal chromatin regulates whether or not proteins that contribute to essential transcriptional functions can gain access to specific genomic loci. The addition of covalent post-translational

modifications in the residues of histone tails or on DNA, can counter the nature of chromatin allowing or preventing access to DNA (Chookajorn *et al.*, 2007).

A key aspect in epigenetics includes the recognition of certain nucleic acid sequences homologous at both DNA and RNA levels. These DNA-DNA and RNA-RNA interactions induce gene silencing or activation and RNA degradation, respectively, by modifications of the chromatin via epigenetic mechanisms (Wolffe & Matzke, 1999). The events of gene activation and silencing are connected with genomic and cytoplasmic cell defense systems that protect the organism under environmental pressures or in pathogen invasion mechanisms. These mechanisms are maintained and passed on during cell replication cycles and through cell generations as long as the cell remains viable. Epigenetics is commonly involved in the variable expression of gene families which determine erythrocyte invasion and virulence processes in *P. falciparum*. These genes do not undergo recombination but are marked by histone modifications for the activation of transcription processes (Merrick & Duraisingh, 2010).

Epigenetic control of gene expression and the levels of expression can be considered in normal cell development which requires the stable regulation of activation and repression of genes at different cell developmental cycles/phases. However, in circumstances such as drug pressure, effects of the immune system, or altered nutritional environments, epigenetic changes have been highlighted across an array of organisms and even reported in cancer cell lines, (Chookajorn *et al.*, 2007; Hoey, 2010) where they mediate non-genomic behavioral phenotypic transmissions. These changes are however reversible, and once the pressures are removed the normal phenotype is restored. This highlights the importance of these epigenetic mechanisms and changes to allow an organism to quickly adapt to new environments although just for a short time before more permanent measures are sought such as DNA mutations in long term exposure situations (Edwards & Myers, 2007).

Epigenetics in *P. falciparum* has been majorly studied in the *var* gene with regards to the *P. falciparum* Erythrocyte Membrane Protein-1 (*Pf*EMP-1), (Ralph & Scherf, 2005). With increasing interest in the aspects of drug resistance, key epigenetic mechanisms of acetylation and methylation demonstrated in the chromatin component of the *var* gene locus, have been hypothesized (Jiang, Xu, Wang, Zhao, & Chen, 2010) to also occur in the same manner in different gene loci of *P. falciparum* including, the multidrug resistance 1 (PfMDR1), chloroquine resistance transporter (PfCRT), dihydrofolate reductase (PfDHFR) and dihydropoteroate synthase (PfDHPS) genes, which are known to confer drug resistance to chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) once mutations occur (Figueiredo *et al.,* 2008; R. N. Price *et al.,* 1999). In the *Plasmodium* asexual blood stage life cycle, alterations in the parasites transcriptional profile and their transcriptomes at different points in the life cycle leads to varying developmental and morphological forms. This involves multiple histone modifications acting in combinations or in a sequential manner on one or more histone tails to affect these downstream functions (Hakimi & Deitsch, 2007).

The ability of the epigenetic changes to provide non-genetic basis of adaptation, with reversible phenotypic variations, can cause the phenomenon of anti-malarial drug sensitivity reversal once the drug pressure is withdrawn. Also, the reversal of drug resistance to compounds such as chloroquine has been demonstrated with the use of related compounds such as verapamil, desipramine and nonylphenolethoxylates (Basco & Le Bras, 1990; Ciach, Zong, Kain, & Crandall, 2003; van Schalkwyk, Walden, & Smith, 2001), and such reversal to chloroquine sensitivity has also been reported in field isolates of *P. falciparum* in clinical studies in Malawi (Kublin *et al.,* 2003; Mita *et al.,* 2003). This may be related in part to some of these epigenetic changes that enhance the reversal process. These epigenetic mediated gene regulation mechanisms may therefore allow *P. falciparum* to switch to phenotypes that enable it to express drug resistance.

Assessing these modifications in relation to levels of gene expression is thus important to determine whether or not there is a relationship. Such a positive association may be manipulated to circumvent the development of drug resistance. Given these possibilities, a lot of effort is currently being put in to analyze the epigenetic machinery of *Plasmodium* with specific interest on the enzymes responsible for the introduction of modifications to DNA at the chromatin and genomic levels (Merrick & Duraisingh, 2010). Since these epigenetic mechanisms are much more flexible than genetic changes, they permit fast and reversible adaptation, and the altered expression of these genes does not affect the capacity of the parasite to grow and replicate (Chookajorn *et al.*, 2007).

1.2 Statement of the Problem

Plasmodium falciparum has been noted to adapt quickly and does so extremely well to a wide range of anti-malarial's. As a result, resistance has developed to a number of highly effective drugs and drug combinations including chloroquine (CQ), sulphadoxine and pyrimethamine (SP) and artemisinin. This has limited the scope of chemotherapy and become a major concern in the development of fast and long acting drugs. Investigations that shed light on mechanisms underlying the development of drug resistance and specifically transient resistance, may provide clues for the reversal of epigenetic changes that mediate drug resistance. The involvement of epigenetics in cellular development, invasion and virulence processes of *P. falciparum* is well appreciated. On the contrary, its role in development of drug resistance remains largely uninvestigated. This study seeks to understand the contribution of epigenetic processes in the development of resistance in chloroquine in *P. falciparum* parasites.

1.3 Justification

Drug treatment is an essential component of control efforts against malaria. This is curtailed by the development of drug resistance. The increase in cases of drug resistance by the parasite to past and current drug treatment strategies in severely endemic areas has prompted increased interest in the mechanisms that mediate resistance. Better

understanding of these mechanisms and their subsequent manipulations may enable reversal of drug resistance to allow for the re-introduction of drugs which were once highly effective in the control of malaria. The development, loss and recovery of chloroquine drug resistance as noted in an *in vivo* experimental model (Peters, 1965), shows that the resistance to chloroquine is unstable and may be lost in the absence of drug pressure. This resistance was shown to recur after reintroducing increasing drug pressure. These changes indicate that there exist underlying mechanisms associated with transient/temporary drug resistance and possibly genes that regulate this process. This study therefore seeks to determine whether epigenetic mechanisms contribute to altered phenotypic and genotypic effects during the development of drug resistance in *P. falciparum* under anti-malarial drug pressure affecting drug sensitivity.

1.4 Research Questions

1. Is resistance to CQ mediated by SNPs or by other mechanisms such as epigenetic changes in *P. falciparum* under anti-malarial drug pressure?
2. In what order and manner do these changes occur, during the development of resistance to CQ?

1.5 Hypothesis

Epigenetic mechanisms mediate drug sensitivity changes prior to genetic fixation of mutations that cause drug resistance in *P. falciparum* under anti-malarial drug pressure.

1.6 Objectives

1.6.1 General Objective

To determine the involvement of epigenetic and genetic mechanisms in generation of chloroquine tolerant phenotype clones of *Plasmodium falciparum*.

1.6.2 Specific Objectives

1. To determine variations in CQ sensitivity of *P. falciparum* continuously cultured at stepwise increased CQ drug concentrations.
2. To determine SNP genotypic changes at PfCRT and PfMDR1 genes in the drug exposed cultures.
3. To determine changes in clonal genetic diversity in the drug exposed cultures at MSP1, MSP2 and GLURP genes.
4. To determine epigenetic changes by evaluating global DNA methylation in the drug exposed cultures.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Plasmodium falciparum* Life Cycle

Malaria is a mosquito borne infectious disease caused by a eukaryotic parasite of the genus *Plasmodium*. In humans, malaria is caused by *P. falciparum, P. malariae, P. ovale, P. vivax* and *P. knowlesi*. *P. falciparum* is the most virulent species and is responsible for the majority of malaria deaths, especially in tropical and subtropical regions. *P. falciparum* malaria accounts for 80% of all human malarial infections and 90% of the deaths (Snow, Guerra, Noor, Myint, & Hay, 2005). Malaria caused by *P. vivax, P. ovale* and *P. malariae* causes milder disease in humans that is not generally fatal. A fifth species *P. knowlesi* is a largely zoonotic and causes malaria in macaques but can also infect humans (Singh *et al.,* 2004). The *Plasmodium* parasites infect a diverse range of hosts which include birds, reptiles, monkeys, chimpanzees and rodents (Escalante & Ayala, 1994).

The life cycle of *Plasmodium* parasites is split between a vertebrate host and an insect vector as highlighted in figure 2.1. *Plasmodium* parasites infect susceptible female Anopheles mosquitoes during a blood meal on an infected vertebrate hosts. Within the insect mid-gut, gametocytes are rapidly activated to produce gametes. Fertilization follows, leading to formation of a motile ookinete, the only diploid stage in the life cycle of the parasite, which penetrates the epithelial cell monolayer surrounding the gut lumen. The ookinete ceases its migration upon reaching the basal lamina separating the mid-gut and hemocoel. Here the ookinete differentiates into an oocyst, which grows over a period of days and produces sporozoites; ultimately these are released into the hemolymph and migrate to the salivary glands from where they are passed to vertebrate hosts as the mosquito blood-feeds (Touray, Warburg, Laughinghouse, Krettli, & Miller, 1992). The sporozoites enter the blood stream and within 30 to 45 minutes invade the liver cells (hepatocytes) becoming multinucleated hepatic schizonts. The schizonts then

produce thousands of merozoites that are released out of the liver cells into the bloodstream. Released merozoites invade the red blood cells, undergoe a trophic period (rapid growth) and asexual replication to form trophozoites which mature into erythrocytic schizonts. These schizonts rupture the red blood cells in a period of 48 hours for *P. falciparum* to release new merozoites. The merozoites proceed to invade new red blood cells and the cycle starts again. The release of merozoites from ruptured schizonts is a synchronized event and is responsible for the cyclic chills and fever classically associated with clinical malaria. After several erythrocytic schizogony cycles (8-15 days for *P. falciparum*), some merozoites differentiate into sexual forms (male or female gametocytes). During a blood meal by a female Anopheles mosquito, the sexual forms are taken up into the mosquito's gut completing the life cycle (Touray *et al.*, 1992).

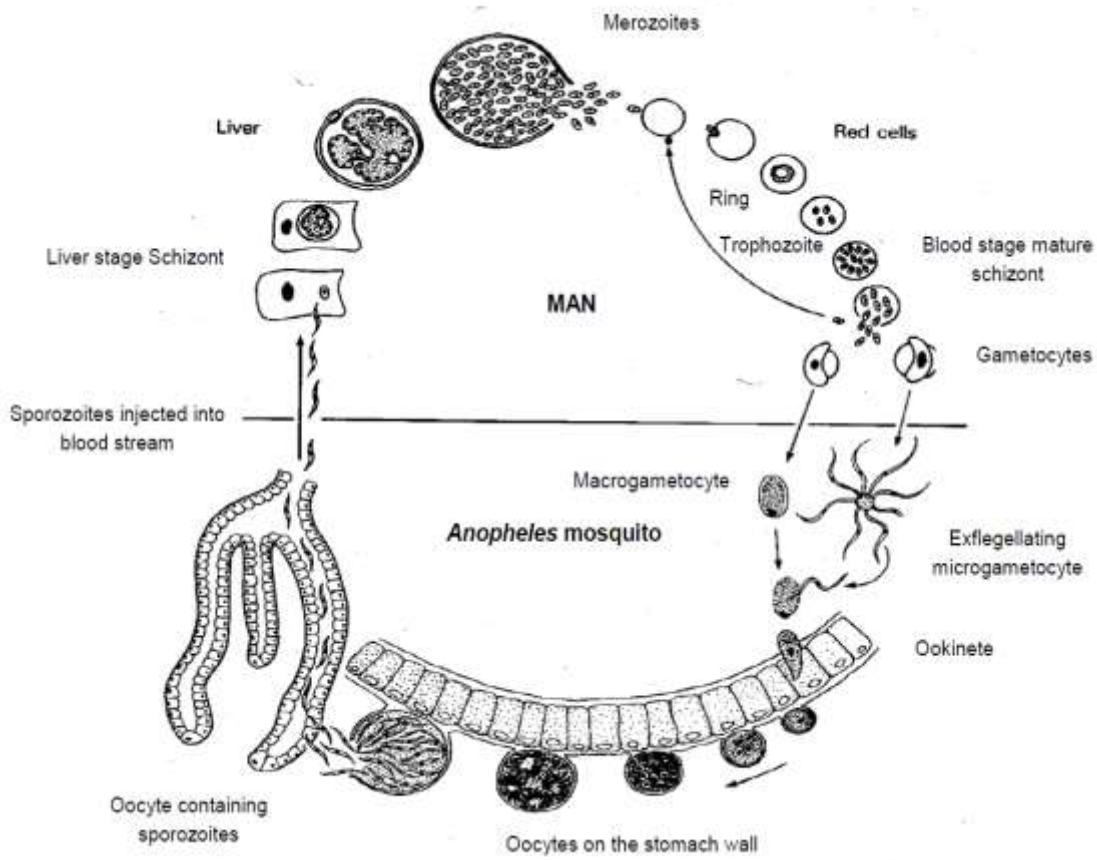

Figure 2.1: *Plasmodium* life cycle. Adapted from (Touray *et al.*, 1992)

2.2 Chemotherapy and Drug Resistance

The emergence of *P. falciparum* resistance to widely used anti-malarial drugs such as CQ and SP has made malaria control and treatment much more difficult. This is particularly so in Africa, where a few effective, affordable and safe alternatives are available. Many factors have contributed to the development and spread of resistance. Drug pressure has been identified as one of the key factors for the selection and spread of resistance in *P. falciparum* contributed to by the extensive use and misuse of these anti-malarial drugs over prolonged periods of time (Wernsdorfer, 1994). Intermittent preventive treatment for populations at risk could also promote resistance as the anti-malarial drugs are administered to non-cases. Selection pressure depends not only on the drug dosing rate but also on the infection status of the recipient (Hastings *et al.,* 2002). Gene mutations also confer resistance to anti-malarial drugs. This causes the natural selection of 'fit' parasites under a certain drug pressure by single or multiple point polymorphisms in the genome specifically at the target loci of the drug (Wongsrichanalai *et al.,* 2002).

2.2.1 Chloroquine and Drug Resistance

The first reports of *P. falciparum* resistance to CQ were reported in the early 1960's from South America and South East Asia, where direct or indirect mass drug administration had been implemented (Moore & Lanier, 1961; Wernsdorfer & Payne, 1991). Intermittent preventive treatment (IPT) conducted in populations at risk regardless of their current infection status in malaria endemic areas contribute to the high rates of spread in resistance (Greenwood, 2004). In Africa, *P. falciparum* resistance to CQ was reported from the eastern regions of the continent in the late 1970's and spread progressively west (Campbell, Chin, Collins, Teutsch, & Moss, 1979; Fogh, Jepsen, & Effersoe, 1979). CQ resistance is not evenly distributed and important differences can be found within and between countries. CQ resistance seems to have spread more rapidly in East than in West Africa, and this may be due to factors such as intensity of transmission, population immunity or population movements (Hastings &

D'Alessandro, 2000). Such factors may help in understanding and devising strategies to contain the spread of drug resistance.

Molecular studies have identified genetic markers indicative of CQ resistance. Polymorphisms at two major gene loci are associated with resistance. The PfCRT gene located on chromosome 7 codes for a vacuolar membrane transporter protein. The substitution of threonine for lysine at codon 76 of the gene is strongly associated with the development of resistance to CQ (Wellems, Walker-Jonah, & Panton, 1991). This mutation is essential for the resistant phenotype by allowing efflux of CQ from the lysosome (Djimde *et al.,* 2001). Other lesser polymorphisms in PfCRT codons C72S, M74I, N75E, H97Q, A220S, Q271E, N326S, I356T and R371I are however also required as well as the involvement of several other genes (Fidock *et al.,* 2000). The PfMDR1 gene located on chromosome 5 that codes for a P-glycoprotein homologue (Pgh) of the mammalian multidrug resistance gene is also involved. The change of aspartic acid for tyrosine at codon 86 is associated with CQ resistance together with other mutations at codons Y184F, S1034C, N1042D and D1246Y to a lesser degree (Hayward *et al.,* 2005; Reed, Saliba, Caruana, Kirk, & Cowman, 2000). The PfCRT Thr76 and PfMDR1 Tyr86 polymorphisms are thus important for expression of a resistant phenotype and may be the reason for the slow development of CQ resistance.

2.2.2 Sulphadoxine-Pyrimethamine and Drug Resistance

The development of CQ resistance led to the shift to SP, a combination of two synergistic drugs, as the first line drug by 1998 (Amin *et al.,* 2007). SP and mefloquine are readily absorbed drugs with a long half-life thus permitting effective single dose treatment of malaria and the following chemo-prophylactic period prevents infection for several weeks and may be important in recovery from anemia. However, these drugs do not yield the predicted useful therapeutic life time which is noted to be short, probably because of their prolonged half-life. This causes a higher probability of selecting resistant strains due to exertion of undesirable drug pressure for a long time once their

concentrations drop below the critical threshold and as a consequence leads to the fast development of resistance (Nzila & Mwai, 2000). In a Kenyan case study on pyrimethamine drug efficacy, (Clyde & Shute, 1954) monthly mass drug administration with pyrimethamine initially decreased the parasite prevalence but was followed by the emergence of parasites showing increased tolerance to the drug. The pyrimethamine sensitive parasites were rapidly replaced by resistant strains that disappeared when the monthly administration of pyrimethamine was stopped. This resulted in a very high selection pressure that induced an almost instantaneous *P. falciparum* resistance to pyrimethamine.

The molecular basis of resistance to SP is widely characterized. Sulphadoxine and pyrimethamine act synergistically to inhibit the enzymes dihydropteroate synthetase (DHPS) and dihydrofolate reductase (DHFR) respectively, which are involved in folate synthesis. Gene polymorphisms at codons S436A, A437G, K540E, A581G and A613S of PfDHPS confer resistance by decreasing the binding affinity of the enzyme. In PfDHFR, mutations at codons A16V, N51I, C59R, S108N and I164L reduce the drug-binding affinity of the enzyme (Plowe *et al.,* 1997). A change of the codon 108 is the key polymorphism for pyrimethamine resistance and additional changes at codons 51, 59 and 164 occur sequentially to progressively increase the degree of resistance.

2.2.3 Artemisinin-based Combination Therapy

To protect drugs from resistance, there is now clear evidence that combining drugs with two different modes of action and elimination profiles can improve their efficacy without increasing their toxicity (Olliaro & Taylor, 2010). The development of highly effective artemisinin derivatives has renewed hope for the treatment of malaria in the form of artemisinin-based combination therapy (ACT). In 2001, the World Health Organization recommended ACTs as the first-line treatment for uncomplicated malaria and by 2004 it become the first line drug in Kenya (Amin *et al.,* 2007). ACTs protect the individual drugs from resistance by relying on the principle of combining two drugs with

different mechanisms of action (White, 1999). The fast acting artemisinin derivative rapidly clears the main parasite load within the few hours that it remains at therapeutic levels and thus reduces subsequent gametocyte carriage (Sutherland *et al.,* 2005), while the partner drug, which is generally longer lasting, remains to clear the rest of the parasites. The combination of artesunate and mefloquine has been used with success in Southeast Asia (R. N. Price *et al.*, 1999). In sub-Saharan Africa, most countries have now adopted either artemether-lumefantrine (AL), or artesunate-amodiaquine (ASAQ) as their first-line ACT. Treatment success with an ACT will depend largely on the parasite's existing level of tolerance to the partner drug.

Despite the promise of ACTs, evidence of resistance against artemisinin derivatives has been detected, characterized by slow parasite clearance rates (Dondorp *et al.,* 2009). In Thailand, where treatments with artesunate and mefloquine are the first line regimen, concerns over potential artemisinin resistance were voiced when efficacy failure against the two day standard regimen were reported (Stepniewska *et al.,* 2010). Although slower parasitological clearance does not constitute direct evidence that ACT regimens are currently failing, these altered parasitological responses are regarded as an early warning to drug resistance. Resistance to artemisinin has now been shown to have spread to other neighboring regions of Cambodia and Myanmar (Phyo *et al.,* 2012) with recent reports of declined responsiveness of *P. falciparum* to ACTs in the Kenyan coast (Borrmann *et al.*, 2011). Underlying molecular markers for artemisinin resistance have not been fully elucidated with the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) type protein encoded by the PfATPase6 gene being the major chemotherapeutic target of artemisinin. Mutations at codons 263, 431, 623 and 769 alter the enzymatic activity of PfATPase6, with the 769 codon located within the cytoplasmic nucleotide binding domain being essential to structural transitions needed for the progress of the ATPase cycle and calcium binding and preventing artemisinin derivatives from interfering with these conformational changes (Uhlemann, Ramharter, Lell, Kremsner, & Krishna, 2005).

2.3 Epigenetic Mechanisms and Genome Expression

Epigenetic mechanisms can lead to the activation of certain regions of the genome to increase the expression of particular genes. The result is protein expression levels allowing the cell to respond to signals generated under external and internal influence that regulate growth and differentiation. Mechanisms must also exist to silence parts of the genome whose expression products are no longer required by the cell to sustain growth. Silencing of specific genes can be simply achieved by reversing the activating processes or by directly changing the chemical nature of DNA to create a silencing effect. Epigenetic modifications of histone modifications and DNA methylation (Figure 2.2), occur at the nucleosome which is the primary determinant of genome activity due to its positioning on DNA and also because of the chemical structure of the histone proteins associated with it (Chookajorn *et al.*, 2007). It is the major factor determining the degree of packaging of chromatin.

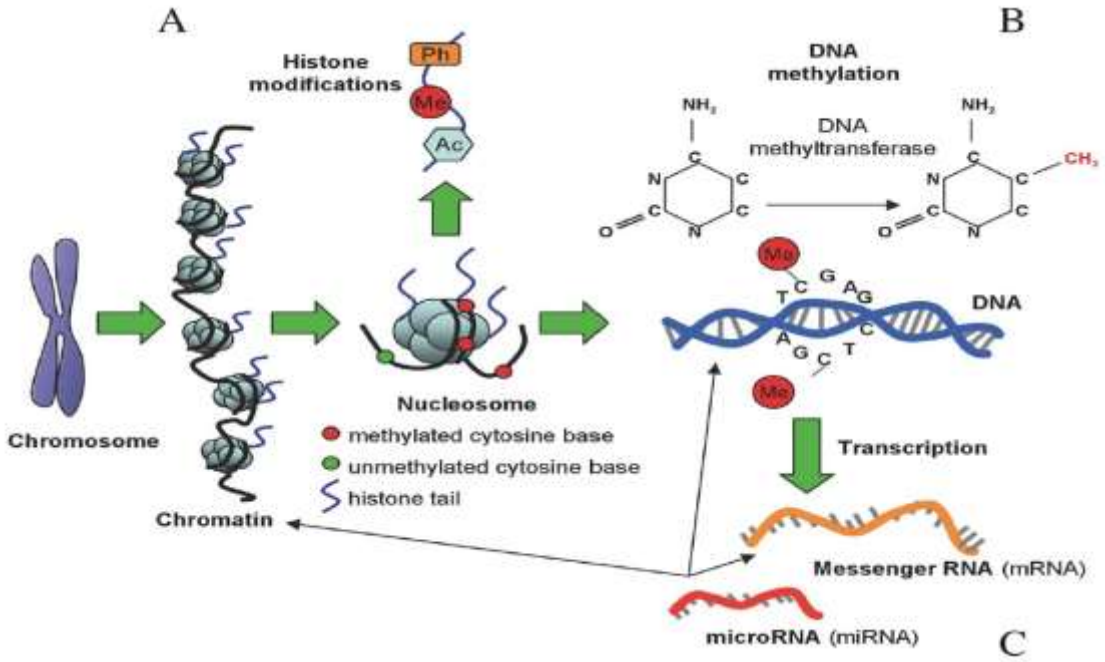

Figure 2.2: Types of epigenetic modifications. (A) Histones can undergo phosphorylation (Ph), methylation (Me), and acetylation (Ac), among other chemical modifications involved in chromatin remodeling and transcriptional regulation. (B) DNA molecules are methylated by the addition of a methyl group to carbon position 5

on cytosine bases, catalyzed by DNA methyltransferase, which maintains repressed gene activity. (C) mRNA is translated into a protein product, but this process can be repressed by binding of microRNAs (miRNA), a class of noncoding RNA (ncRNA) (Relton & Smith, 2010).

2.3.1 Histone Modifications

Histone proteins can undergo various types of modification, the most important of which is histone acetylation, the attachment of acetyl groups to lysine amino acids in the N-terminal regions of histones. This modification forms tails that protrude from the nucleosome core octamer (H2A, H2B, H3, and H4). This acetylation reduces the affinity of the histones for DNA and also reduces the interaction between individual nucleosomes that leads to formation of the 30 nm chromatin fiber. Histone acetylation causes chromatin to decompact causing exposure of the genes and thus allowing access to proteins and enzymes that mediate the activation of transcription (Merrick & Duraisingh, 2010).

Histone acetylation is achieved by action of the enzyme histone acetyltransferase (HAT) (Pennisi, 1997). Some proteins that have important influences on genome expression have been shown to have HAT activity, such as the *Tetrahymena* protein, p55, a homolog of a yeast protein, GCN5, which is known to activate the assembly of the transcription initiation complex (Brownell *et al.*, 1996). This observations and the fact that different types of cell display different patterns of histone acetylation, are clear indications that histone acetylation plays a prominent role in regulating genome expression. Histone acetylation occurs in conjunction with nucleosome remodeling in regulating genome activity. Acetylation is not only restricted to histones but also to other proteins involved in genome expression, such as the general transcription and enzymes. In addition to local modifications to histone proteins in the regions surrounding expressed genes, HATs can also carry out more general modifications throughout the entire genome (Berger, 2000).

Silencing of the genome is achieved by reversing the Histone acetylation process. This is achieved by removing the acetyl groups from histone tails, and hence reversing the transcription activating effects of HAT. This role is carried out by the enzyme histone deacetylase (HDAC). HDAC activity and gene silencing was recognized when mammalian HDAC1, related to the yeast protein Rpd3, was shown to be a repressor of transcription (Taunton, Hassig, & Schreiber, 1996). This deacetylation increases the affinity of the histones for DNA causing better packaging and also increases the interaction between individual nucleosomes that leads to further compaction and formation of the 30 nm chromatin fiber. The proteins and enzymes that mediate the activation of transcription are restricted access to the genes leading to a repression of expression levels. The association between histone deacetylation and repression occurs in an opposite effect as histone acetylation and activation.

Other histone modifications include methylation, phosphorylation and ubiquitination, where the tails of the core histones are attached to methyl, phosphate groups and the protein ubiquitin, respectively. These modifications can also influence chromatin structure and have a significant impact on cellular activity. Phosphorylation of histone H3 and of the linker histone, H1, has been shown to be associated with formation of metaphase chromosomes (Bradbury, 1992), and the ubiquitination of histone H2B plays a role in the control of the cell cycle (Robzyk, Recht, & Osley, 2000). Methylation of lysine 9 forms a binding site for the HP1 protein which induces chromatin packaging and further compaction of the chromatin mediating silencing of gene expression with reduced transcription levels (Bannister *et al.,* 2001; Lachner, O'Carroll, Rea, Mechtler, & Jenuwein, 2001), while methylation of lysine 4 has the opposite effect and promotes an open chromatin structure. Lysine-4 methylation is noted to be closely correlated with acetylation of histone H3 (Litt, Simpson, Gaszner, Allis, & Felsenfeld, 2001), and these two types of modification may work hand in hand to activate regions of chromatin.

2.3.2 DNA Methylation

This is the enzymatic addition of methyl groups at cytosine bases by DNA methyltransferases to form 5-methylcytosines (5-mC) that leads to repression of gene activity. This methylation is however limited to cytosine in the sequence 5'-CpG-3'. Maintenance methylation, a truly epigenetic mechanism that occurs after gene replication, is responsible for adding methyl groups to newly synthesized DNA strands at positions opposite methylated sites on the parent strand (Merrick & Duraisingh, 2010). Another form of DNA methylation is the *de novo* methylation that adds the methyl groups to new positions in the genome changing the inherited pattern in a localized region of the genome.

DNA Methylation results in the repression of gene activity. This was illustrated in experiments in which methylated or unmethylated genes were introduced into cells by cloning and their expression levels measured (Jones, 1999). Gene expression was shown not to occur if the DNA sequence is methylated. The link between gene expression and DNA methylation is also apparent in chromosomal DNA, showing that active genes are located in unmethylated regions. Cellular housekeeping genes have unmethylated CpG sequences, whereas tissue specific genes are unmethylated only in those tissues in which the gene is expressed. The methylation pattern is maintained after cell division with information specifying which genes should be expressed being inherited by the daughter cells, ensuring that in a differentiated cell the appropriate pattern of gene expression is retained even though the cell is being replaced or added to by new cells. Methylation influences genome expression by providing binding domains for methyl-CpG binding proteins which are components of histone deacetylase complexes. Methylated CpG sequences are the target sites for attachment of HDAC complexes that modify the surrounding chromatin in order to silence the genes (Jones, 1999).

A variation of DNA methylation, 5-hydroxymethylcytosine, is formed from 5-methylcytosine via addition of a hydroxy group. In mammals, it can be generated by

oxidation of 5-methylcytosine, a reaction mediated by the Tet family of enzymes (Iyer, Tahiliani, Rao, & Aravind, 2009), and is especially important in the central nervous system where it is found in very high levels (Globisch *et al.*, 2010). 5-hydroxymethylcytosine plays a role in global DNA demethylation reversing the effect of DNA methylation in repression of gene activity thus activating gene expression (Hackett *et al.*, 2013). Additionally, 5-formylcytosine, an oxidation product of 5-hydroxymethylcytosine and possible intermediate of an oxidative demethylation pathway has been detected in DNA from embryonic stem cells (Pfaffeneder *et al.*, 2011).

2.4 *Plasmodium falciparum* Genotyping Methods

2.4.1 DNA Sequencing

DNA sequencing is described as the gold standard, allowing the accurate determination of the presence of alleles and their exact position within a particular locus. This makes direct sequencing dependable for the identification of novel alleles and haplotypes which cannot be accomplished by other methods many of which require prior knowledge of the polymorphism. However existence of multiple infections necessitates PCR cloning prior to sequencing and primer design for A-T rich segments. This makes this technique extremely laborious. Pyrosequencing has been used reliably to quantify alleles in mixed malaria infections. It is a real time sequencing method that detects release of pyrophosphate during nucleotide incorporation by an enzyme cascade that generates light proportional to the amount of nucleotide incorporated. This technique allows sequencing of short stretches of nucleotides (10-20 bp) surrounding known polymorphisms without sequencing the rest of the conserved sequence. Pyrosequencing software can quantify the proportion of each alternative nucleotide at each alternative SNP site based on relative peak heights (Takala *et al.*, 2006).

2.4.2 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP*)*

PCR-RFLP is a DNA finger printing technique that uses restriction endonucleases to cut DNA into fragments on the basis of abundance of a restriction site, varying due to polymorphisms that add or remove a restriction site. First an amplicon of the locus under investigation is generated by a primary PCR reaction. The specific allele is then amplified in a secondary PCR, and finally, the amplification product is digested with appropriate enzymes and the fragments separated by agarose gel electrophoresis. Digestion is usually based on presence or absence of the restriction site. This method was applied effectively to genotype drug resistant SNPs of malaria parasite genome (Takala *et al.*, 2006).

2.4.3 Real Time PCR

Real Time PCR works like conventional PCR but employs the use of fluorogenic probes to detect allele polymorphisms. During the course of PCR amplification the probe binds to the amplicon and emits fluorescence proportional to the product. This allows for the "real time" monitoring of the amplification reaction. This method has rapid cycling reaction which provides results within short period of time. It is also sensitive, specific and highly reproducible. However substantial optimization is required prior to sample analysis and this type of PCR amplification is not suited for multiplexing. Quantitative real time PCR has found many applications including measuring mRNA expression levels, DNA copy number, transgene copy number and expression analysis, allelic discrimination, and measuring viral titers (Ginzinger, 2002). Calculation of the initial amount of the nucleotide template is achieved either by absolute quantitation (involving comparison with a standard curve with serial dilutions of standards of known concentration) or more often by relative quantitation using an internal control (for normalization) and a reference calibrator that yields fold differences between the target and the reference (Ginzinger, 2002). Absolute quantitation is preferred for viral load determination while relative quantitation using a calibrator is routinely applied in gene

expression studies. Another approach that is also used for mRNA quantitation is the comparative Ct ($2^{-\Delta\Delta}$CT) method (Livak & Schmittgen, 2001).

2.4.4 Genome Wide Association Studies

Genome-wide association studies (GWAS) is a unique analysis tool that is used to determine the presence of genetic markers throughout the parasite genome. It identifies the genomic regions or parasite genes that are responsible for causing resistance to anti-malarial drugs (Alam *et al.,* 2011). It distinguishes the resistant gene distributed across the *P. falciparum* genome. In this case, parasite genotypes in different patients have been reported to have the same clearance half-life (Takala *et al.,* 2006). Using this tool, variability within samples can generate robust genotype data in patients showing delayed parasite clearance. This gives a broad coverage of SNPs so as to monitor drug resistance closely (Takala *et al.,* 2006). In addition to that, high heritability has been associated with slow clearance rates therefore GWAS helps to show the role parasite genotypes play on parasite clearance half-life. This generates more information on mechanism of resistance and validates markers for drug resistance surveillance to contain its spread (Anderson *et al.,* 2010).

2.4.5 Microsatellite Marker Analysis

Microsatellites are regions comprising eight or more consecutive repeats of any sequence of 1–8 bp (Alam *et al.,* 2011). Over 50% of the *P. falciparum* microsatellites display length polymorphisms which enables the distinguishing of different isolates on the basis of alleles consisting of different numbers of repeat units. Replication slippage (also known as slip-strand mis-pairing) occurs during DNA replication and generates these repeat units. Detection of microsatellites typically involves the use of primers flanking to amplify the repeat locus by PCR followed by analysis by electrophoresis on sequencing grade polyacrylamide gels (Liljander *et al.,* 2009).

Multilocus genotyping has been used to study the population structure of *P. falciparum* (Anderson *et al.,* 2010), in antimalarial drug trials to define treatment outcome following antimalarial drug administration (Witkowski *et al.,* 2010), and in vaccine trials to assess the molecular impact of the vaccine on parasite multiplicity (Waitumbi *et al.,* 2009). This technique involves a nested PCR amplification of the loci of interest. The primary PCR uses primer pairs corresponding to the conserved sequences, spanning the polymorphic regions of block 2 of MSP-1 gene and block 3 of MSP-2 gene. Amplicons from the primary PCR are used as templates for the nested reaction. Allele specific primer sets are used to amplify the K1, MAD20, and RO33 alleles of the MSP-1 gene and the FC27 and IC3D7 alleles of MSP-2 alleles. Allelic discrimination is usually achieved by running the PCR amplicons in agarose gel electrophoresis whereby amplicons are distinguished from each other based on fragment size. However, due to limited resolving power of gel electrophoresis, adequate discrimination of alleles especially in high transmission settings where the MOI is high may not occur (Gupta, Dorsey, Hubbard, Rosenthal, & Greenhouse, 2010).

When compared to conventional gel electrophoresis, capillary electrophoresis (CE) was found to provide better resolution of *P. falciparum* alleles (Gupta *et al.,* 2010). The reverse primers used in capillary electrophoresis are labeled with fluorescent dyes. Besides the high resolution, CE has been recommended by the World Health Organization for allele discrimination and sizing due to its high throughput, ability to multiplex by using different fluorescent dyes for different alleles, thereby reducing cost and time. Sample loading, separation and size calling are automated. The utility of CE technique has been validated for a variety of applications including the analysis of multiple-locus variable-number tandem repeat (Liljander *et al.,* 2009). A part from fragment sizing, CE also calculates the peak height and area for each amplicon. These two parameters were evaluated to add another dimension when studying the population structure of malaria parasite clones.

2.5 DNA Methylation Methods

2.5.1 Reverse-phase High Performance Liquid Chromatography

Reverse-phase HPLC is a large-scale genome wide method for assessing changes in cytosine methylation levels. This method relies on the quantitative hydrolysis of DNA using DNase I and nuclease P1, or snake venom phosphodiesterase, followed by alkaline phosphatase treatment (Gomes & Chang, 1983). The liberated deoxy-ribonucleotides are then separated by standard reverse phase HPLC and the different bases identified by monitoring their UV absorbance at 254 and 280 nm. Further specificity may be achieved by combining HPLC separation with mass spectrometry so as to provide positive identification of the separated bases (Del Gaudio, Di Giaimo, & Geraci, 1997).

2.5.2 Enzyme Linked Immunosorbent Assays

This is a genome-wide approach to the study DNA methylation utilizing a highly specific reaction between monoclonal antibodies and 5-mC. The test DNA samples are denatured and immobilized onto diethylaminoethanol membranes and then incubate them with a monoclonal antibody directed against 5-mC (Oakeley, 1999). The antibody-DNA complex is then detected using a fluorescein isothiocyanate (FITC) linked secondary antibody, followed by fluorescence scanning. The intensity of the staining is proportional to the degree of methylation in the DNA. Such immunological approaches have also been used to visualize the chromosomal patterns of DNA methylation in individual cells by fluorescence microscopy (Bužek *et al.*, 1998). The major limitation with antibody labelling of DNA is that it is only quantitative when the 5-mC is not base paired, a situation that is very difficult to achieve in condensed chromosomes. To resolve this, the DNA samples are treated with sulfuric acid which depurinates the DNA and makes it less likely, for 5-mC to be involved in base pairing.

2.5.3 Restriction Enzyme Digestion

Restriction endonucleases have different sensitivities to cytosine methylation and provide a simple tool for the study of methylation changes within their recognition sites.

The methylation of DNA will create a site that will render it insensitive to cleavage by one enzyme, but not to the other. This will give rise to differentially cleaved products with size difference that can be easily observed and scored (Oakeley, 1999). This procedure is extremely popular due to its relative simplicity, low cost, and ease of interpretation. This method is however only useful for probing a very limited number of potential methylation sites, so the amount of useful information that can be gained from it is limited. This technique can be made more sensitive by the use of the polymerase chain reaction (PCR) which amplifies products from small quantities of digested DNA (Singer-Sam *et al.,* 1990).

2.5.4 Sodium Bisulphite Reaction

The reaction between pyrimidines and sodium bisulphite has provided a rapid method for the identification of 5-mC in any DNA sequence. Reversible sulphonation destabilizes the amino group at position 4 of cytosine and 5-mC so that these bases will then deaminate to either uracil or thymine, respectively, with a pH optimum of 5.8 (Wang, Gehrke, & Ehrlich, 1980). The deamination reactions of sulphonated cytosine and 5-mC proceed at very different rates, such that the deamination of cytosine will be complete before substantial deamination of 5-mC has occurred (Frommer *et al.*, 1992). This reaction is used to distinguish between cytosine and 5-mC in DNA.

2.6 Research Gap

New effective chemotherapies or immunotherapeutic solutions for malarial control can be developed only following an understanding of the mechanisms driving or enhancing pathogenicity. Parasites are forced to utilize rapid adaptive mechanisms in order to survive and thrive under the stresses of host immunity and drug pressure. The role of epigenetic mechanisms in the early development of drug resistance has not been examined exhaustively. The enzymes involved in DNA methylation and the alterations of chromosomal packing may be potential targets for the development of new therapeutic and diagnostic strategies against malarial infections. Finally, it should be

noted that the experimental approach might have a much broader application spectrum, especially in the case of other neglected tropical diseases. This study seeks to contribute towards a more complete understanding of the role of epigenetic mechanisms in the development of drug resistance in malaria parasites.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 *In vitro* Culture of *Plasmodium falciparum*

Plasmodium falciparum chloroquine sensitive strain 3D7 obtained from the Walter Reed Army Institute of Research (Silver Spring, MD, USA), was maintained in continuous culture according to standard techniques (Trager & Jensen, 1976). Briefly, the culture was maintained in a 5% suspension of Human O^+ erythrocytes in 6 mL of culture media RPMI 1640 (Sigma-Aldrich, MO, USA), supplemented with 10% Human pooled AB^+ Serum, 2 g/L $NaHCO_3$ and 50 µg/mL Gentamycin, at 37 °C in a 90% N_2, 5% CO_2 and 5% O_2 environment and incubated for 24 hours with daily complete media change. Parasitemia was determined via a thin blood film smear, fixed with absolute methanol and stained with 10% giemsa stain for 15 minutes. Infected erythrocytes were enumerated per 10,000 erythrocytes to get a parasite density count.

3.1.1 *Plasmodium falciparum* Cloning

Plasmodium falciparum cloning was achieved using the limiting dilution technique (Butterworth *et al.*, 2011) to obtain one parasite/µL for continuous culture. Parasitemia was determined as described and total red blood cell count determined via a complete blood count on Ac.T 5diff CP coulter (Beckman Coulter, CA, USA). 20 µL of the culture was picked and diluted in 180 µL of complete culture media RPMI 1640 to obtain a 1:10 dilution. This was subsequently repeated till a $1:10^7$ dilution was obtained. Each of the dilutions was cultured in a 5% suspension of Human O^+ erythrocytes in 3 mL of complete culture media RPMI 1640 and incubated at 37 °C in a 90% N_2, 5% CO_2 and 5% O_2 environment and checked after every 72 hours for complete media change and determination of growth via thick and thin blood film smears.

3.1.2 Drug Susceptibility Assay

A SYBR Green I based *in vitro* anti-malarial drug susceptibility fluorassay was employed to determine the IC_{50} of the CQ unexposed and exposed *Plasmodium* cultures (Akala *et al.,* 2011). The parasitemia of the culture was adjusted to 0.8% and a hematocrit of 2%, and 100 µL added to a pre-dosed 96 well flat bottomed plate containing 12.5 µL of serially diluted CQ ranging from 1000-1.95 ng/mL. The plates were incubated for 72 hours at 37 °C in a 90% N_2, 5% CO_2 and 5% O_2 environment after which, 100 µL of lysis buffer (5 mM EDTA, 15.76 g Tris-HCL, 0.008% w/v Saponin, and 0.08% v/v Triton X-100 per L) containing SYBR Green I (Invitrogen, CA, USA) was added and the plates read at a fluorescent excitation and emission wavelength of 485 and 530 nm, respectively on a Tecan GENios microplate fluorescence plate reader (Tecan Group, CH) and the IC_{50} determined using a non-linear regression log[inhibitor] versus response-variable slope equation in GraphPad Prism v5.01 (GraphPad Software, CA, USA). Based on the $IC_{50,}$ drug susceptibility was classified as follows: $IC_{50} \leq 10$ ng/mL – chloroquine sensitive, $10 < IC_{50} \leq 30$ ng/mL moderate resistance to chloroquine, and $IC_{50} > 30$ ng/mL chloroquine resistant (Akala *et al.,* 2011). The log[inhibitor] versus response graph was used to extrapolate suboptimal concentrations at 10% and 30% to be used in the drug exposed cultures as described below.

3.1.3 *In vitro Plasmodium falciparum* Drug Cultures

Chloroquine (sourced from Walter Reed Army Institute of Research, Silver Spring, MD, USA) was dissolved in 70% ethanol to obtain a stock concentration of 5 mg/mL. Cultures maintained at 5% hematocrit and 2% parasitemia were sequentially exposed to 4.51 ng/mL, 5.99 ng/mL and 7.15 ng/mL of CQ, corresponding to 10%, 30% and 50% inhibitory concentrations (IC) of the parental population. This occurred in continuous culture with sustained drug pressure and daily media change, till adaptation of the parasite population at the particular drug concentration was noted and the drug exposure concentration stepwise increased. Cell growth rates, morphology and death were assessed by microscopy, in order to score adaptation rates at different drug

concentrations compared to a non-exposed control culture. The drug cultures were run in triplicates to observe variable changes under drug pressure.

3.2 SNP Genotyping

3.2.1 Genomic DNA Isolation

Total genomic DNA was isolated using the QIAamp® DNA Mini Kit (QIAGEN, CA, USA) according to the manufacturer's instructions. Briefly, 20 µL of QIAGEN protease (proteinase K), was pipetted into a 1.5 mL micro centrifuge tube and 200 µL of the culture sample added. Approximately 200 µL of lysis buffer AL was added, the mixture vortexed for 15 seconds and incubated on a heating block for 15 minutes at 56 °C. Absolute ethanol, 200 µL, was then added and vortexed for 15 seconds. The lysate was then carefully applied onto the QIAamp spin column in a 2 mL collection tube and centrifuged for 1 minute at 6000 xg and the filtrate discarded. The column was then transferred into a clean 2 mL collection tube and 500 µL of wash buffer AW1 added. This was then centrifuged at 6000 xg for 1 minute and the filtrate discarded. 500 µL of wash buffer AW2 was then added into the spin column in a clean 2 mL collection tube and centrifuged for 3 minute at 20000 xg. Finally, the purified DNA preparation was eluted into 1.5 mL micro centrifuge tube from the spin column by addition of 110 µL elution buffer AE. This was incubated for 1-5 minutes at room temperature and centrifuged at 6000 xg for 1 minute. The eluted DNA was aliquoted and stored at -20 °C.

3.2.2 PCR and RFLP for PfCRT Codons 220, 271, 326, 356, and 371

The isolated DNA was amplified by conventional nested PCR and profiled by RFLP to determine SNP at codons 220, 271, 326, 356, and 371 in the PfCRT gene which are known to and play a role in conferring resistance to chloroquine (Fidock *et al.*, 2000).

The primary and secondary PCR reactions of the nested PCR were performed in a 25 µL reaction containing; 1X PCR Buffer, 2 mM $MgCl_2$, 200 µM dNTPs 0.06 U/µl

AmpliTaq® Gold DNA Polymerase (Applied Biosystems, CA, USA), 0.2 µM of each primer (Table 3.1) and 2 µL DNA template. For the secondary PCR, 0.4 µM of each primer (Table 3.1) was used and 0.5 µL of the primary PCR product used as the DNA template. For the primary amplification, the following cycling conditions were used; primary denaturation at 95 °C for 5 minutes, followed by 40 cycles of denaturing at 92 °C for 30 seconds, annealing at 45 °C for 30 seconds and extension at 65 °C for 45 seconds. Final extension was carried out at 72 °C for 15 minutes. The secondary nested PCR was performed as per the following cycling conditions; primary denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturing at 92 °C for 30 seconds, annealing at 45 °C for 30 seconds and extension at 65 °C for 30 seconds. Final extension was carried out at 72 °C for 15 minutes (Amor *et al.,* 2012). Amplification was performed using a Veriti 96 well Thermal cycler PCR machine (Applied Biosystems, CA, USA). Gel electrophoresis was performed to visualize the PCR amplification products. 5 µL of the PCR products were mixed with 1 µL of 6X tracking dye and loaded onto a 2% agarose gel (Sigma-Aldrich, MO, USA) stained with 2 µL Ethidium bromide (10 mg/mL) in 1X Tris-Acetate-EDTA buffer. The electrophoresis was run at 80 volts for 1 hour. DNA was visualized using the AlphaImager MINI® ultraviolet transillumination (Cell Biosciences, CA, USA), and the expected PCR product band sizes determined by comparison with a standard 1 Kilo base pair DNA ladder (Invitrogen, CA, USA).

Table 3.1: Primary and Secondary PCR Primer Pairs for PfCRT 220, 271, 326, 356 and 371

Target SNP	Primary PCR Primers	size (bp)	Secondary PCR Primers	size (bp)
PfCRT 220	Sense 5'-ACAATTATCTCGGAGCAGTT-3' Antisense 5'-CCCAAGAATAAACATGCGAAAC-3'	720	Sense 5'- TATTTATTTATTTATATATTTTGTTTTCTTGCCATTAAGG-3' Antisense 5'-TATTGTTGTAACAATAGC-3'	132
PfCRT 271	Sense 5'-ACAATTATCTCGGAGCAGTT-3' Antisense 5'-CCCAAGAATAAACATGCGAAAC-3'	720	Sense 5'-GGCACATTCATTTATTTATTTTTTCTTTCCTAATTAATGAATACGTT-3' Antisense 5'-GGCTATGGTATCCTTTTTCC-3'	121
PfCRT 326	Sense 5'-CCTTGGCATTGTTTTCCT-3' Antisense 5'-CCAAAGTTACGAAATCTAATAATCTTGG-3'	529	Sense 5'-CCTTTTTATTCTTACATAGCTGGTTATTGAATTATCAC-3' Antisense 5'-TGGCATTGTTTTCCTTCT-3'	66
PfCRT 356	Sense 5'-CCTTGGCATTGTTTTCCT-3' Antisense 5'-CCAAAGTTACGAAATCTAATAATCTTGG-3'	529	Sense 5'-ATATATATGGCTAAGAATTTAAAGTAATAAGCAGTTGCT-3' Antisense 5'- AATTATCGACAAATTTTCTACC-3'	102
PfCRT 371	Sense 5'-CCTTGGCATTGTTTTCCT-3' Antisense 5'-CCAAAGTTACGAAATCTAATAATCTTGG-3'	529	Sense 5'-TATTATTTTTACTTTTTAATTTTATAGGGTGATGTCTTAA-3' Antisense 5'-AAGTTACGAAATCTAATAATCTTGGTTC-3'	70

RFLP to determine SNP profile at PfCRT gene codons 220, 271, 326, 356, and 371, utilized restriction endonucleases specific to each polymorphism (Table 3.2). This was carried out in a 20 µL reaction using 8 µL of secondary PCR product containing 1 U/µL of the enzyme (New England Biolabs, MA, USA), 1X NEBuffer 3 (or NEBuffer 4) and supplemented with 1X Bovine Serum Albumin (BSA) where necessary (UMSM, 2002; Amor *et al.,* 2012). The reaction was incubated at 37 °C for 16 hours (overnight). Digestion products were examined by gel electrophoresis on a 2% MetaPhor™ agarose gel (Lonza, NJ, USA), stained with 2 µL Ethidium bromide (10 mg/mL) in 1X Tris-Acetate-EDTA buffer. The electrophoresis was run at 80 volts for 1 hour. Visualization was done and the expected digestion product scored as a mutant or wild type based on the band sizes determined by comparison with a standard 1 Kilo base pair DNA ladder

and from standard controls of *Plasmodium falciparum* strains D6 (Sierra Leone, CQ sensitive) and W2 (Indochina, CQ resistant), with known genotype profiles.

Table 3.2: Restriction Endonucleases and Cleavage Target and Products

Target	**Restriction Endonuclease**	**Cleaved Phenotype**	**Product Sizes (bp)**	**Genotype**
PfCRT 220	BglI	Alanine/A	95 and 37	Wild type
PfCRT 271	XmnI	Glutamic Acid/E	78 and 43	Mutant
PfCRT 326	MseI	Asparagine /N	21 and 45	Wildtype
PfCRT 356	AlwNI	Threonine/T	68 and 34	Mutant
PfCRT 371	AflII	Isoleucine/I	34 and 36	Wild type

3.2.3 Sequencing PfCRT 72-76 Haplotype

Sequencing was done to confirm the PfCRT 72-76 haplotype using sequencing PCR primers pairs 5'-TGTGCTCATGTGTTTAAACTT-3' and 5'-CAAAACTATAGTTACCAATTTTG-3'. The 145 bp PCR product was then cleaned using ExoSAP-IT (Affymetrix, CA, USA) according to the manufacturer's instructions, and a cycle sequencing reaction setup using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA), containing a 1:10 mixture of dNTPs and ddNTPs to achieve chain termination. A 10 µL reaction volume was prepared constituting of 1 µL BigDye terminator ready reaction mix, 2 µL BigDye sequencing buffer (5X), 1 µL of each primer (0.4 µM) separately, 4 µL PCR water and 2 µL of purified PCR product. The reaction was run under the following cycling conditions: 95 °C for 5 minutes, 35 cycles of denaturing at 95 °C for 15 seconds, annealing at 55 °C for 30 seconds, extension at 68 °C for 2 minutes and 30 seconds, and a final extension at 68 °C for 3 minutes (Alam *et al.,* 2011). The cycle sequencing PCR product was then cleaned using Ethanol/EDTA precipitation and re-suspended in 10 µL Highly deionized (HI-DI) formamide and loaded onto the 3130 genetic analyzer (Applied Biosystems,

CA, USA) for resolution via capillary electrophoresis. Sequences were retrieved and analyzed using BioEdit Sequence Alignment Editor v7.1.3.0 (Hall, 1999).

3.2.4 Probe Based qPCR for PfMDR1 Codons 86, 184, 1034, and 1042

SNP determination at codons 86, 184, 1034, and 1042 of PfMDR1 was done by probe based qPCR (Kamau *et al.,* 2012). In a 0.2 mL MicroAmp optical tube, a final reaction volume of 10 µL was constituted consisting of 0.5 µL AB TaqMan-MGB genotyping primer-probe mix (20X) (Table 3.3), 5.0 µL AB TaqMan genotyping master mix (2X), 2.5 µL DNase free water and 2.0 µL DNA template. Amplification was performed using a 7300 Real-Time PCR System (Applied Biosystems, CA, USA). A pre-read was made at 60 °C for 1 minute followed by cycling conditions that included primary denaturation at 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. A post-read was made at 60 °C for 1 minute. The allelic discrimination scores for each sample were made using the Sequence Detection Software, SDS, v1.3 (Applied Biosystems, CA, USA).

Table 3.3: Primers and Probes for PfMDR1 86, 184, 1034, 1042 and 1246

PfMDR1 Codon	Primer Sequence	Probe Sequence
86	Forward- 5'-AGGAGGAACATTACCTTTTTTTATATCTGTGT-3' Reverse- 5'-ATTGTACTAAACCTATAGATACTAATGATAATATTATAGGAT-3'	5'FAM- CATCACCTAAATACATGTTC -3'MGB 5'VIC- CATCACCTAAAT**T**CATGTTC -3'MGB
184	Forward- 5'-GGTACGAAATTTATAACAATTTTTACATATGCCAGTT-3' Reverse- 5'-AAAAACGCAAGTAATACATAAAGTCAAACGT-3'	5'FAM-CCTTTTTAGGTTTATATATTTG-3'MGB 5'VIC-CCTTTTTAGGTTTATT**T**TATTTG-3'MGB
1034	Forward- 5'-GACAAAAAAGAAGAATTATTGTAAATGCAGCTT-3' Reverse- 5'-AGGATCCAAACCAATAGGCAAAACT-3'	5'FAM- TTTGACAGAATCCC-3'MGB 5'VIC-CTTTGAC**T**GAATCCC-3'MGB
1042	Forward- 5'-GGATTCAGTCAAAGCGCTCAATTA-3' Reverse- 5'-GTACCTCTTTTAATTAAGAAGGATCCAAACCA-3'	5'FAM-ATAGGCAAAACTATTAATAAA-3'MGB 5'VIC-TAGGCAAAACTAT**G**AATAAA-3'MGB

The probes consist of a 5' Fluorophore reporter dye and 3' none fluorescent Quencher (FAM for the wild type and VIC for the mutant probe. Nucleotides differing from the wild type sequence are as highlighted in bold.

3.2.5 PfMDR1 Copy Number Estimation

Estimation of PfMDR1 copy number was performed by qPCR using the $2^{-\Delta\Delta Ct}$ method of relative quantification (Livak & Schmittgen, 2001). Amplification was done in a 10 µL reaction consisting of 1X TaqMan® Universal Master Mix (Applied Biosystems, CA, USA), 0.3 µM of each primer, 0.1 µM probe (Table 3.4) and 2.0 µL DNA template. Amplification was done on a 7500 Fast Real Time PCR system (Applied Biosystems, CA, USA) with conditions of primary denaturation at 95 °C for 10 minutes, and 40 cycles of 95 °C for 15 seconds and 58 °C for 1 minute (Ric N. Price *et al.*, 2004). *P. falciparum* reference clone Dd2 was used as a multiple copy control for PfMDR1 and *P. falciparum β*-tubulin as a housekeeping gene to normalize the quantitative data. Briefly, ΔΔCt = (Ct, PfMDR1 - Ct, *P. falciparum β*-tubulin)x − (Ct, PfMDR1 - Ct, *P. falciparum β*-tubulin)y, where x is the test isolate and y is the reference clone. The results were then calculated as n-fold changes in PfMDR1 gene copies, normalized to *P. falciparum β*-tubulin, relative to the copy number of PfMDR1 in the reference clone using the $2^{-\Delta\Delta Ct}$ equation. The samples were analyzed in triplicates and the average cycle threshold (Ct) values used to determine the copy number rounded to the nearest integer.

Table 3.4: Primer and Probes for PfMDR1 and *P. falciparum β*-tubulin Genes

Gene	Primer Sequence	Probe Sequence
PfMDR1	Forward 5'-TGCATCTATAAAACGATCAGACAAA-3' Reverse 5'-TCGTGTGTTCCATGTGACTGT-3'	5'FAM-TTTAATAACCCTGATCGAAATGGAACCTTTG-3' TAMRA
***β*-tubulin**	Forward 5'-TGATGTGCGCAAGTGATCC-3' Reverse 5'-TCCTTTGTGGACATTCTTCCTC-3'	5'VIC-TAGCACATGCCGTTAAATATCTTCCATGTCT-3' TAMRA

3.3 Clonal Genetic Diversity Analysis

Genotyping to detect the population structure was achieved using conventional nested PCR to amplify the polymorphic regions of block 2 of MSP1 gene (K1, MAD20, and RO33 alleles), block 3 of MSP2 gene (FC27 and IC3D7 alleles), and GLURP. Alleles

for each of these genes were discriminated by fragment analysis via high resolution capillary electrophoresis (Gupta *et al.,* 2010; Liljander *et al.,* 2009).

The primary PCR reaction for the nested PCR was performed in a 25 µL reaction containing a final concentration of 1× PCR buffer, 2 mM $MgCl_2$, 125 µM dNTPs, 0.02 U/µL AmpliTaq® Gold DNA polymerase (Applied Biosystems, CA, USA), and 0.25 µM each of the flanking primer pairs for MSP1 and MSP2 (Table 3.5). 3 µL of extracted DNA was used as the template. The cycling conditions were an initial denaturation at 95 °C for 5 minutes followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 58 °C for 2 minutes, extension at 72 °C for 2 minutes, with a final round of amplification at 58 °C for 2 minutes and 72 °C for 5 minutes.

For the secondary PCR, a 25 µL reaction containing final concentrations of 1× PCR buffer, 1 mM $MgCl_2$, 125 µM dNTPs and 0.02 U/µL of AmpliTaq® Gold DNA polymerase, and 0.125 µM of the respective MSP1 and MSP2 allelic type-specific primers (Table 3.5). However, for the IC3D7 allele, 0.05 U/µL of AmpliTaq® Gold DNA polymerase and 0.3 µM of allele specific primers was used. One microliter of product from each primary reaction was used as a template for secondary PCR amplification, except for the IC3D7 nested reaction where 2 µL product from each primary reaction was used.

The cycle conditions for the amplification of the MSP1 allelic types were as follows: initial denaturation at 95 °C for 5 minutes followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 61 °C for 2 minutes, extension at 72 °C for 2 minutes, and a final step at 61 °C for 2 minutes and 72 °C for 5 minutes. For the MSP2 allelic types, the cycle conditions were: initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 58 °C for 1 minute, extension at 72 °C for 2 minutes and a final round at 58 °C for 1 minute and 72 °C for 5 minutes.

The PCR products were wrapped in aluminum foil to avoid quenching of the fluorescent dyes and stored at -20 °C. Gel electrophoresis was performed to visualize aliquots of the PCR amplification products on a 2% agarose gel (Sigma-Aldrich, MO, USA) at 80 volts for 1 hour. The expected PCR product band sizes were determined by comparison with a standard 1 Kilo base pair DNA ladder and positive control samples previously confirmed to amplify K1, MAD20, RO33, FC27 and IC3D7 alleles.

Table 3.5: Primary and Secondary PCR Primer Pairs for MSP1 and MSP2

Target Gene	Primary PCR Primers	Target Allele	Secondary PCR Primers
MSP1	Sense 5'-CTAGAAGCTTTAGAAGATGCAGTATTG-3'' Antisense 5'-CTTAAATAGTATTCTAATTCAAGTGGATCA-3'	K1	Sense 5'-AAATGAAGAAGAAATTACTACAAAAGGTGC-3'' Antisense 5'-GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3'-NED (yellow)*
		MAD20	Sense 5'-AAATGAAGGAACAAGTGGAACAGCTGTTAC-3'' Antisense 5'-ATCTGAAGGATTTGTACGTCTTGAATTAAC-3'-PET (red)*'
		RO33	Sense 5'-TAAAGGATGGAGCAAATACTCAAGTTGTTG-3'' Antisense 5'-CATCTGAAGGATTTGCAGCACCTGGAGATC-3'-VIC (green)*
MSP2	Sense 5'-ATGAAGGTAATTAAAACATTGTCTATTATA-3' Antisense 5'-CTTTGTTACCATCGGTACATTCTT-3'	FC27	Sense 5'-AATACTAAGAGTGTAGGTGCARATGCTCCA-3' Antisense 5'-TTTTATTTGGTGCATTGCCAGAACTTGAAC-3'-6FAM (blue)*'
		IC3D7	Sense 5'-AGAAGTATGGCAGAAAGTAAKCCTYCTACT-3'' Antisense 5'-GATTGTAATTCGGGGGATTCAGTTTGTTCG-3'-VIC (green)*'
GLURP	Sense 5'-ACATGCAAGTGTTGATCCTGAAG-3' Antisense 5'-GTGTCTTTGTAGGTACCACGGGTTCTTGTGG-3'	GLURP	Sense 5'-gtgtcttTGTAGGTACCACGGGTTCTTGTGG-3' Antisense 5'TGTTCACACTGAACAATTAGATTTAGATCA-3'-6FAM (blue)*

*Fluorophore modification of the primers used in capillary electrophoresis based method.

Fragment analysis was done via capillary electrophoresis as follows; a reaction mix was prepared containing 0.5 µL of LIZ standard (GeneScan-500LIZ® for fragments less than 500 bp and GeneScan-1200LIZ® for fragments between 500-1200 bp) in 9 µL of HI-DI formamide. The mix was pulse vortexed and centrifuged at 6000 xg and 1 µL of

amplified product from the nested PCR reaction added, vortexed and then briefly centrifuged at 2000 xg. Samples were denatured at 95 °C for 5 minutes and then chilled on ice to maintain the single strands prior to loading in the 3130 genetic analyzer (Applied Biosystems, CA, USA) for resolution via capillary electrophoresis. Fragment sizing and analysis was done using GeneMapper® Software version 4.0 (Applied Biosystems, CA, USA).

3.4 DNA Methylation Analysis

The detection and quantification of global DNA methylation was achieved using a DNA Enzyme Linked Immunosorbent Assay (ELISA) kit (Zymo Research, CA, USA) with a monoclonal antibody, anti-5-methylcytosine, specific and sensitive to 5-methylcytosine (5-mC) (Chaudhry & Omaruddin, 2012). One hundred nanograms of the DNA sample was brought to a final volume of 100 µL with 5-mC coating buffer in a PCR tube and denatured at 98 °C for 5 minutes and then transferred immediately to ice. The denatured DNA was then transferred to the wells of the ELISA plate and incubated in the dark at 37 °C for 1 hour. The coating buffer was then blotted out and the wells washed 3 times with 200 µL of 5-mC ELISA buffer. A further 200 µL of the wash buffer was added and the plate incubated in the dark at 37 °C for 30 minutes. The wash buffer was then blotted out and 100 µL mix of both anti-5-methylcytosine and secondary antibody in 5-mC ELISA buffer added and the plate incubated in the dark at 37 °C for 1 hour. Finally, the antibody mix was blotted from the wells and washed 3 times with the wash buffer before addition of 100 µL of the HRP developer to each well. The plate was incubated at room temperature for 10-60 minutes to allow for color development and absorbance read at 405-450 nm on a VMax® kinetic ELISA microplate reader (Molecular Devices, CA, USA). The absorbance for each sample was plotted as a function of absorbance at 405 nm (Y-axis) versus % 5-mC (X-axis), and the equation % 5-mC = $e^{\{(\text{absorbance} - \text{y intercept})/\text{slope}\}}$, derived from the logarithmic second-order regression, used to determine the 5-mC percentage for unknown DNA samples based on their absorbance.

3.5 Ethical and Scientific Considerations

The study was approved by the Ethical Review and Scientific Steering Committees of the Kenya Medical Research Institute, Nairobi (SSC #2112). The *Plasmodium falciparum* strain 3D7 used was obtained from cryopreserved cloned samples from the National Institute of Health (NIH, MD, USA) via the Walter Reed Army Institute of Research (Silver Spring, MD, USA).

Human blood for *in vitro* culture was obtained from willing and consenting adults under the human blood collection for the *in vitro* culture of malaria parasites protocol approved by the Walter Reed Army Institute of Research Institutional Review Board and Kenya Medical Research Institute Ethical Review Committee (#1919). The study posed minimal risk and confidentiality of the donors was maintained.

3.6 Data and Statistical Analysis

Data collected and generated in the study was entered and stored in MS Excel. Results were presented in forms of graphs and tables. Statistical analysis was done in Graphpad Prism v5.01 (GraphPad Software, CA, USA), where a student's t test was employed to compare the results.

CHAPTER FOUR

4.0 RESULTS

The results obtained from the experiments to understand the epigenetic and genetic mechanisms involved in generation of chloroquine tolerant phenotype clones of *Plasmodium falciparum* are described in this chapter. Here the phenotypic characteristics are described in terms of parasite morphology changes during the course of drug exposure as well as adaptation rates during continuous exposure to the drug. Genotypic data describes underlying molecular changes that were observed during the drug exposure period including single nucleotide polymorphisms, gene copy number, allelic structure and DNA methylation.

4.1 Growth characteristics of *P. falciparum* 3D7 prior to and after exposure to CQ

The growth profile and characteristics of the *P. falciparum* 3D7 before and during sustained exposure to CQ was observed over the time course of the experiment. As shown in Figure 4.1, the parasitemia of the unexposed population doubled every 48 hours. At drug exposure equivalent to IC_{10}, the parasites had comparable growth rate to that of the unexposed population. At CQ concentration equivalent to IC_{30}, the parasite growth was retarded and only reached 4% in 5 days. At CQ concentration equivalent to IC_{50}, the parasites failed to recover. It was noted that there was an overall drop in survival rates of the parasite with introduction of the drug. In respect to cell morphology, parasites at the unexposed and at the IC_{10} drug exposure level were healthy and had normal cell morphology (Figure 4.2). At the IC_{30} drug exposure level, gametocyte forms were evident indicating that the parasites were stressed, and at the IC_{50} drug exposure level, cell death was noted with degenerative and distorted cell morphology.

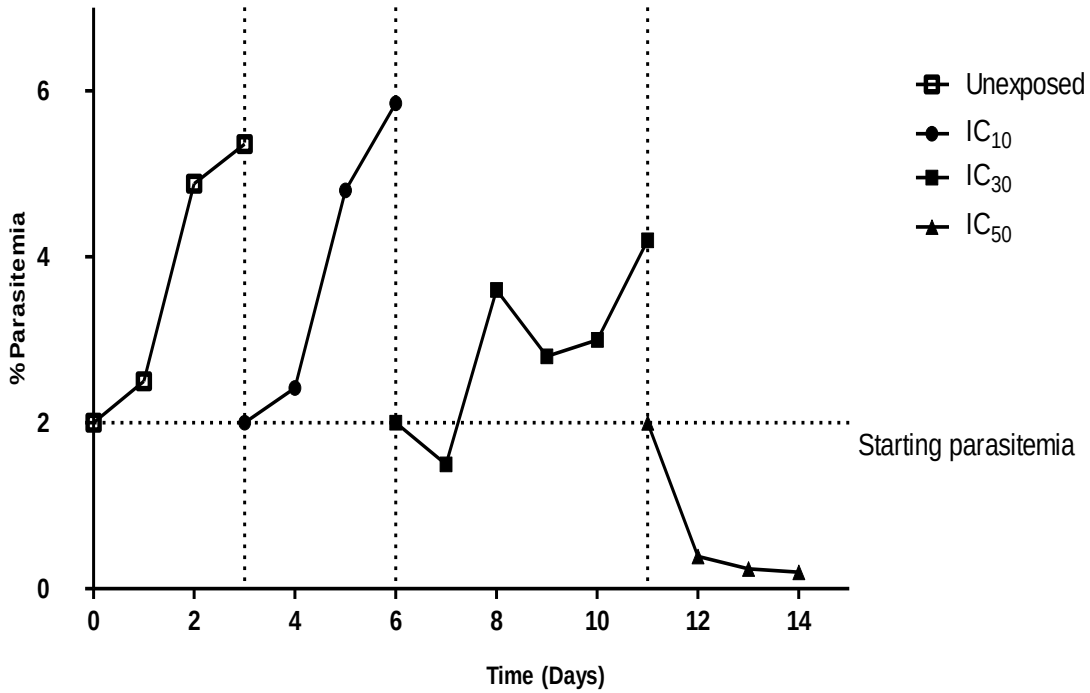

Figure 4.1: Growth profile of the *P. falciparum* 3D7 strain before and after continuous exposure to chloroquine (CQ). The parasitemia of the CQ unexposed population doubled every 48 hours reaching 5% parasitemia in 3 days. At drug exposure equivalent to IC10, the parasites had growth rate comparable to that of the unexposed population (attaining 5% parasitemia in 3 days). At CQ exposure equivalent to IC30, parasite growth was retarded and only reached 4% in 5 days. At CQ equivalent to IC50, the parasites failed to recover.

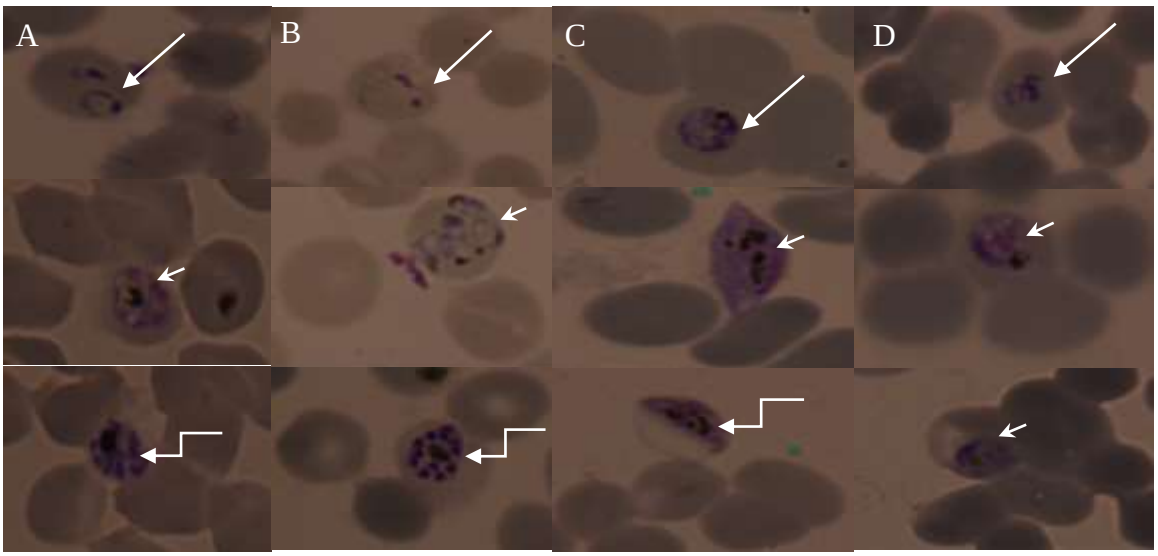

Figure 4.2: **Giemsa stained malaria parasites before and after exposure to CQ** showing; A - normal healthy morphological features of the unexposed population; normal healthy rings (arrow), mature trophozoites (arrow head) and schizont (elbow arrow), B – healthy morphological features at IC_{10} chloroquine (CQ) exposure level; normal ring (arrow), erythrocyte infected with multiple trophozoites (arrow head) and schizont (elbow arrow), C – gametocytes at IC_{30} CQ exposure level; young (arrow), growing (arrow head) and a mature gametocyte (elbow arrow), and D - degenerative forms at IC_{50} CQ exposure level; distorted morphology (arrow) and clearing cytoplasm (arrow head).

4.2 Drug Sensitivity Changes

Increase in CQ tolerance in 3D7 strain of *P. falciparum* after sustained drug pressure were noted as are shown in Figure 4.3. The mean IC_{50} of CQ unexposed parental population was 7.03±1.37 ng/mL (n=9) and increased significantly to 10.55±1.55 ng/mL (n=6, p=0.0016) and 15.05±1.38 ng/mL (n=8, p <0.01) at concentration equal to IC_{10} (4.51 ng/mL) and IC_{30} (5.99 ng/mL) respectively. At 7.15 ng/mL of CQ (equal to IC_{50}), the parasites failed to reach the minimum parasitemia (0.8%) required for the SYBR Green I *in vitro* drug sensitivity assay. This is an indicator of increased drug tolerance and possibly the development of drug resistance within the culture population.

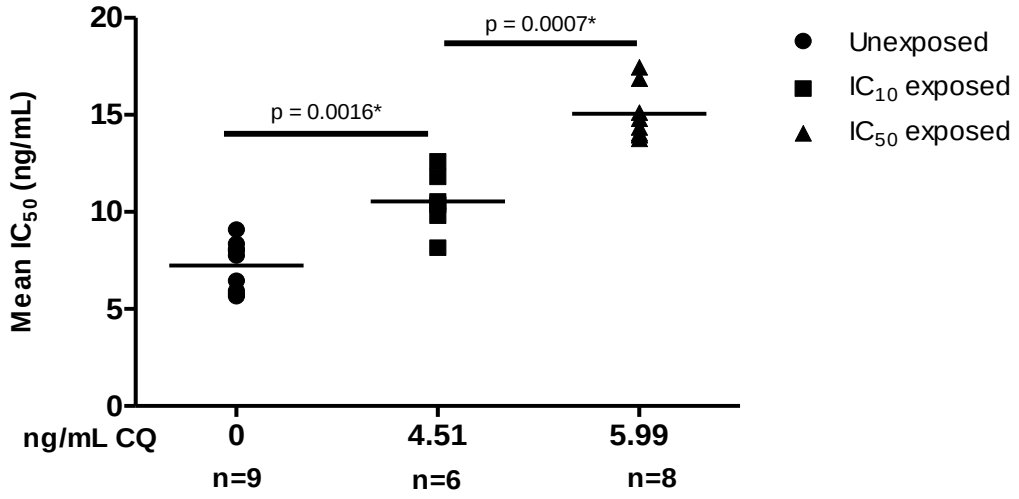

Figure 4.3: Scatter plot of mean IC_{50} values for *P. falciparum* 3D7 before and after continuous exposure chloroquine (CQ). The mean IC_{50} for the unexposed parental population was 7.03±1.37 ng/mL and increased significantly to 10.55±1.55 ng/mL and 15.05±1.38 ng/mL at CQ concentration equal to IC_{10} (4.51 ng/mL) and IC_{30} (5.99 ng/mL). p* values indicate significant differences between the means, n is the number of repeats.

4.3 PfCRT SNP profiling; codons 220, 271, 326, 356 and 371 by PCR-RFLP

The SNP profiles of the *P. falciparum* 3D7 cultures before and after CQ exposure were monitored for any genetic mutation changes arising during the culturing and drug exposure process in the PfCRT gene. For codons 220 (A), 271 (Q), 326 (N), 356 (I) and 371 (R) no genotypic changes occurred and the SNP profiles remaining the same as wild type gene (Figures 4.4 – 4.5).

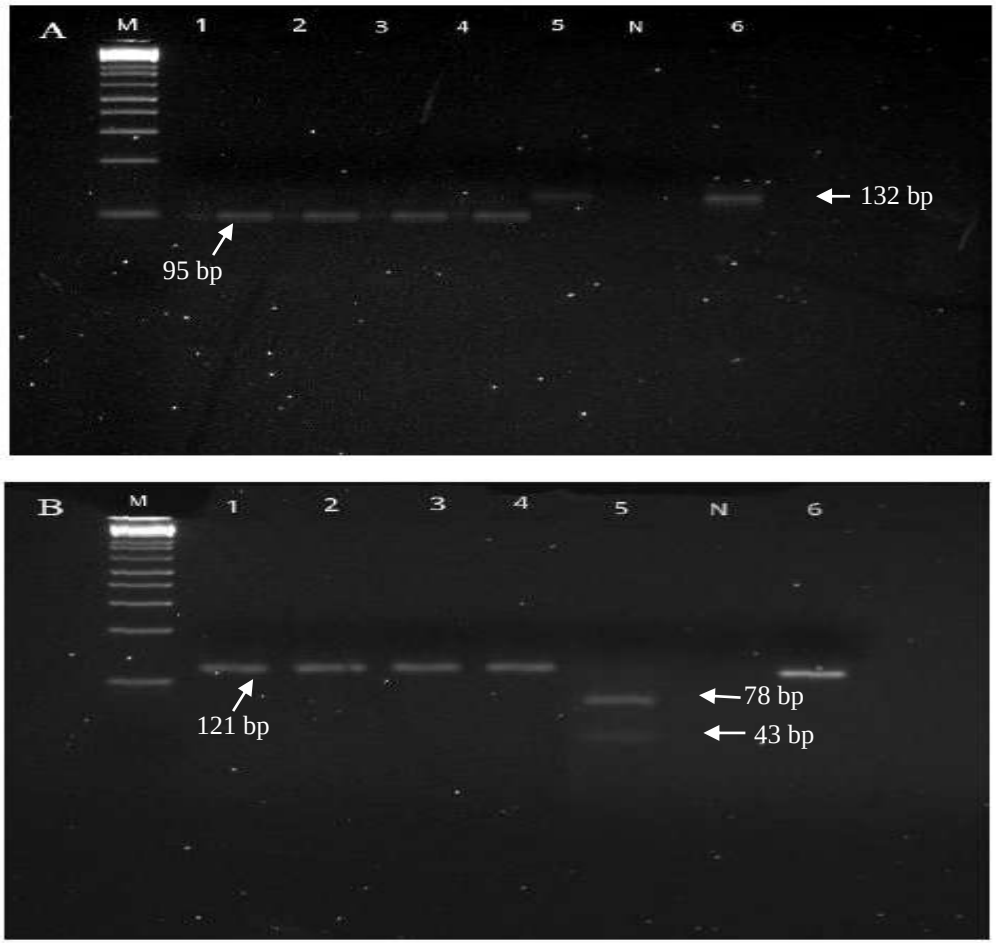

Figure 4.4: RFLP at PfCRT codons 220 (A) and 271 (B). In A, lanes 1-4 show the digested wild type 95bp product in the unexposed, IC_{10} exposed, IC_{30} exposed, and IC_{50} exposed, respectively, while lanes 5-6 show the undigested 132bp product in mutant (W2) and undigested control samples, using restriction enzyme BglI. In B, lanes 1-4 and 6, show the 121 bp undigested wild type product in the unexposed, IC_{10} exposed, IC_{30} exposed, IC_{50} exposed, and the undigested control sample respectively, while lane 5 shows the digested 78 bp and 43 bp product in mutant control (W2), using the restriction enzyme XmnI. M is the 1 Kb plus DNA ladder and N a negative template control.

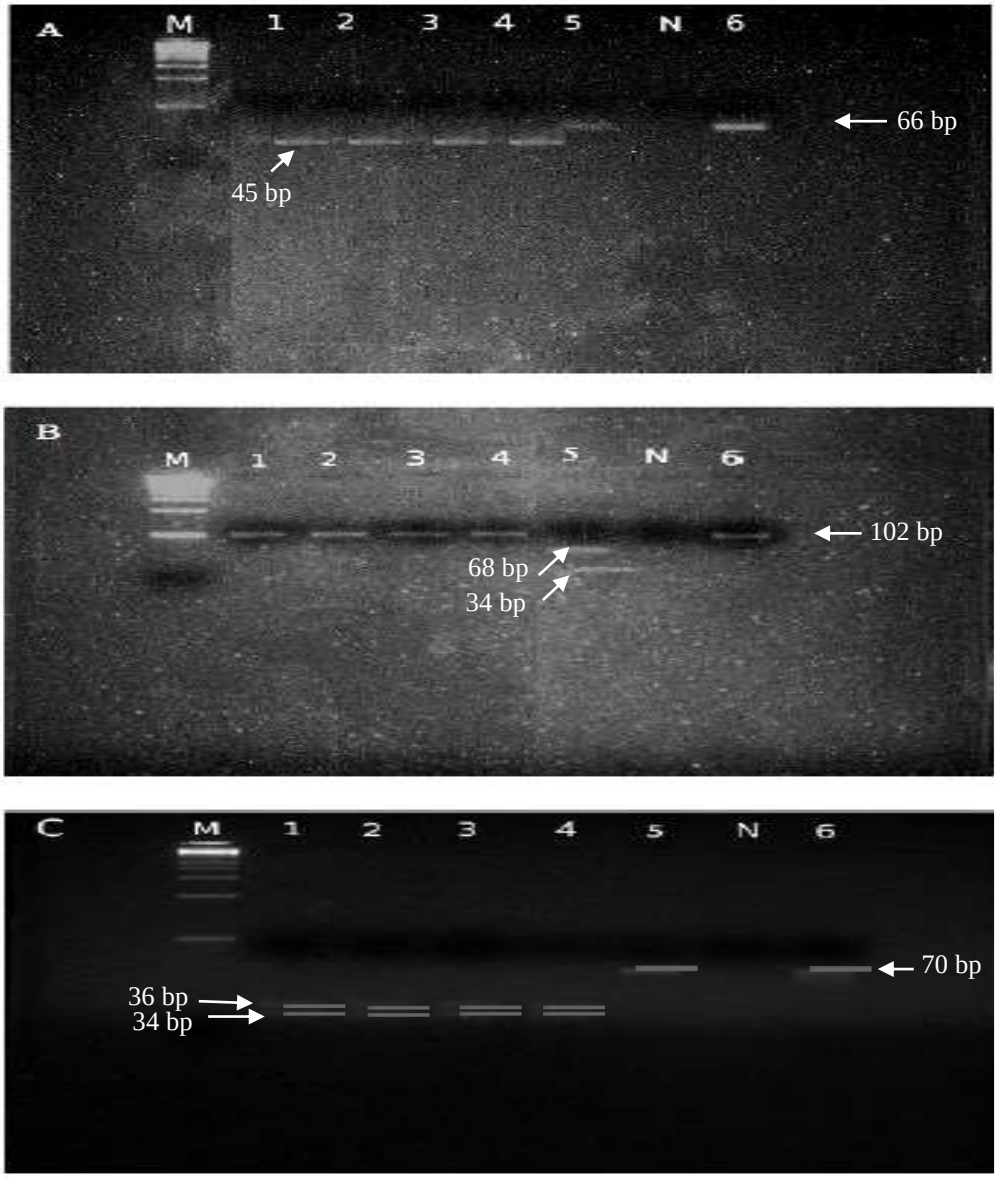

Figure 4.5: RFLP at PfCRT codons 326 (A), 356 (B) and 371 (C). In A, lanes 1-4 show the digested wild type 45bp product in the unexposed, IC_{10} exposed, IC_{30} exposed, and IC_{50} exposed, respectively, while lanes 5-6 show the undigested 66 bp product in mutant (W2) and undigested control samples, using restriction enzyme MseI. In B, lanes 1-4 and 6, show the 102bp undigested wild type product in the unexposed, IC_{10} exposed, IC_{30} exposed, IC_{50} exposed, and the undigested control sample respectively, while lane 5 shows the digested 68 bp and 34 bp product in mutant control (W2), using restriction

enzyme AlwNI. In C, lanes 1-4 show the digested wild type 36 and 34 bp product in the unexposed, IC_{10} exposed, IC_{30} exposed, and IC_{50} exposed, respectively, while lanes 5-6 show the undigested 70bp product in mutant control (W2) and undigested control samples, using restriction enzyme AflII. M is the 1 Kb plus DNA ladder and N a negative template control.

4.4 PfCRT 72-76 SNP haplotype

The PfCRT codons 72-76 were sequenced to determine the haplotype of this variable region. PfCRT codon 76, one of the important mutations associated with CQ resistance, was maintained in its wild type genotype. There was no change in the different drug exposure levels with the wild type CVMNK (TGTGTAATGAATAAA) haplotype being maintained (Figure 4.6).

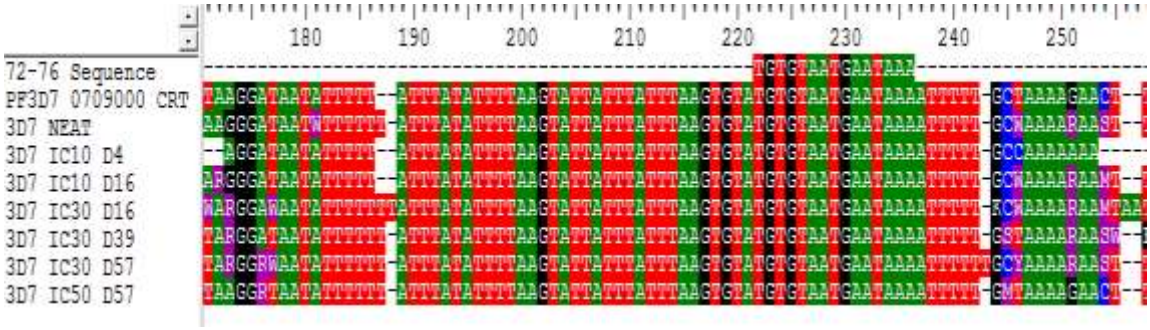

Figure 4.6: Multiple sequence alignment of the PfCRT 72-76 region in *P. falciparum* 3D7 before and after CQ exposure. The 72–76 sequence highlights the region of interest in the multiple sequence alignment while the PF3D7 0709000 CRT is the wild type reference sequence. 3D7 Neat is the unexposed population, while the exposed populations are 3D7 IC_{10}, 3D7 IC_{30} and 3D7 IC_{50}.

The complete PfCRT SNP profile for the exposed and unexposed *Plasmodium falciparum* 3D7 culture samples is as summarized in table 4.1.

Table 4.1: SNP analysis at PfCRT codons 72-76, 220, 271, 326, 356 and 371, in *P. falciparum* 3D7 before and after CQ exposure

Sample	**CQ exposure concentration level**	**PfCRT codon**									
		72	73	74	75	76	220	271	326	356	371
Wild Type allele		TGT [C]	GTA [V]	ATG [M]	AAT [N]	AAA [K]	GCC [A]	CAA [Q]	AAC [N]	AAT [N]	AGA [R]
Mutant allele		**A**GT [**S**]	GTA [V]	AT**T** [**I**]	**G**A**A** [**E**]	A**C**A [**T**]	**T**CC [**S**]	**G**AA [**E**]	A**G**C [**S**]	A**T**T [**T**]	A**T**A [**I**]
P. falciparum 3D7	No Exposure	TGT [C]	GTA [V]	ATG [M]	AAT [N]	AAA [K]	GCC [A]	CAA [Q]	AAC [N]	AAT [N]	AGA [R]
	IC_{10}	TGT [C]	GTA [V]	ATG [M]	AAT [N]	AAA [K]	GCC [A]	CAA [Q]	AAC [N]	AAT [N]	AGA [R]
	IC_{30}	TGT [C]	GTA [V]	ATG [M]	AAT [N]	AAA [K]	GCC [A]	CAA [Q]	AAC [N]	AAT [N]	AGA [R]
	IC_{50}	TGT [C]	GTA [V]	ATG [M]	AAT [N]	AAA [K]	GCC [A]	CAA [Q]	AAC [N]	AAT [N]	AGA [R]

Residues that differ from the wild-type sequence are shown in bold

Amino acid represented in the [Square brackets]

4.5 PfMDR1 copy number and SNP analysis; codons 86, 184, 1034, 1042 and 1246

At the unexposed parental population, and at all CQ exposure levels (IC_{10}, IC_{30} and IC_{50}), the PfMDR1 codon usage remained wild type for codons 86 (N), 184 (Y), 1034 (S), 1042 (N) and 1246 (D), with a single copy of the gene at all instances (Table 4.2). SNP analysis was performed by allelic discrimination using probe based PCR resulting in a genotyping plot that discriminated between wild type and mutant alleles (Figure 4.7).

Table 4.2: PfMDR1 SNP analysis at codons 86, 184, 1034, 1042 and 1246, and gene copy number

Sample	**CQ exposure concentration level**	**PfMDR1 codon**					***copy number***
		86	184	1034	1042	1246	
Wild Type allele		AAT [N]	TAT [Y]	AGT [S]	AAT [N]	GAT [D]	1
Mutant allele		**T**AT [**Y**]	T**T**T [**F**]	**T**GT [**C**]	**G**AT [**D**]	**T**AT [**Y**]	2 – 4
P. falciparum 3D7	No Exposure	AAT [N]	TAT [Y]	AGT [S]	AAT [N]	GAT [D]	1
	IC_{10}	AAT [N]	TAT [Y]	AGT [S]	AAT [N]	GAT [D]	1
	IC_{30}	AAT [N]	TAT [Y]	AGT [S]	AAT [N]	GAT [D]	1
	IC_{50}	AAT [N]	TAT [Y]	AGT [S]	AAT [N]	GAT [D]	1

Residues that differ from the wild-type sequence are shown in bold

Amino acid represented in the [Square brackets]

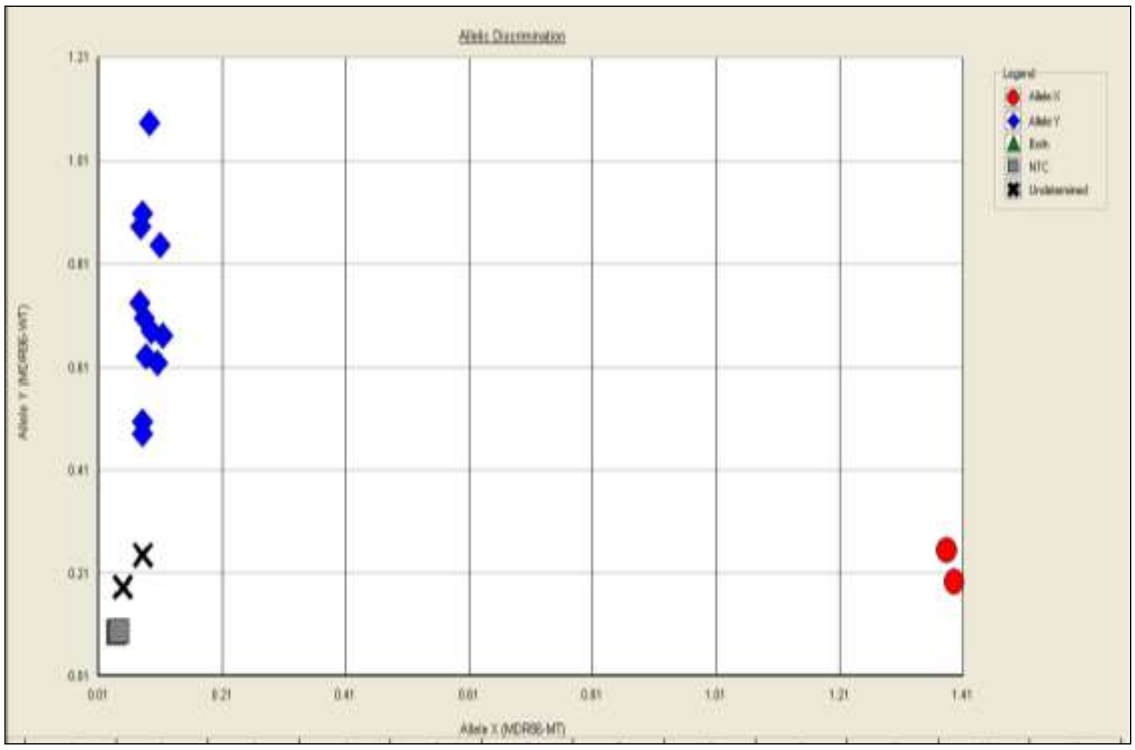

Figure 4.7: Allelic discrimination SNP genotyping plot for PfMDR1 codon 86 showing the mutant (●), and wild type (♦) alleles (■ - No Template Control, X – Undetermined).

4.6 Allelic Diversity Analysis

The allelic structure of the *P. falciparum* 3D7 CQ unexposed parental population had one K1 allele of 248 bp, two IC3D7 alleles of 482 bp and 596 bp, and a GLURP allele of 800 bp. Following exposure to CQ, clonal selection was evident with marked reduction of parasite population (measured by peak height and area) carrying the 248 bp K1 and 596 bp IC3D7 alleles at IC10. This reduction continued at CQ exposure concentrations equal to IC30 and by IC50, parasites with these alleles were completely lost (Figure 4.8).

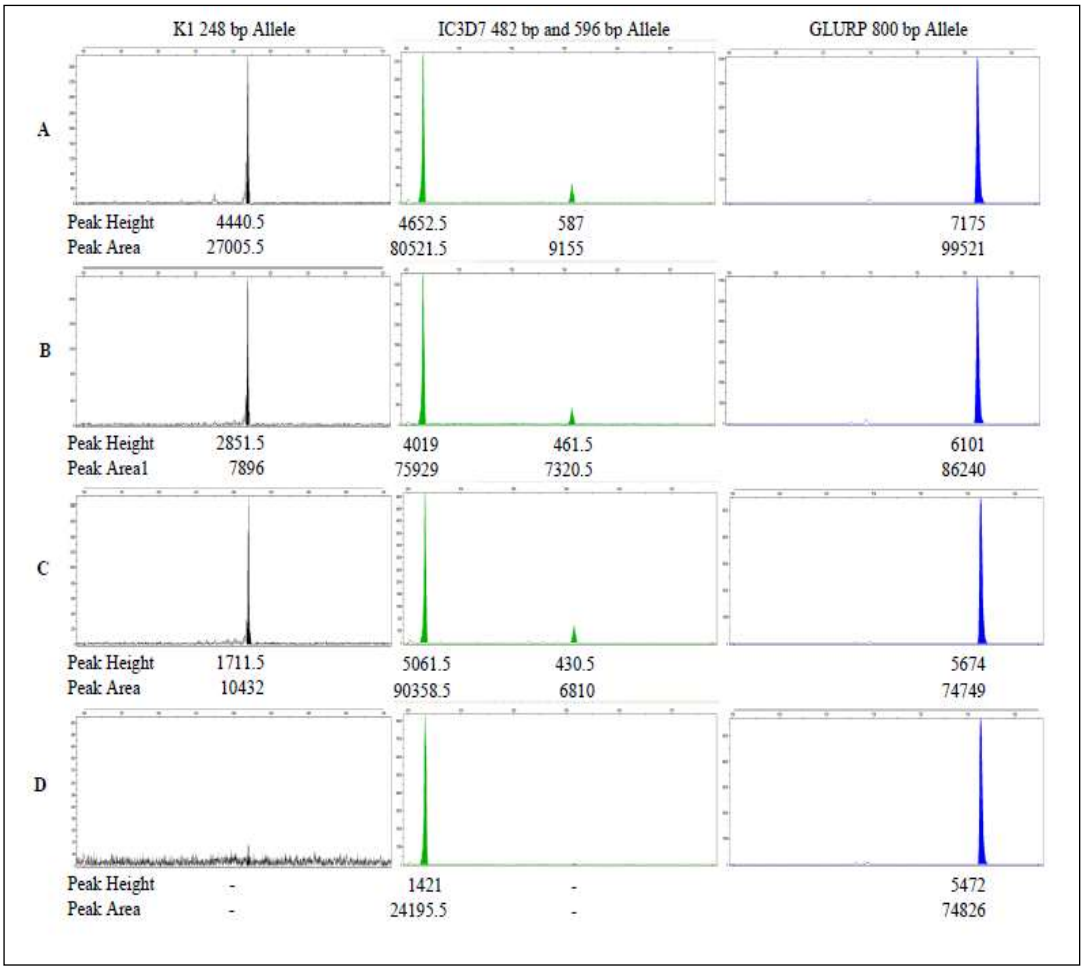

Figure 4.8: Electropherogram showing MSP1 (K1), MSP2 (IC3D7) and GLURP alleles before and after sustained exposure to different concentrations of chloroquine (CQ). Panel A: CQ unexposed population had a 248 bp K1, two IC3D7 allelic types (482 bp and 596 bp fragments) and an 800 bp GLURP. At CQ concentration equivalent to IC_{10} and IC_{30} (Panels B and C respectively), despite the presence of the same alleles observed in CQ unexposed parasites, a change in population structure is indicated by increasing reduction in peak height and peak area for parasites carrying the 248 bp K1 and 596 bp IC3D7 alleles. At CQ concentration equal to IC_{50} (Panels D), the 248 bp and 596 bp allelic forms were completely lost, with only the 482 bp IC3D7 and 800 bp GLURP alleles being retained.

4.7 DNA Methylation Analysis

Global 5-mC DNA methylation was not observed in the CQ unexposed or exposed parasite populations by DNA ELISA. The 5mC-DNA ELISA results had a net optical density (OD) values similar to that of the 0% methylation control (Table 4.3).

Table 4.3: Optical densities values obtained for the *P. falciparum* 3D7 before and after CQ exposure by 5mC-DNA ELISA.

Sample	**OD value (405 nm)**	**% 5-mC**
Positive Control (100 % 5-mC)	0.325	100 %
Positive Control (75 % 5-mC)	0.319	75 %
Positive Control (50 % 5-mC)	0.295	50 %
Positive Control (25 % 5-mC)	0.256	25 %
Positive Control (10 % 5-mC)	0.187	10 %
Positive Control (5 % 5-mC)	0.142	5 %
Negative Control (0 % 5-mC)	0.07	0 %
Blank	0.066	0 %
P. falciparum 3D7 No CQ Exposure	0.067	0 %
P. falciparum 3D7 IC_{10} CQ Exposure	0.068	0 %
P. falciparum 3D7 IC_{30} CQ Exposure	0.069	0 %
P. falciparum 3D7 IC_{50} CQ Exposure	0.071	0 %

CHAPTER FIVE

5.0 DISCUSSION

The mechanisms leading to the development and establishment of drug resistance in *P. falciparum* are not fully elucidated, but have been shown to arise due to gene mutations in drug target genes, changes in gene expression caused by alterations in cytoplasmic or environmental factors affecting these selected genes, and subsequent selection of strains that can withstand drug pressure (Beale, 1980). However, the development of these drug resistant states occurs at an evolutionary fitness cost where normal growth and survival is affected, raising the question whether there are selective advantages of maintaining resistant genotypes in the absence of drug pressure. The phenomena of non-genetic and probably temporary changes causing physiological adaptations of the parasites to anti-malarials under drug pressure however may alleviate this fitness cost allowing the parasite to regain normal function and survival once the drug pressure is gone. In this study, evolution of tolerance to CQ was investigated by evaluating changes in allele structure, genetic polymorphisms of PfCRT and PfMDR1 transporter genes and global DNA methylation at 5-methylCytosine.

5.1 Growth characteristics after drug exposure

The growth characteristics of CQ sensitive 3D7 strain of *P. falciparum* used in this study was assessed before and during sustained CQ exposure. The CQ unexposed parental population had a typical growth profile, doubling every 48 hours. Under CQ pressure equivalent to IC_{10}, the growth rate was similar to the unexposed parental population. At $IC_{30,}$ growth rate declined, gametocytes started appearing and morphologically degenerative asexual forms were noted. This study demonstrated an increase in gametocytogenesis concurring with other studies which observed the same findings following CQ administration which stresses the parasite (Buckling, Ranford-Cartwright, Miles, & Read, 1999). At CQ concentration equivalent to IC_{50}, the parasites failed to recover. The inability of the parasite population to recover could be due to the fact that

at IC_{50} a therapeutic/curative concentration is used that rapidly clears the parasite population under sustained pressure and disrupts the life cycle for multiplication of merozoites to infect new red blood cells.

5.2 Variation in Chloroquine Sensitivity

Chemo sensitivity to CQ was in the parental and exposed parasite populations was determined with tolerance increasing significantly from an IC_{50} of 7.03±1.37 ng/mL (sensitive) for the unexposed parental population to 15.05±1.38 ng/mL (moderate resistance). Parasites could not be maintained to attain higher IC_{50} than this. Other studies have reported induction of CQ tolerance in *P. falciparum* sensitive strain of up to 19 ng/mL (Cooper *et al.,* 2002). In *ex vivo* field isolates, IC_{50} values of up to 100 ng/mL have been reported (Eyase *et al.,* 2013). It is not clear why in the current study, adaptation to CQ concentration beyond 15.05±1.38 ng/mL was not possible, but it could be attributed to the methodology used.

An alternative approach of short exposures to high drug concentrations instead of sustained exposure could have overcome this limitation, as was used for derivation of highly resistant mefloquine lines (Peel, Merritt, Handy, & Baric, 1993). In addition, the current study used a clonal population as opposed to mixed population. The reduction in repertoire could have limited the plasticity of clones to adapt to higher drug concentration levels. A recent study in Thailand reported that polyclonal parasites, with two or more alleles at either MSP1, MSP2 or GLURP, had a higher likelihood to develop resistance as opposed to monoclonal parasites (Gosi *et al.,* 2013). Changes in the temporary growth, arrest and dormancy profile of the parasites may infer selection of this tolerant phenotype. Developmental arrest and the selection of dormant parasite blood stages, especially rings and schizonts, offer a more general defense mechanism of parasites against drugs (Witkowski *et al.,* 2010). This induces a resistant state phenotype which recovers from dormancy once the drug pressure is alleviated.

5.3 SNP genotypic changes at PfCRT and PfMDR1

CQ resistance in *P. falciparum* is associated with change in amino acid, and SNPs are used as genetic markers of drug resistance. In this study, variations in SNPs at the PfCRT and PfMDR1 genes were not detected despite the increase in tolerance to CQ. The changes from K76T and N86Y in the PfCRT and PfMDR1 genes respectively, are particularly important in development of chloroquine resistance (Djimde *et al.,* 2001; Hayward *et al.,* 2005). The CVMNK haplotype is also key as progression to drug resistance starts with mutations around the K76T position, especially codons 72, 74 and 75, with variations of CVIET, CVIDT, CVMNT and SVMNT noted in drug resistant populations (Takahashi *et al.,* 2012). Other mutations that play a role in this process are located in codons 220, 271, 326, 356 and 371 of PfCRT, and codons 184, 1034, 1042 and 1246 of PfMDR1, and are seen as lesser mutations which tend to occur sequentially either after or before the occurrence of K76T and N86Y mutations (Fidock *et al.,* 2000; Hayward *et al.,* 2005). Possibilities exist that apart from PfCRT and PfMDR1, other genes that the current study did not evaluate are involved, contributed by the fact that the development of chloroquine resistance may be controlled by a number of unrelated metabolic processes controlled by different genes (Beale, 1980). Loci that have been implicated include amplification of chromosome 3 and 12, which neither PfCRT nor PfMDR1 are located (Lim & Cowman, 1996), and positive selection of microsatellite loci flanking the PfCRT and PfMDR1 genes (Alam *et al.,* 2011). By use of next-generation sequencing approaches, whole genome transcript analysis, and genome wide association studies, other changes in the *Plasmodium* genome associated with evolution of drug resistance may be explored.

5.4 Clonal genetic diversity

The only genetic change that was noted following induction of CQ tolerance was in allelic structure. Subtle changes in clonal selection appeared early, with exposure of parasites to CQ equivalent to IC_{10} that was evident as allele reduction in parasites carrying the 248 bp K1 and 596 bp IC3D7 alleles. This trend was enhanced by increase

in concentration of CQ level, and at a dose equivalent to IC_{50} of parental population, parasites with these alleles were completely lost. This being the only noticeable change observed, it is tempting to speculate that the development of CQ tolerance starts by clonal selection. Such changes have also been noted in clinical cases of severe malaria where drug treatment has been administration (A-Elbasit *et al.,* 2007). The high allelic variation in the MSP-1, MSP-2 and GLURP genes, and the absence of specific resistance associated alleles (Gosi *et al.,* 2013) may point toward changes in the parasite genome relating to increased tolerance to drug exposure. Further genomic studies would help establish whether this is the case.

The appearance of *P. falciparum* parasites with decreased *in vitro* sensitivity but no measurable resistance markers to anti-malarials has raised the urgent need to characterize the resistance phenotype and the mechanisms that cause this phenomenon. An aspect of these would be reduced parasite clearance rates observed after drug treatment (Witkowski *et al.,* 2010). Studies have shown that reduced clearance is a phenotypically hereditable trait, a hallmark of epigenetics, and this variation in clearance time is determined by genetic factors, with variations in gene copy number and expression associated (Anderson *et al.,* 2010).

5.5 DNA Methylation

The role of epigenetics in facilitating temporary phenotypic adaptation before mutations fixation could occur via gene expression or repression. Modifications of 6-methyl-Adenine (6-mA) and 5-methyl-Cytosine (5-mC) have been noted in eukaryotic organisms and in lower prokaryotes (Gomez-Diaz, Jorda, Peinado, & Rivero, 2012). However, no 6-mA has been demonstrated in *P. falciparum* despite the fact that its genome is AT rich (Pollack, Kogan, & Golenser, 1991), leaving the possibility of utilization of 5-mC for modification. In this study, global 5-mC in *P. falciparum* DNA was assessed using a monoclonal antibody specific and sensitive to this DNA

modification. Global 5-mC DNA methylation was not observed in the initial parental and derived CQ tolerant populations of 3D7 *P. falciparum* parasites.

Earlier studies, using methylation specific restriction enzymes, had observed partial methylation of cytosine residues in CpG islands at a specific site for the gene coding for dihydrofolate reductase-thymidylate synthase (DHFR) associated with drug resistance to pyrimethamine (Pollack *et al.,* 1991). More recently, genome wide mapping of the *Plasmodium* genome has found presence of methylated cytosine residues and also a functional DNA methyltransferase that may mediate this modification (Ponts *et al.,* 2013). Results presented here on the absence of 5-mC DNA methylation are similar to other studies where absence of 5-mC DNA methylation in *P. falciparum* was noted using by liquid chromatography/electron spray ionization mass spectrometry (LC/ESI-MS) (Choi, Keyes, & Horrocks, 2006). Further evidence from bioinformatics analysis of predicted proteins of the *Plasmodium* genome show that no homologues for methyltransferases or any methyl DNA binding protein domains are present (Coulson, Hall, & Ouzounis, 2004). This absence of detectable 5-mC may be based on the fact that the genome of *P. falciparum* is AT rich and the theory that G and C nucleotides are less favored by natural selection in obligate pathogens because GTP and CTP nucleotides cost more energy and there is less availability of GTP and CTP in the cell (Rocha & Danchin, 2002). Also, reducing G and C in the genome would reduce the innate immune response as toll-like receptor 9 specifically recognizes non-methylated CpG dinucleotides (Dalpke, Frank, Peter, & Heeg, 2006).

CHAPTER SIX

6.0 CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

6.1 Conclusions

This study demonstrated increased tolerance to chloroquine by *P. falciparum* 3D7 strain in continuous culture with prolonged drug exposure. Morphological changes due to physiological stress were observed in the parasite population. This increase in tolerance was neither associated with any SNP changes in the PfCRT or PfMDR1 genes, nor with an increase in gene copy number of the PfMDR1 genes. However, a change in clonal genetic diversity was noted in the parasite population that developed tolerance to CQ, which involved variations observed in the highly polymorphic regions of MSP1 and MSP2 genes. No epigenetic changes were observed when global 5-mC DNA methylation was evaluated.

These results thus suggest that development of CQ tolerance starts by clonal selection and that epigenetic mechanisms do not mediate early changes in drug sensitivity prior to genetic fixation of mutations that cause drug resistance in *P. falciparum* under anti-malarial drug pressure.

6.2 Study Limitations

Global DNA methylation profiling approach was used to detect the overall effect on drug sensitivity changes on the entire genome. Although this is a good approach to elucidate the polygenic nature of drug resistant phenotypes, a more localized, gene specific approach may have been used that may have identified minor changes in DNA methylation that may not have been detected using a global approach. Secondly, DNA methylation was not investigated in a mutant parasite population that had developed from a sensitive line. The approach taken here tried to elucidate the stage development DNA methylation process towards drug resistance, achieving drug resistant phenotypes with no detectable mutation changes. The lack of proper laboratory infrastructure and

cost implications in conducting such in depth genetic studies also limits the extent to which the study objectives may be achieved. The use of different approaches to solve the issue and validate the results require extensive funding and specialized equipment which could not be realized in this study.

6.3 Recommendations

Alternate approaches that are recommended for achieving more insight into this area of research will include using a different drug exposure method that includes short exposures to high drug concentrations instead of sustained exposures at low drug concentrations to achieve highly drug resistant strains. Also, the use of a mixed population contributing different repertoires in terms of drug resistant alleles should be investigated to detect if this would contribute to faster adaptation and development of drug resistant phenotypes and expression of genotypic markers. The multifactorial nature of the drug under investigation, may require more intensive tools for the detection of minor changes in the genotype of the resistant populations by utilizing tools such as genome wide association studies or whole genome deep sequencing to identify all associated variants arising from increased tolerance to the drug. It is hoped that with next-generation sequencing approaches, genome wide association studies, including sulfur sequencing, may reveal hitherto unknown variation in *Plasmodium* genome that are associated with evolution of drug resistance. It will also be important to investigate other epigenetic changes such as reversible histone modifications and RNAi as potential mechanisms for allowing survival following temporary drug pressures.

Future recommendations and insights into *P. falciparum* epigenetics would include but not be limited to:

i. Gene specific DNA methylation profiles at drug resistance genes under drug pressure versus the global DNA methylation.

ii. Analysis of protein interactions at the chromatin level especially epigenetic mechanisms of histone modification at genes specific for drug resistance in *P. falciparum*.

iii. Functional role of RNAi including noncodingRNA (ncRNA) and microRNA (miRNA) in the development of drug resistant phenotypes.

A better understanding of the actions of anti-malarial drugs and mechanisms of drug resistance will lead to more effective therapeutic combinations as well as improved molecular assays to detect and track drug-resistant parasites.

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APPENDICES

Appendix 1. KEMRI SSC Approval Letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

ESACIPAC/SSC/9636 **11th August , 2011**

Martin Wahome

Thro'
Director, CBRD
NAIROBI

REF: **SSC No. 2112 (Revised) – Epigenetic control of gene expression levels in *plasmodium falciparum* under chloroquine pressure**

Thank you for your letter dated 10th August, 2011 responding to the comments raised by the KEMRI SSC.

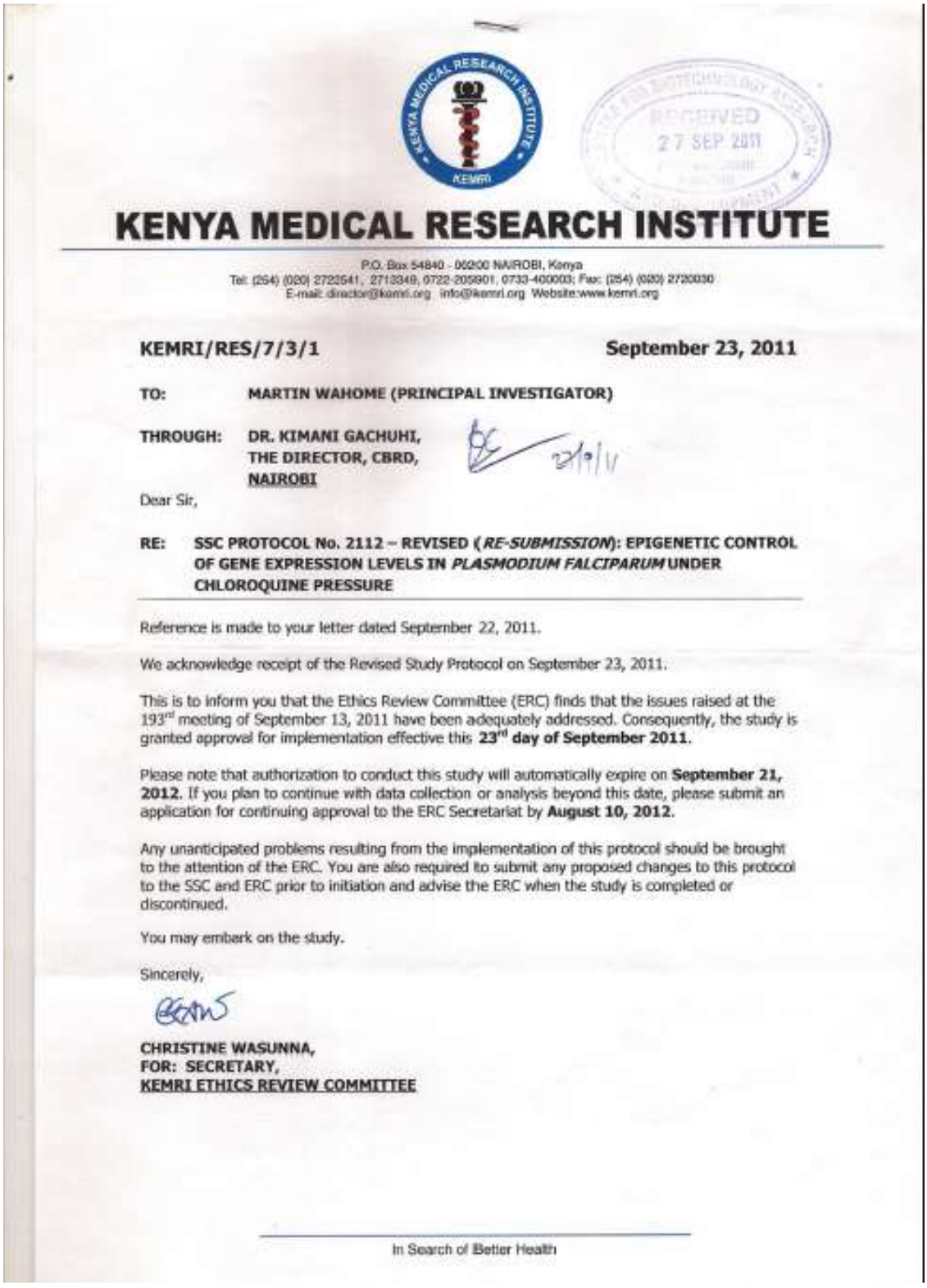
I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval

Fol: Sammy Njenga, PhD
SECRETARY, SSC

In Search of Better Health

Appendix 2. KEMRI ERC Approval Letter



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KEMRI/RES/7/3/1 **September 23, 2011**

TO: **MARTIN WAHOME (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. KIMANI GACHUHI,**
THE DIRECTOR, CBRD,
NAIROBI

Dear Sir,

RE: **SSC PROTOCOL No. 2112 – REVISED (*RE-SUBMISSION*): EPIGENETIC CONTROL OF GENE EXPRESSION LEVELS IN *PLASMODIUM FALCIPARUM* UNDER CHLOROQUINE PRESSURE**

Reference is made to your letter dated September 22, 2011.

We acknowledge receipt of the Revised Study Protocol on September 23, 2011.

This is to inform you that the Ethics Review Committee (ERC) finds that the issues raised at the 193rd meeting of September 13, 2011 have been adequately addressed. Consequently, the study is granted approval for implementation effective this **23rd day of September 2011.**

Please note that authorization to conduct this study will automatically expire on **September 21, 2012.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **August 10, 2012.**

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study.

Sincerely,

CHRISTINE WASUNNA,
FOR: SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix 3. Abstract of Published Manuscript

American Journal of Biology and Life Sciences
2014; 2(5): 100-107
Published online September 30, 2014 (http://www.openscienceonline.com/journal/ajbls)

Chloroquine tolerance in 3D7 strain of *P. falciparum* was associated with change in allelic structure and not *Pf*crt and *Pf*mdr1 transporter genes nor DNA methylation

Martin Maina Wahome[1, 2], Josphat Nyandieka Nyataya[1], Hosea Miima Akala[1], George Odhiambo Awinda[1], Beth Kinya Mutai[1], Rebecca Waihenya[3], Edwin Kamau[1], Sabah Ahmed Omar[4], John Njenga Waitumbi[1, *]

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Abstract

Development of anti-malarial resistance occurs at a fitness cost to the parasites. It makes sense to assume that *P. falciparum* would first use alternative mechanisms to survive transient drug pressure, and only resort to genetic fixation if the pressure is sustained. In this study, development of chloroquine (CQ) tolerance was assessed by DNA methylation, allelic diversity and genetic changes at *Pf*crt and *Pf*mdr1. A CQ sensitive 3D7 strain of *P. falciparum* was cloned by limiting dilution and the derived population exposed to increasing CQ concentrations of 4.51 ng/mL, 5.99 ng/mL and 7.15 ng/mL corresponding to 10%, 30% and 50% inhibitory concentrations (IC) of the parental population. The surviving parasite density at each drug level was determined by SYBR Green I fluorassay. Allelic diversity of CQ unexposed and those surviving drug pressure were assessed by nested PCR that targeted the polymorphic regions of msp1, msp2 and glurp. Global DNA methylation at 5-methylCytosine (5-mC) was assessed by ELISA. Mutations at *Pf*crt and *Pf*mdr1 genes were assessed by single nucleotide polymorphisms (SNPs). The CQ unexposed population had an IC_{50} of 7.03±1.37 ng/mL, one K1 allele (248 bp), two IC3D7 (482 bp and 596 bp) and one 800 bp glurp. *Pf*crt and *Pf*mdr1 were wild type. 5-mC DNA methylation was not detectable. Post CQ exposure at 4.51 ng/mL and 5.99 ng/mL, IC_{50} increased to 10.5 ng/mL and 15.05 ng/mL respectively. Parasite growth at 7.15 ng/mL of CQ was minimal IC_{50} could not be determined. At 5.99 ng/mL of CQ, change in parasite structure was marked by allele reduction in parasites carrying the K1 and the 596 bp IC3D7 alleles. At 7.15 ng/mL of CQ, parasites with these two alleles were lost, but the 482 bp IC3D7 and 800 bp glurp clones survived. CQ tolerant populations remained wild type at *Pf*crt and *Pf*mdr1. 5-mC DNA methylation was not observed in any of the derived parasite populations. These data suggest that, development of CQ tolerance starts by clonal selection. In absence of genetic or epigenetic changes to the surviving clones, further studies are needed to elucidate how CQ induced changes at *Pf*crt and *Pf*mdr1 genes eventually occur.

Keywords

Plasmodium falciparum, DNA Methylation, *Pf*crt, *Pf*mdr1, Single Nucleotide Polymorphisms, Chloroquine Resistance, Genetic Diversity