MOLECULAR ANALYSIS OF ANTIMALARIAL RESISTANCE MARKERS IN PARASITE SAMPLES OBTAINED FROM CHILDREN RECRUITED INTO A DRUG EFFICACY TRIAL IN KWALE, KENYA, 2013.

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DECLARATION

I declare that this research is my own work and has not been submitted for examination in any other university.

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LIST OF ABBREVIATIONS

uRBC	uninfected Red Blood cells
ITNs	Insecticide Treated Nets
IRS	Indoor Residual Spraying
ART	Artemisinin
ACTs	Artemisinin-based Combination Therapies
CQ	Chloroquine
AL	Artemether-Lumefantrine
LM	Lumefantrine
DP	Dihydroartemisinin-Piperaquine
SP	Sulphadoxine-Pyrimethamine
CQR	Chloroquine Resistance
DNA	Deoxyribonucleic acid
SNP	Single Nucleotide Polymorphism
PfMDR1	Plasmodium falciparum multidrug resistance gene
PfCRT	Plasmodium falciparum chloroquine resistance transporter
MSP2	Merozoite Surface Protein 2
GLURP	Glutamate Rich Protein
K13	Kelch 13
ACPR	Adequate Clinical Parasitological Response
PCR	Polymerase Chain Reaction

ABSTRACT

Artemisinin-based combination therapies (ACTs) have resulted to a significant drop in malaria incidences by 18% from 2010 to 2016. Unfortunately, ACT resistance reported in Western Cambodia in 2008 poses a consequential threat to the efficacy of artemisinin (ART). Furthermore, ART-resistance has sprouted in several countries in Southeast (SE) Asia. Thus far, ACT resistance has not been reported in Kenya but the main concern is the possible spread of resistant parasites from SE Asia to Sub-Saharan Africa. This study therefore set out to distinguish recrudescent from new infections and analyze known drug resistance markers, *k13*, *Pfcrt* and *Pfmdr1* in pre- and post-treatment dried blood spot (DBS) samples, obtained from children, recruited into an antimalarial drug efficacy trial of AL (Artemether-lumefantrine) and DP (dihydroartemisinin-piperaquine), in Msambweni constituency, Kwale County, on the coast of Kenya in 2013. Parasite DNA was extracted for molecular genotyping on days 0, 1, 2, 3, 7, 14, 21, 28 and 42. The parasite DNA was used to: 1) genotype *glurp* and *msp2* genes to differentiate re- from recrudescent-infections by gel electrophoresis and to calculate the PCR-corrected adequate clinical parasitological response (ACPR) and 2) genotype the drug resistance markers by PCR and capillary sequencing. In total, 363 children were recruited into the trial; 162 (45%) and 201 (55%) receiving AL and DP respectively. 49 participants were malaria slide-positive after treatment; 1, 1, 8, 13 and 26 on days 7, 14, 21, 28 and 42, respectively. The *msp2* and *glurp* genotype analysis performed on samples from 39 of the 49 participants. This revealed 8 (20.5%) recrudescent infections and 31 (79.5%) re-infections. The day 42 PCR-corrected ACPR was 97.5% and 99.5% in the AL and DP arm respectively. The *Pfcrt* 76T mutation was seen to decrease from 27% (day 0) to 9.7% (day 42) as part of the CVIET haplotype. For *Pfmdr1,* there was a slight increase in the 86Y mutant frequency from 11% (day 0) to 12% (day 1), however, the mutant allele was cleared in all the subsequent days. The prevalence of the 184F mutation remained stable at 41% (day 0, n=208), 56% (day 1, n=116) and 40% (days 2 to 42, n=20) and the mutant 1246Y allele was observed only on day 0 (7%). In the *k13* propeller domain only one synonymous mutation was observed on day 42 at codon 487 from a GTA -> GTG encoding the amino acid Valine. Our results suggest that ACTs were still effective at the study site in 2013, since no *k13* mutations were observed and due to the high PCR-corrected ACPR recorded in both AL and DP. The high re-infection rate suggests a need for continued drug resistance surveillance.

CHAPTER ONE

INTRODUCTION

Prior to the use of artemisinin-based combination therapies (ACTs) in treating uncomplicated malaria, sulphadoxine-pyrimethamine (SP) and chloroquine (CQ) were effective antimalarials (1). Chloroquine resistance was reported in 1950 in both SE Asia and South America and by 1980 it was rendered ineffective (2). In Africa, CQ resistance emerged in 1970 and by 1980 the level of resistance had increased rapidly in both the eastern and southern part of Africa (3). In Senegal, CQ resistance was first reported in 1990 and by 1995 45% of the infections demonstrated moderate to high levels of resistance (4). Several studies have confirmed that mutations in the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene is the marker for CQ-resistance (5). Mutation on the *pfcrt* gene on codon 76 from a Lys to Thr (K76T) has been associated with CQ resistance (5). Following the spread of CQ resistance in Africa, in 1993, Malawi took the forefront as the first African country to change the treatment policy from CQ to an antifolate combination, SP, since by this time the efficacy of CQ was below 50% (6). Other countries such as Kenya, Uganda and Tanzania followed suit (7). Resistance to SP is strongly correlated to the parasite having a gradual build-up of non-synonymous-mutations in the genes encoding the drug target enzymes, dihydropteroate synthase (codons 437, 540 and 581) and dihydrofolate reductase (codons 51, 59, 108 and 164) (8,9). Following the spread of *P. falciparum* resistance to mono-therapies, the efficacy of ACTs in treating uncomplicated malaria has seen a decline in mortality rates by 33% (10). ACTs are co-formulations of a potent ART component that eliminates the bulk of the parasite load plus a partner drug that eliminates the remaining parasites, due to its longer plasma drug half-life (11). Unfortunately, artemisinin resistance, phenotypically displayed >48 hours parasite clearance times or simply as slow parasite clearance, is persistently being reported in SE-Asia (12–14). Specific non-synonymous mutations in the kelch propeller domain of *Plasmodium falciparum* chromosome 13 (*k13)* mutations have been identified as the suitable marker for artemisinin resistance (15). A number of SE Asian countries have identified the mutations associated with delayed parasite clearance such as Cambodia, Vietnam and Myanmar (16). In Africa artemisinin resistance has not been reported, as shown in Figure 1-1. *In vivo* drug trials have reported rapid clearance times of parasites as well as low frequency

K13 propeller mutations in most African countries (16–19). Majority of the mutations identified in Africa are different from those observed in SE Asia.

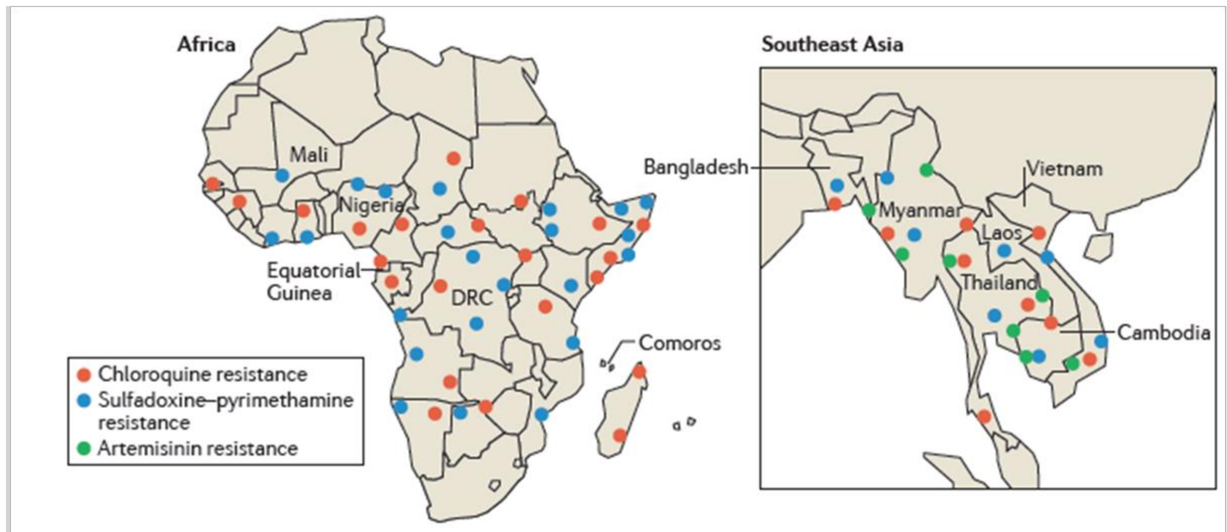

Figure 1-1: **A map of both Africa and Southeast Asia showing the distribution of *P. falciparum* resistance to CQ, SP and ART**. Adapted from (20). CQ and SP resistance has been reported in Southeast Asia and Africa. Artemisinin resistance which is defined as having both the delayed parasite clearance phenotype and mutations in the propeller domain of *Pfk13* gene has not yet been identified in Africa.

1.2 Problem Statement

Malaria infects approximately 200 million people annually with the major tool for treatment being a combination of antimalarial drugs known as ACTs (21). A unique feature of artemisinins is that they rapidly clear parasites as compared to other antimalarials such as quinine (22). Unlike most drugs that only target the late erythrocytic stages, artemisinins also target early erythrocytic stages by killing the ring stages. Unfortunately, ART resistance was first identified in 2008 after artesunate monotherapy on the Thailand-Cambodia border (12,13). Artemisinin resistance presenting as delayed parasite clearance after a 3-day treatment is highly correlated with greater recrudescent rates *in vivo*. This suggests that parasites surviving artemisinin exposure will resume their normal development after artemisinin elimination from the body. Since the Greater Mekong has been the main epicenter of the

emergence of *P. falciparum* resistance starting with CQ as well as SP, it is likely that artemisinin resistance will spread to other malaria-endemic continents (23).

1.3 Justification

Despite a recorded 60% decrease in mortality rates between 2000 and 2015, the development and spread of drug resistant malaria parasites has proven to be one of the greatest challenges to malaria control. This is partly owing to the use of low quality drugs or counterfeit drugs and poor patient-treatment compliance. Following the recommendation of ACT use in 2006 by WHO, the increasing deployment of ACTs is one of the major factors behind the reduced incidences of malaria cases. Unfortunately, continued affirmation shows there is an indigenous emergence and spread of artemisinin resistance within SE-Asia which has for years been considered the epicenter of drug resistance; sulphadoxine-pyrimethamine, mefloquine chloroquine, proguanil and piperaquine has emerged there and has spread globally. Even though a number of African studies have reported promising efficacies of artemisinin, it is important that the efficacy of the ACTs is monitored as part of the malaria control programs. WHO (2015) recommends continued monitoring of resistant markers after every 2 years for early detection of artemisinin resistance, to ensure the first-line treatments are still effective and for prompt changes in national treatment policies. When selecting the best possible ACT for a specific region, antimalarial resistance markers along with the results of clinical trials and *in vitro* studies provide useful information. The use of molecular markers such as *Pfk13*, *Plasmodium falciparum* multidrug resistance gene 1 (*Pfmdr1*) and *Pfcrt* is essential for malaria surveillance programs.

1.4 RESEARCH QUESTION

Is there a difference in the frequency of *Pfk13*, *Pfmdr1* and *Pfcrt* mutations before and after treatment with AL and DP?

1.5 HYPOTHESIS

There are no differences in the frequencies of *Pfk13*, *Pfmdr1* and *Pfcrt* mutations before and after treatment.

1.6 Objectives

1.6.1 Main Objective

To determine the difference in drug resistance allele frequencies in *Pfcrt, k13 and Pfmdr1* genes in parasites collected before and after ACT treatment.

1.6.2 Specific Objectives

1. To distinguish recrudescence from new infections using highly polymorphic *P. falciparum* merozoite surface protein 2 (*Pfmsp2)* and glutamate rich protein (*glurp*) genes before and after treatment.
2. To determine the frequency of mutations in *Pfcrt*, *Pfmdr1* and *k13*, drug resistance markers, in samples obtained before treatment.
3. To determine if there are any changes in allele frequencies of the known drug resistance markers before (day 0) and after (days 3, 7, 14, 21, 28 and 42) ACT treatment.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria

Malaria is transmitted by the female *Anopheles* mosquito that is harbors the intracellular protozoan parasite of the genus *Plasmodium*. Of the many *Plasmodium* species there are five known to cause malaria in humans: *P. falciparum, P. vivax, P. malariae, P. knowlesi* and two ovale subspecies, i.e. *P. ovale curtisi* and *P. ovale wallikeri* (24). The first two types are the most common species causing malaria with *P. falciparum* being the most frequent in sub-Saharan Africa and the most virulent of the four. Infection with *P. falciparum* can lead to death, whereas the other species cause illness but are rarely life-threatening (25,26). The *P. falciparum* genome is composed of 22.8 megabases (Mb) spread across 14 chromosomes, a nucleotide content of 80% A+T that rises to 90% in introns and approximately 5300 proteins (27). Comparative genome analysis shows that there is synteny between the different *Plasmodium* species with the exception of genes located in the telomeric regions that are known to be involved in antigenic variation and immune evasion.

2.2 The Epidemiology of Malaria

It is a challenge to workout the burden of malaria especially in low and middle-income countries due to the inconsistencies in data collection and also because not all infections progress to disease manifestation. In 2002, 515 million cases of clinical *P. falciparum* malaria globally with the majority of these cases concentrated in sub-Saharan Africa (70%), were reported (25). A recent World Malaria Report (28) estimates that there were 212 million malaria cases and 42,900 deaths in 2015. Infection rates were higher in children under five years, pregnant women, HIV/AIDS patients and migrants or mobile populations who have limited access to prevention tools and diagnostic tests.

2.3 Life Cycle of *Plasmodium falciparum*

2.3.1 Exo-erythrocytic Cycle

The life cycle of *P. falciparum* involves two cycles: the asexual and sexual cycles that occur in the human and *Anopheles* mosquito, respectively as shown in Figure 2-1. Malaria begins when a *P. falciparum*-infected female anopheles mosquito bites the human host, during this step the

mosquito intends to acquire a blood-meal and in the process injects viable sporozoites into the human host (29,30). The highly motile sporozoites travel from the bite site to the liver via the bloodstream and start their intracellular development in the hepatocyte in a process known as exoerythrocytic schizogony. Sporozoites in the hepatocyte begin dividing into schizonts. Each schizont raptures to release >1000 merozoites, which are then delivered into the bloodstream. This is a clinically silent stage that leads to a 10,000-fold rise in the parasitemia and may take approximately 2-10 days, depending on the parasite species (31). Both *P. ovale* and *P. vivax* remain dormant in the liver and are reactive days to weeks after the primary infection (32,33).

2.3.2 Erythrocytic Cycle

Mature merozoites from hepatocytes invade the red blood cells marking the start of clinical disease. Over a period of 44-72 hours, the merozoites undergo development from ring stages to mature trophozoites and finally schizonts. Each mature schizont in the erythrocyte burst to release 10-20 merozoites that circulate the blood stream. These circulating merozoites re-invade other uninfected red blood cells (uRBC) in the end leading to an exponential increase in parasite densities as high as 10^{10} parasites per host.

Asexual erythrocytic parasites are primarily responsible for febrile illness associated with fever and chills but may develop into severe disease affecting organs. After at least two asexual cycles, 1% of the merozoites develop into macrogametes (female) and microgametes (male) sexual forms in a process known as gametocytogenesis. These sexual forms of the parasite are known to be infective to mosquitoes and develop as a result of host environmental stress or antimalarial drug usage.

2.3.3 Mosquito Stage

The sexual cycle starts after a female *Anopheles* mosquito feeds on an infected human host. Following a blood meal, the mosquito ingests micro- and macrogametes that are taken to the mosquito's midgut. The male gamete fertilizes the female gamete forming a diploid zygote that undergoes genetic recombination, which is an important factor in the generation of multiple drug resistant phenotypes (34). The zygote further matures into an ookinete that penetrates the midgut cell to become an oocyte. The oocyte undergoes several mitotic divisions to form sporoblasts after which budding takes place 10-14 to the produce sporozoites in a process called sporogony. The midgut sporozoites are seen to be present in the mosquito's

salivary gland making them available for inoculation into the human host during a blood meal, initiating another life cycle.

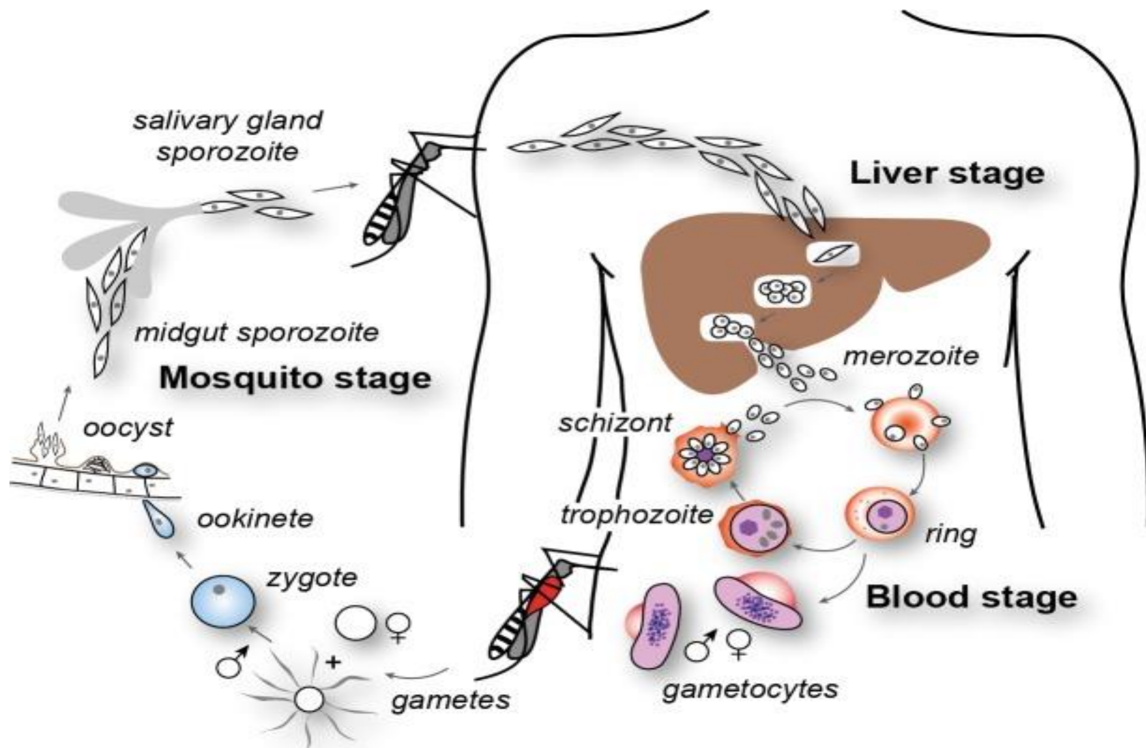

Figure 2-1: The life cycle of the *P. falciparum* parasite occurring in both the human host and Anopheline mosquito. Adapted from Cowman et al., 2012 (35)**.**

2.4 Malaria Disease

P. falciparum malaria can either cause asymptomatic infection, uncomplicated malaria or severe malaria. In malaria-endemic areas, repeated exposure to *P. falciparum* infection early in life results in non-sterile development of clinical immunity later in life. In such environments it is therefore common to find adults with circulating parasites but without any evident symptoms. These kinds of infections are known as asymptomatic infections. This is in contrast to malaria infections in low-endemic areas, where the population has unchallenged immunity and *P. falciparum* infection leads to symptomatic disease in all age groups. Young children are however prone to symptomatic infections mainly due to their underdeveloped humoral response to pathogens. Approximately 99% of infected individuals have uncomplicated malaria with non-specific symptoms such as malaise, joint pains and headaches at 48 hour intervals which can be easily confused with other diseases (36). Due to reasons such as

delayed or inappropriate treatment and low immunity, approximately 0.2-0.5% of *P. falciparum* infections develop into a more severe form of disease (37). Common features of severe malaria involve major systems like the brain (cerebral malaria), pulmonary system (respiratory failure) and hematopoietic system (severe anaemia) or even lead to a coma (36).

2.5 Interventions for Control and Elimination of Malaria

2.5.1 Prevention of malaria

Current malaria control strategies depend on the adoption of insecticide-treated nets (ITNs), indoor residual spraying (IRS) and ACTs. These strategies have resulted in reduced mortality rates by 60% in Africa between the years 2000 and 2015 (38), not excluding the positive impact of improved quality of health systems and improved case management due to enhanced diagnostics.

The most effective malaria prevention strategies recommended for sub-Saharan Africa are ITNs and IRS. ITNs are known to repel and kill mosquitoes because they have embedded insecticides. There is evidence that a wide scale use of ITNs provides some extended level of protection to non-ITN users, as it helps reduce the overall malaria transmission (39). IRS involves spraying insecticides on the ceilings, walls and other indoor resting places of mosquitoes. In most cases, sleeping and living rooms of a household are targeted for spraying. Countries adopting the use of ITNs and IRS have reported significant declines in malaria incidences (40,41).

2.5.2 Chemotherapy for Treatment

For many years, chloroquine (CQ) was used as the major antimalarial for the treating uncomplicated malaria. The appearance of CQ resistance (CQR) in Southeast Asia and its spread to Africa and Latin America led to the introduction of sulphadoxine-pyrimethamine (SP) to which parasites developed an even more rapid resistance (42). To reduce the pace of selection of resistance, WHO recommends artemisinin (ART) -based combination therapies (ACTs) for treatment of uncomplicated malaria. ACTs are a co-formulation consisting of a potent artemisinin component that rapidly clears the majority of the asexual parasites paired with a partner drug that eliminates the residual parasites (11). WHO recommends the following ACTs: artemether-lumefantrine (AL), artesunate-amodiaquine, artesunate-mefloquine, dihydroartemisinin-piperaquine (DP), artesunate-pyronaridine and artesunate-

sulphadoxine-pyrimethamine. The choice of ACT to be used is based on the efficacy of the drug against the region's circulating strains of *P. falciparum*. In Kenya, AL is the first line antimalarial for treating uncomplicated malaria.

2.6 Artemisinin Based Combination Therapies

Artemisinin, *qinghaosu*, is derived from the Chinese herb *Artemisia annua*. The antimalarial activity of *A. annua*, with its active ingredient being the sesquiterpene lactone (43), was discovered through screening of an array of traditional medicines that cured monkeys and mice that had simian and rodent malaria, respectively (44,45). Artemisinin has a poor bioavailability that limits its efficacy. Semisynthetic derivatives of artemisinin (Figure 2-2): artemether, artesunate and dihydroartemisinin have been developed with a modified chemical structure and improved pharmacological properties. In humans, these derivatives rapidly achieve optimum plasma levels and have been shown to have elimination half-lives of approximately 1-3 hours. The 3-day ACT treatment course reduces the number of asexual parasites by approximately 10^7-fold with an added bonus of having gametocytocidal activity which is important in reducing transmissibility of the parasite (46).

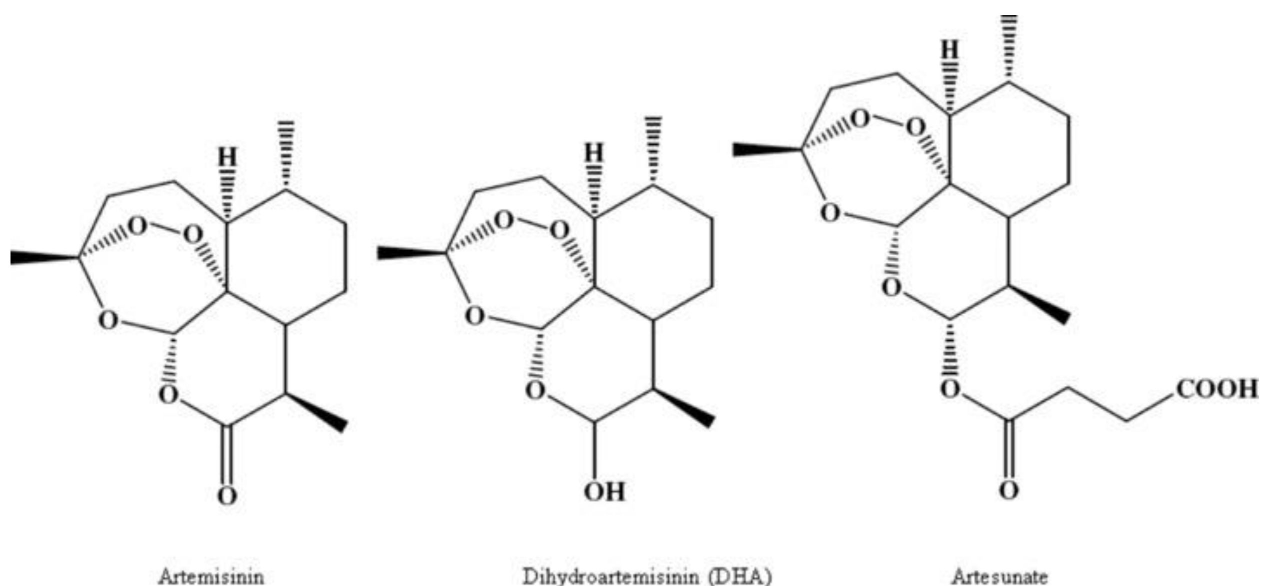

Figure 2-2: The chemical structure of the three artemisinin derivatives. Adapted from Waknine-Grinberg et al., 2010 (47).

2.6.1 Mechanism of Action of ACTs

Artemisinins target immature and mature trophozoite stages leading to a 10,000 fold reductions in parasitemia by 48 hours (21). Artemisinin is activated by Fe^{2+} heme that is produced in the process of hemoglobin digestion in the parasite food vacuole, forming heme-artemisinin compounds *in vivo* (48,49). Activated artemisinin releases carbon-centered free radicals that alkylate parasite biomolecules leading to cell death, as shown in Figure 2-3. Dead parasites are cleared from the patient's blood stream in a process known as pitting (50,51). Pitting occurs in the spleen, with the removal of the intraerythrocytic parasites from the RBC as it traverses the endothelial wall of the spleen (52). However, a population of trophozoites dormant and have been seen to resume growth weeks to days after (53).

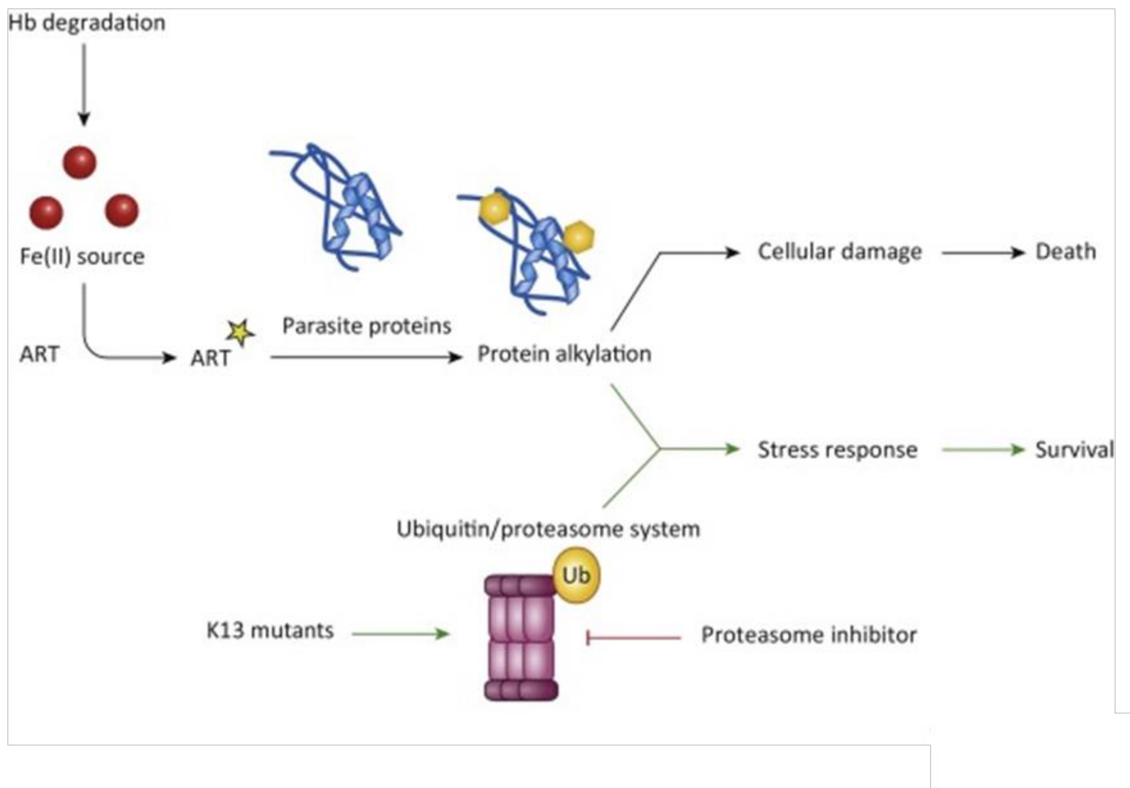

Figure 2-3: Diagram showing events that can either lead to parasite death or survival following ART treatment. Adapted from, Tilley et al., 2016 (54).

2.7 Dynamics of ACT resistance

Drug resistance limits the efficacy of many antimalarials, posing a threat to malaria control programs. Parasite resistance is the potential of the parasite to resume its normal metabolic

process despite the presence of the antimalarial within limits of host tolerability. The key driver of antimalarial resistance is the genetic diversity of the malaria parasite. Antimalarial resistance is a two-step process involving an initial genetic event that selects a favorable mutant clone followed by the frequency of the arising mutant. Such resistant-mutant parasites would be further selected upon administration of antimalarials. In some cases, a change in the first line treatment has reported a resurgence in the sensitive phenotypes, implying that the mutant parasites are less fit and get outcompeted by the wild-type parasites (55,56). For this reason, WHO recommends continued monitoring of the efficacy of ACTs by *in vivo* tests, *in vitro* tests and molecular genotyping of known antimalarial resistant markers for early detection of resistance(57).

This study attempts to genotype a number of antimalarial resistant markers from field isolates obtained during an *in vivo* study. In order to determine the efficacy *In vivo* studies are used to assess the clinical efficacy of antimalarial drugs. According to the standard World Health Organization protocol, patients with uncomplicated malaria are recruited into the study where enrollment occurs on before treatment (day 0), after treatment (days 1, 2, 3, 7, 14, 21, 28) and whenever the patient presents with an illness. Other studies have suggested a 42-day follow-up to adequately capture the treatment failures in cases where the antimalarial drug has a long plasma half-life (58).

2.8 The role of molecular markers

Identification of suitable molecular markers of resistance has greatly enhanced prompt discovery of antimalarial resistance. Such markers can inform researchers on the origins as well as the spread of antimalarial drug resistance, providing a better understanding of the population dynamics of drug resistant genotypes. Such drug resistant genotypes can be linked to the slow clearance phenotypes owing to the complete genome sequencing and annotation of the *P. falciparum* genome. The presence of polymorphisms, which have been selected due to drug pressure, have been exploited as markers of drug resistance. Such polymorphisms include: microsatellites, single nucleotide polymorphisms (SNP) and insertions or deletions (indels). In this study, we mainly focus on determining the pre- and post-treatment frequencies of four resistance-markers: *k13* propeller-domain, *Pfcrt* and *Pfmdr1*.

2.8.1 Kelch 13 (k13)

The *k13* gene is found on chromosome 13 with one exon encoding the K13 protein with 726 amino acids and a molecular weight of 83.66kDa. On the C-terminus end of the K13 protein there are six motifs, Figure 2-4, where each motif consists of 50 amino acids that are seen to be organized into a secondary structured beta-sheets. The six Kelch motifs are seen to fold into a propeller domain that harbors multiple protein-protein interaction sites (59). In SE-Asia non-synonymous mutations in *k13*-propeller domain have been shown to be the genetic correlates of artemisinin resistance using both *in vitro* and *in vivo* studies (60).

The first cases of artemisinin resistance were displayed as prolonged parasite clearance times of >90 hours, which is higher than the recorded 52 hours in patients who were cured (13). A significant breakthrough in understanding the genetic architecture of artemisinin resistant parasites came as a result of understanding whole genome sequence data of African ART-resistant and clinical isolates from Cambodia. A study done in SE-Asia was able to identify specific non-synonymous K13- propeller domain mutations in *vitro* and *in vitro* parasites displaying slow clearance (60). Non-synonymous mutations at codons Y493H, R539H, I543T and C580Y were observed in the K13 propeller domain and were associated with higher ring-stage parasite survival ($RSA_{0\text{-}3h}$ survival assay) rates as compared to the wild type. Additional gene editing studies using CRISPR-Cas9 were then carried out to validate the role of the C580Y mutation. The C580Y mutation was associated with increased ring stage parasite survival of ~13.5% (61), almost similar to the rate previously reported for the Cambodian resistant parasite isolate (60).

Several epidemiological studies conducted in Southeast Asia have identified multiple occurrences of mutations in the K13 propeller domain that result in drug resistance (14,62–65). Non-synonymous K13 mutations have also been identified in Africa but at very low frequencies, known as singletons (62), that have no impact on ART efficacy. However, a recent study conducted in China reported a *P. falciparum* K13-variant infection from a migrant worker from China coming to Equatorial Guinea with delayed parasite clearance phenotype following several rounds of ACT treatment (66).

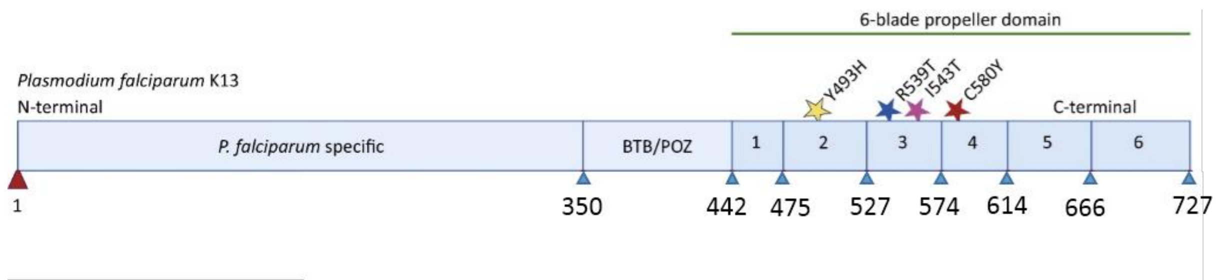

Figure 2-3: The structure of the Kelch protein showing the six propeller domains and the four non-synonymous mutations associated with higher ring-stage parasite survival rates. Adapted from Ariey et al., 2013 (60)**.**

2.8.2 *P. falciparum* multidrug resistance protein 1 (*Pfmdr1*)

The *Pfmdr1* gene is sits on chromosome 5 encoding the P-glycoprotein homolog 1 (Pgh-1) protein which has 1419 amino acids with a mass of 62.25kDa, as shown in Figure 2-5. The Pgh-1 protein is a membrane-bound ATP-binding cassette (ABC) transporter found in the digestive vacuole with two domains each consisting of 6 helical transmembrane domains. Pgh-1 has been suggested to regulate intracellular drug concentrations (67). Studies using fluorescein derivatives (Fluo-4) provide supporting evidence that PfMDR1 plays a role in movement of solutes as well as antimalarial drugs into and out of the parasite's food vacuole (68). In humans, a similar P-glycoprotein homolog polymorphisms has been linked to resistance to cancer drugs (69). Polymorphisms and copy number variations of *Pfmdr1* gene are a major determinant of parasite resistance or susceptibility to a number of antimalarials (70). The main mutations in PfMDR1 can be grouped into two: amino-terminal mutations that include N86Y (asparagine changing to a tyrosine), Y184F (tyrosine to a phenylalanine) and a carboxyl-terminal mutation D1246Y (aspartic acid to a tyrosine).

Resistance to amodiaquine (71), mefloquine (72), lumefantrine (73) and artemisinin (72) has been reported on a number of studies to be influenced by PfMDR1 polymorphisms in both *in vivo* and *in vitro* studies.

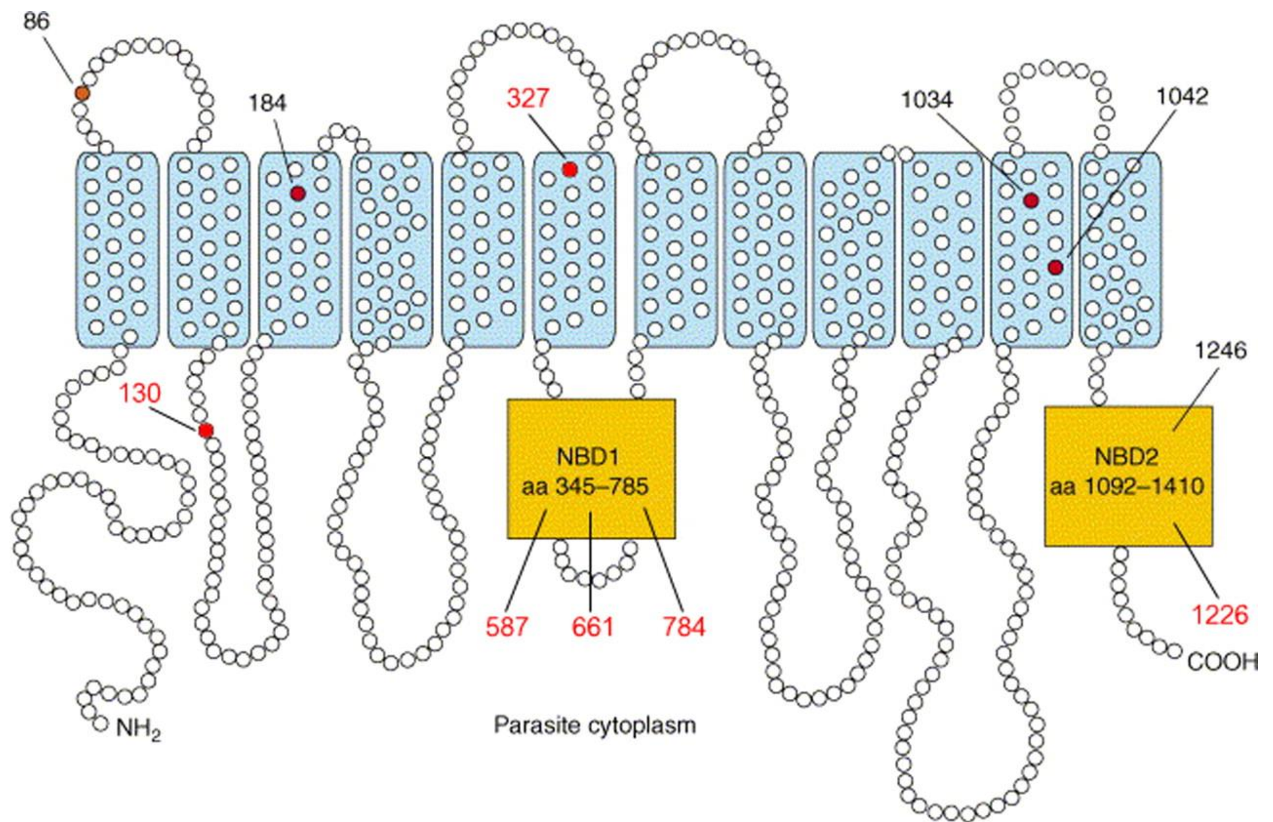

Figure 2-4: The Structure of PfMDR1 showing the 12 transmembrane domains (shaded blue) and the known point mutations indicated as shaded circles. Adapted from Valderramos et al., 2006 (69).

2.8.3 *P. falciparum* chloroquine resistant transporter (*pfcrt*)

The *Pfcrt* gene is localized on the 7th chromosome and encodes the PfCRT protein having 424 amino acids with a mass of 48.6kDa (74). The PfCRT is a transmembrane protein found in the food vacuole having 10 domains (see Figure 2-6) (75). Mutations in the *Pfcrt* gene are a key determinant of CQ resistance (CQR) both *in-vivo* and *in-vitro*(74,76). *In* vitro studies that involve genetic recombination of the CQ-sensitive HB3 isolate and the CQ-resistant Dd2 isolate have clearly provided conclusive evidence that *pfcrt* is the primary determinant of CQR (77). Further studies to have been able to show less CQ accumulation inside the parasite vacuole of mutant *pfcrt*-genotypes as compared to the wild-types (78,79). CQR is linked to 15 different polymorphisms in the *pfcrt* gene. Mutations on codons 72-76 have been used to distinguish the two different geographical haplotypes: CVIET (South-East Asia and Africa)

and SVMNT (South America and Southeast Asia) (80). The causal mutation in *pfcrt* a change from Lysine (K) to Threonine (T) at codon 76 is used as the molecular marker for CQR(80).

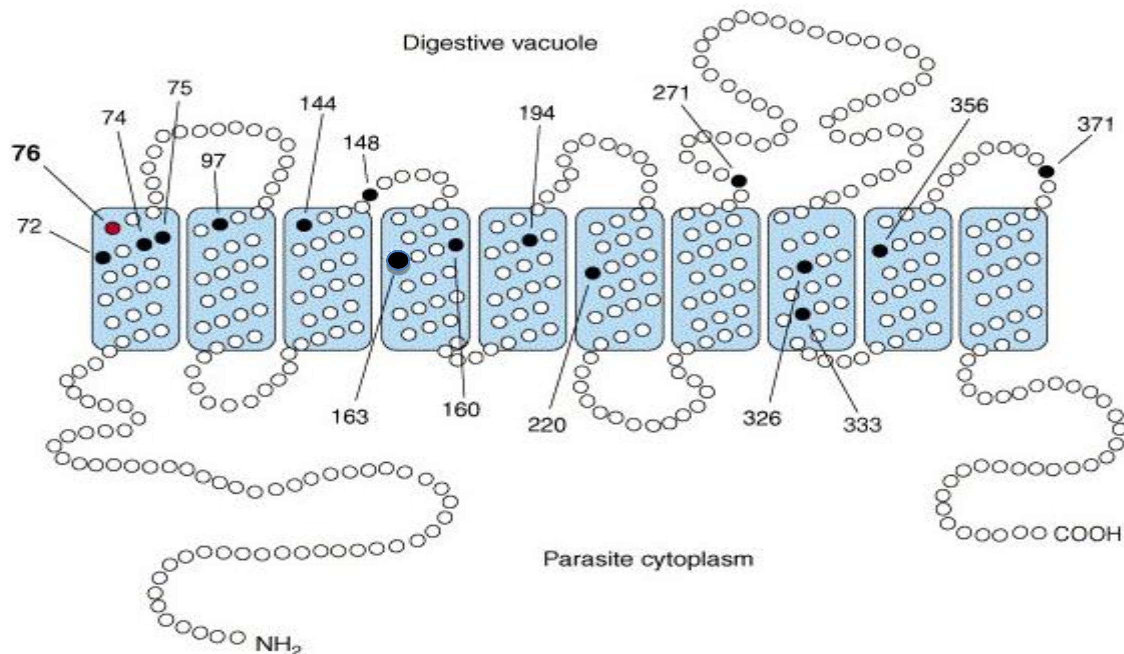

Figure 2-5: The structure of PfCRT showing the 10 transmembrane domains (shaded blue) and the known point mutations indicated as shaded circles. Adapted from Valderramos et al., 2006 (69)**.**

2.9 The role of other molecular markers

A number of *P. falciparum* genes are known to be highly polymorphic. The occurrence of such genes has been exploited in assessing parasite populations in the human host. The probability of a patient, especially in a malaria endemic area, being infected with the same *P.* falciparum genotype to the former is low (81). The genetic diversity of *P. falciparum* is usually assessed by genotyping polymorphic antigenic markers such as RII repeat of the glutamate rich protein (GLURP) and the merozoite surface proteins (*msp1* and *msp2*) (82). Therefore, by comparing the genotypes of such loci at baseline, before treatment, and the time of parasite recurrence, should provide a distinction between recrudescent and new infections (81). A number of drug trials have used this strategy to correct the outcomes of drug efficacy trials (83–85). However, it is important to note that the discriminatory power of these loci is dependent on the allelic diversity, the frequency of each allele within the population and the

genotyping protocol used (86,87).

2.9.1 Merozoite surface protein 2 gene (*msp2*)

The merozoite surface protein-2 is a 45kDa glycoprotein anchored in the merozoite surface by a glycophosphatidylinositol (GPI) anchor and due to its ability to elicit immune response it is considered to be an abundant antigen at the surface of the merozoite (88). Sequence variation has been detected in the *msp2* gene for both laboratory maintained (89) and field *P. falciparum* isolates (90–92). The comparison of the *msp2* sequences from the two isolate classes reveals highly conserved 5' and 3' sequences that flank a central variable region, block 3 that includes both repetitive and non-repetitive sequences.

The non-repetitive allelic families are classified into two forms that define two allelic families, FC27 and 3D7/IC (89). Each allelic family has its unique pattern of repetitive sequences. The FC27 allele-family shares a 12-mer and 32-mer repeat region, whereas the 3D7 allele-family share a 4-mer repeat sequence. Both allelic families have an N- and C-terminal conserved region with a GPI-anchor for attachment to the merozoite surface at the C-terminal end (see Figure 2-7).

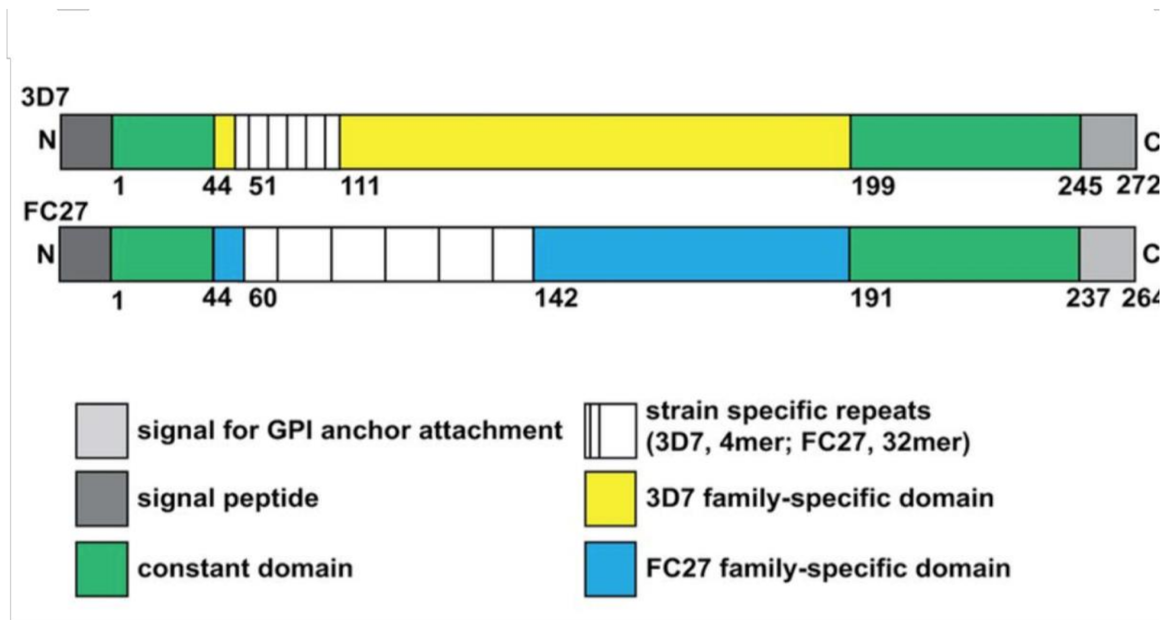

Figure 2-6: **The structure of the FC27 and 3D7 allelic families found on block 3 of the msp2 gene.** Adapted from Boyle et al., 2014 (93)**.**

2.9.2 Glutamate-rich protein gene (*glurp)*

The glutamate-rich protein (*glurp*) encodes a 220kDa protein that is expressed at the pre-erythrocytic and erythrocytic stages of the parasite life cycle (94). GLURP contains an N-terminal non-repeat region (R0), a central repeat region (R1) and the C-terminal immunodominant region (R2), as shown in Figure 2-8. The R0 region has been shown to be highly conserved and elicits stable antibody responses over time (95,96), while the R2 region is genetically heterogenous (97).

The *glurp* gene is known to be less genetically diverse, as reported by studies in different geographic regions, but highly immunogenic. Polymorphisms in the *glurp* gene are characterized as sequence repeats, which in turn affects the gene size and product. Given that a unique variant of the gene-size is found in a human with one parasite, the presence of more than one allele represents a multiclonal infection.

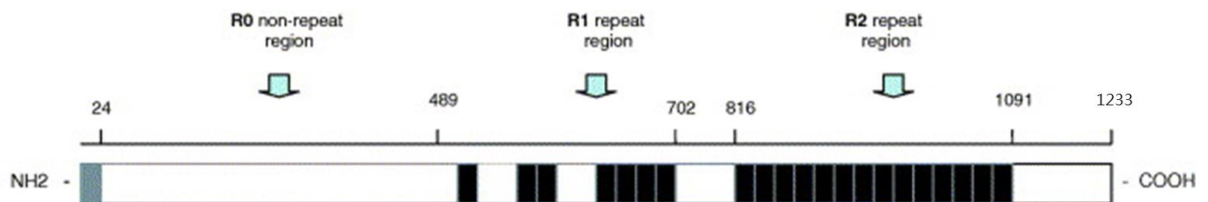

Figure 2-7: **The protein structure of the glutamate rich protein.** Adapted from Maya et al., 2006 (98)**.**

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Site and Study Design

The studies presented in this thesis were conducted at the Kenya Medical Research Institute (KEMRI)/Wellcome Trust Research Programme in collaboration with KEMRI. *P. falciparum* samples were collected following a drug trial conducted in Msambweni constituency in Kwale County, Kenya, as shown in Figure 3-1. The county has a total area of 362.6 km^2 with a population of 124,295 according to the Kenya 2009 census. Recent studies have reported reduced transmission intensities along the coastal parts of Kenya (99) with a *P. falciparum* prevalence of 9%-24% recorded in 2010, as compared to 64% in 1998 (100).

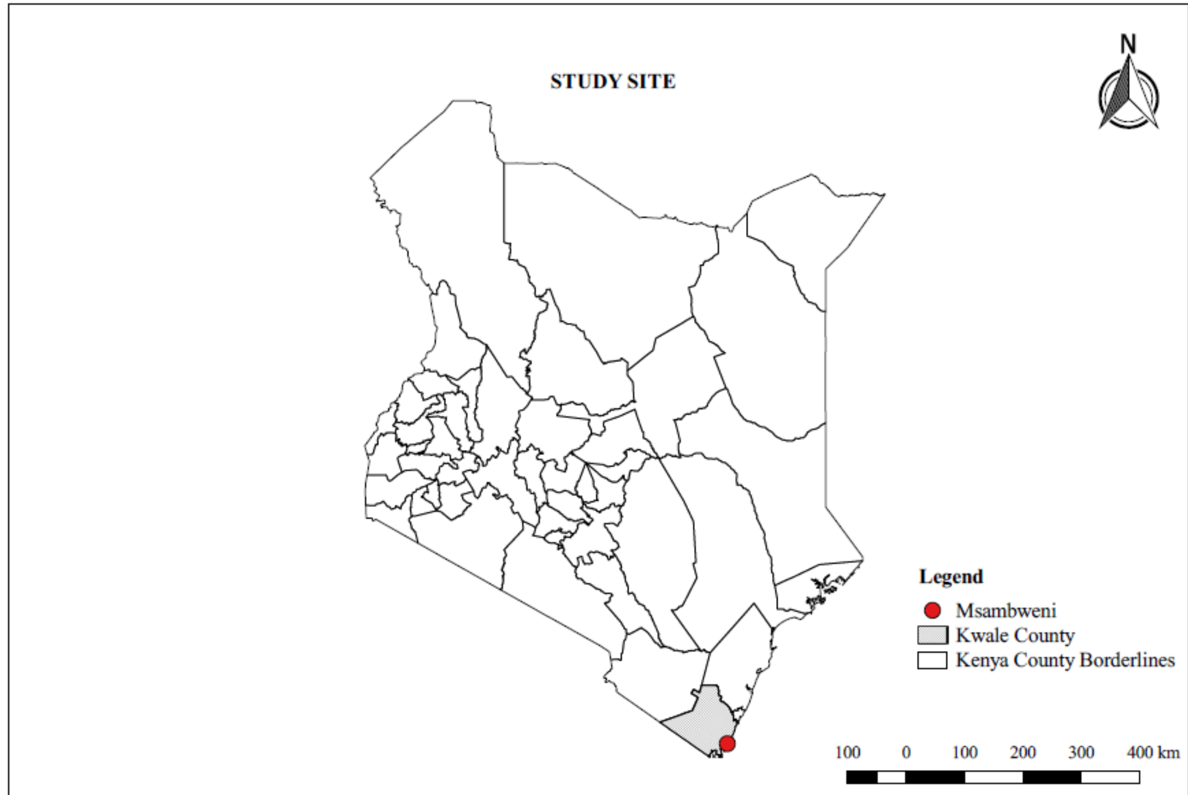

Figure 3-1: Map of Kenya showing Kwale County and Msambweni constituency.

The study was conducted in a cross-sectional, randomized, two arm trial set up designed to analyze the antimalarial markers of resistance to ACTs using molecular biology before and after treatment. Children between 6 months and 5 years were recruited at day 0 of the Kwale study (SSC 2276). Participants reported to the study clinic on days 1, 2, 3, 7, 14, 28 and 42. This study answers the key end-points of the larger multicenter trial that was proposed to assess the efficacy of dihydroartemisinin-piperaquine with artemether-lumefantrine as the comparative drug.

3.2 Randomization and blinding

Using MS-Excel, a randomization list was generated for all participants. Sealed envelopes that were sequentially numbered to contain the treatment arms were prepared. To each participant a study number was assigned and the study nurse or pharmacist administered the treatment by unsealing the envelope corresponding to the treatment number. Only the study nurse and patients were not blinded however the study clinician was blinded to the treatment assignments.

3.3 Study Population

363 children with confirmed *P. falciparum* malaria infection were recruited from the outpatient clinic of Msambweni county referral hospital and were randomized to receive dihydroartemisinin-piperaquine or artemether-lumefantrine.

3.4 Selection Criteria

Inclusion criteria for participants:

Children between 6 months to 5 years of age, absence of severe malnutrition, slide confirmed mono-infection of *P. falciparum*, asexual parasitemia between 2,000-200,000parasites/µl during the high transmission rainy season, a history of fever within past 24 hours, an axillary temperature ≥ 37.5°C, the ability to attend follow-up visits and an informed consent provided by parent or guardian.

Exclusion criteria for participants:

Patients with the following characteristics were excluded from the study: recent history of allergy or patients with danger signs (inability to drink, vomiting in past 24 hours, a recent history of multiple convulsions, unconsciousness, and inability to stand or sit), hypoglycemia,

co-morbidity infections, hyperanaemia, epilepsy, abnormal cardiac rhythm, jaundice and respiratory distress

3.5 Ethical Considerations and Sample Size

The study was approved by KEMRI Scientific and Ethics Review Unit (SERU), as shown in Appendix 1, and utilized all 363 microscopy-positive dried blood spot (DBS) samples collected pre and post treatment between April 2013 and June 2013. Some samples were however lost during follow-up. Table 3-1 shows the total number of samples, microscopy positive samples and their range parasitemia in each day.

Table 3- 1: The number of samples taken from each day of the study, the proportion that was microscopy positive and the range of parasitemia.

DAY	Number of Samples	Number of Microscopy positive (%)	Range parasitemia (Parasites/μl)
0	332	332(100)	1080-195200
1	319	189(59)	40-58400
2	323	23(7)	40-8800
3	321	1(0.3)	160
7	314	1(0.3)	7
14	319	1(0.3)	1560
21	323	8(2.5)	5320-126400
28	315	13(4)	2120-188800
42	286	26(9)	280-76040

3.6 DNA Extraction

3.6.1 Dried Blood Spots

Parasite DNA was extracted from the DBS samples by the Chelex method (33). A clean Harris Uni-core punch was immersed in 5M HCl then soaked in 5M NaOH and briefly rinsed with distilled water. A filter paper was removed from the zip lock bag with forceps and 2mm section of the blood spot was cut directly into a 1.5ml eppendorf tube. After each cut the Harris Uni-core punch was decontaminated with both HCL and NaOH to get rid of any DNA

contamination.

1ml of 0.5% saponin in 1X Phosphate-buffered saline (PBS) was added to each tube and inverted several times to ensure the filter paper is suspended in the solution. The samples were then incubated at 4°C overnight. Using a pipette the brown supernatant was drawn out, discarded and replaced with 1ml of PBS. The tubes were inverted again and incubated at 4°C for 30 minutes.

After discarding the brown solution again, 50µl of 20% Chelex and 150µl DNase/RNase free water was added to the 1.5ml tubes. Each tube was vortexed vigorously for 30 seconds and placed in the heating block at 100°C for 2 minutes. The samples were then centrifuged at 10,000 x g for 2 minutes after which the supernatant was carefully transferred to a fresh 1.5ml eppendorf tube taking care not to transfer the Chelex matrix. The freshly transferred supernatant was again centrifuged for a further 2 minutes at 10,000x g and the supernatant carefully transferred to another 0.5ml microcentrifuge tube. The DNA was then stored at -20°C.

3.6.2 Controls

DNA from laboratory-adapted strains was extracted using QIAamp DNA minikit (Qiagen Inc., Valencia, CA and USA) as per manufacturer's instructions. Prior to this, infected red blood cells (iRBCs) were lysed using saponin. Briefly, iRBCs were washed twice with 1X PBS and the pellet resuspended in PBS containing 0.1% (w/v) saponin (Sigma). The suspension was incubated for 3 minutes at room temperature and centrifuged for 10 minutes at 1440 x g.

Further, protein digestion and lysis of the pellet was performed by incubation in 40µl of the proteinase K and 400µl lysis buffer for 10min at 56°C. 100% ethanol that was responsible for precipitating the nucleic acids was added to each sample and vortexed for 15 seconds. 600µl of the solution was applied to the spin columns and spun at 9000 x g for 1 minute to allow the nucleic acids to be adsorbed onto the QIAamp silica-membrane. The DNA was washed twice, first with 600µl of AW1 and spun at 8000 x g for 1 minute, then with 600µl of AW2 at 13000 x g for 3 minutes. This was done to improve the purity of the extracted DNA by removing any residual contaminants. The extracted DNA was eluted in 100µl elution buffer by spinning at 8000 x g for 1 minute into a 1.5ml eppendorf tube and stored at -20ºC.

3.7 Genetic Diversity of the *P. falciparum* isolates

The number of unique clones per isolate was determined by genotyping the *P. falciparum* merozoite surface protein 2 (*msp2*) and *glurp* by PCR. All reactions were carried out in a final volume of 10µl containing 10mM dNTPs, 10µM primers, 25mM $MgCl_2$ and 0.03U/10µl reaction (AmpliTaq Gold). To check for DNA contamination in any of the amplifications DNase/RNase free water was included as a negative control for each reaction.

Paired samples were genotyped in a stepwise manner first using *msp2* then confirming with *glurp*. A recrudescent infection was defined as the presence of at least one matched allele at either locus.

3.7.1 *msp2* locus

Genotyping of the *msp2* gene was performed in a nested PCR reaction (34) with minor modifications. The primary PCR amplification spanned the entire block 3 region of *msp2* followed by a secondary PCR with separate primer pairs targeting the *msp2* allelic families IC and FC27. Allele specific positive controls for IC parasites (7G8) and FC27 parasites (Dd2) were included in each set of reactions as positive controls. For the primary amplification the following conditions were followed: 5 minutes at 95°C, followed by 30 cycles for 1 minute at 94°C, 2 minutes at 58°C and 2 minutes at 72°C and final extension of 10 minutes at 72°C. For the nested reaction: 95°C for 5 minutes, 30 cycles for 1 minute at 94°C, 61°C for 2 minutes and 72°C for 2 minutes and a final extension of 5 minutes at 72°C. In the primary reaction 1µl of genomic DNA was added as the template. In the nested reaction, 1µl of outer PCR product was used as the template.

3.7.2 *glurp* locus

Genotyping of the glurp gene was done in a single primary reaction. The cycling conditions were set up as follows: 95°C for 5 minutes, followed by 39 cycles of denaturation at 94°C for 1 minute, primer annealing at 55°C for 2 minutes and an extension step at 72°C for 2 minutes. This is followed by final cycle of 55°C for 2 minutes, 72°C for 5 minutes and a final hold step of 20°C for 2 minutes. Purified genomic DNA from 3D7 laboratory strain was amplified and electrophoresed as a positive control in all reactions.

3.8 Genotyping for drug resistance markers

Pfmdr1 (codon 86:720bp, codon 1246: 912bp), *pfmdr1* (codon 72-76: 193bp) and *k13* (886bp) were amplified from genomic DNA by using Expand High Fidelity PCR system (Roche). This system is composed of a unique enzyme with proofreading ability and exonuclease activity. Primers used for PCR were adopted from previously published studies (Table 3-2) and were subjected to in silico PCR using CLC Main Workbench version 9.5 (See Appendix 3) prior to the actual amplification in order to confirm whether the primers would anneal to the template.

All reactions were performed to a final volume of 10µl as shown in table 2-3 and each reaction mixture contained 200µM deoxynucleoside triphosphate, 300nM of each primer, 1X PCR buffer (containing 1.5mM $MgCl_2$, 2.5mM of $MgCl_2$ (buffer 2), 0.035U of Taq and 0.7µl of the template DNA.

Table 3- 2: Amplicon and sequencing primers for pfmdr1, pfcrt and k13 genes

Reference	Gene	Orientation	Sequence	Base-pairs	Annealing temp. (°C)
Okombo et al. 2014	*Pfmdr1_ codon 86*	pfmdr1_F	ATGGGTAAAGAGCAGAAAG	720	54
		pfmdr1_R	TCCCATTAAAGCCTCTTCTA		
	Pfmdr1 codon 1246	pfmdr1_F	ATTGATGTAAGAGATGATGGT	912	53
		pfmdr1_R	TATTCCATCTTGTGCTGATAA		
	Pfcrt	pfcrt_F	GGTGGAGGTTCTTGTCTTGG	194	63
		pfcrt_R	ATAAAGTTGTGAGTTTCGGATG		
Wamae et al., not published	*k13*	k13_F3	GAAGCCTTGTTGAAAGAAGC	886	56
		k13_R3	CGGAGTGACCAAATCTGG		
Snounou et al., 1995	*glurp*	glurp_F	TGAATTCGAAGATGTTCACACTGAAC	600-1100	56
		glurp_R	TGTAGGTACCACGGGTTCTTGTGG		
Zwetyenga et al., 1998	*msp2*	primary_f	ATGAAGGTAATTAAAACATTGTCTATTATA	811	58
		primary_r	ATATGGCAAAAGATAAAACAAGTGTTGCTG		
		FC27_f	GCAAATGAAGGTTCTAATACTAATAG	260-500	61
		FC27_r	GCTTTGGGTCCTTCTTCAGTTGATTC		
		3D7/IC_f	GCAGAAAGTAAGCCTTCTACTGGTGCT	400-610	
		3D7/IC_r	GATTTGTTTCGGCATTATTATGA		

The cycling conditions of the four gene fragments were as follows: 94°C for 2 minutes, 94°C for 15 seconds, 30 cycles at annealing temperatures (X), as shown in Table 3-2, for 30 seconds, 68°C for 2 minutes and a final extension of 7 minutes at 72°C. All annealing temperatures are listed in table 3-2.

3.8.1 Gel Electrophoresis

The 1% w/v Agarose gel was prepared by mixing 1g of Agarose with 100ml of 0.5X Tris base/Boric acid/ EDTA (TBE) buffer. The mixture was heated in a microwave until boiling, and allowed to cool. Red Safe dye (iNtRon Biotechnology) was added to enable visualization of DNA. The gel was then poured into an electrophoresis tank cast and allowed to solidify. 3µl of each PCR product was mixed 2µl loading dye and placed on the gel, and ran at 100Vs for 45 minutes. The gel was visualized under UV transilluminator (Bio-Rad machine). Any sample that had a single band was considered positive upon comparison with the controls and purified for sequencing.

3.8.2 Amplicon Purification

Following amplification, PCR product clean-up was done using Exonuclease 1- Shrimp Alkaline Phosphatase (ExoSAP-IT) (ExoSAP-IT For PCR Product, Affymetrix, Santa Clara, CA). Approximately 6µl of the PCR product was transferred to a 96 well plate to which 1µl of ExoSAP-IT was added. The plate was then loaded to the thermocycler and incubated according to the manufacturer's instructions (37°C for 15 minutes and 80°C for 15 minutes).

3.8.3 Sequencing of *pfk13*, *pfcrt* and *pfmdr1*

Two pairs of primers were used to analyze the 3 mutations at codons 86, 184 and 1246 of *pfmdr1*, 1 primer pair specific for codons 72, 73, 74, 75 and 76 for *pfcrt* and 1 primer pair specific to the propeller domain of *k13* (codon 440-720). Cycle sequencing was carried out using ABI BigDye Terminator v3.1 chemistry (Life Technologies, Carlsbad, CA, USA). The sequencing mix was prepared as follows:

For each reaction (template/primer combination), reagents and primer were mixed in 1.5ml tube

Table 3- 3: The cycle sequencing reaction volumes used

Reagent	Volume per reaction (µl)
Water	4.75
5X Sequencing buffer	1.75
Sequencing primer (5pmol/µl)	1
Big Dye Terminator	0.5
Template	1

Assuming an initial template concentration of 800ng/ µl the template was diluted to 30ng/ µl and used in the cycle sequencing reaction along with the pGEM®-3Zf(+) double-stranded DNA Control Template as per manufacturer's instructions.

The plate was then loaded to the thermal cycler machine. The cycling conditions were set as follows: an initial denaturation of 96°C for 30 seconds, 96°C for 10 seconds, 25 cycles at 50°C for 10 seconds and 60°C for 4 minutes.

3.8.4 Ethanol Precipitation

Precipitation was done to remove any unused primers after cycle sequencing. 1µl of EDTA and 1µl 3M Sodium acetate was added to each well containing DNA making sure these solutions reach the bottom of the well. 25µl ethanol (100%) was added to each well, the plate was then sealed and incubated at room temperature for 15 minutes to precipitate products. The plate was then spun for a minimum of 20 min at 4000 rpm at 20°C. After spinning the supernatant was drained off by gently inverting the plate on a fresh paper towel and spun again at 400rpm for 1 minute. 100µl of 70% ethanol was then added to each well and spun for 5 minutes at 4000 rpm at 20°C. Supernatant was drained off by gently inverting the plate on a fresh paper towel and spun again at 400rpm for 1 minute to get rid of excess ethanol. The plate was then air dried for 60 minutes to ensure ethanol completely dries so as not to interfere with capillary electrophoresis. 10µl of HiDi was added to each well before proceeding to denaturation at 95°C for 3mins. After denaturation the plates were chilled on ice and stored at 4°C before transportation to the sequencing facility. The sequences were read in ABI 3730xl machine at International Livestock Research Institute (ILRI).

3.9 Data Analysis

Data collected in this study was analyzed using R-programming software version 3.4.1 and CLC Main Workbench 9.5. Results from msp2 and glurp genotyping were analyzed and figures generated using R-programming software. The trace files generated by the DNA analyzer were imported into R software to determine the total number of samples with and without chromatograms. Samples with chromatograms were then imported into CLC Main Workbench 9.5 for quality checking, editing, assembling and generation of a final consensus sequence. To determine the changes in the pre-and post-treatment allele frequencies a logistic regression was used and the statistical significance confirmed using a p-trend test. On the matched paired data a McNemar's test was done to determine if there were any significant changes in the allele frequencies after treatment. For both logistic regression and McNemar's test r-programming software was used. The significance level was evaluated at 5% for all analyses.

CHAPTER 4

RESULTS

4.1 Genetic Diversity of the *P. falciparum* isolates

A total of 363 children between the age of 6 months - 125 months were enrolled in the two-arm randomized drug trial between April 2013 and June 2013; 201 (55%) and 162 (45%) receiving AL and DP respectively. The baseline range parasitemia was 1080-195,200 parasites/µl. A 42-day follow-up period consisting of nine time-points including the day of recruitment was carried out, with the number of available samples shown in Table 2-1. 49 participants were reported as being malaria slide-positive after treatment by either AL or DP; 1 (0.3%), 1 (0.3%), 8 (2.5%), 13 (4.1%) and 26 (9%) on days 7, 14, 21, 28 and 42 respectively. The day 42 ACPR of the clinical trial was 89.5% (17/162) by DP and 95.5% (9/201) by AL. The day 28 ACPR was 94.4% (9/162) by DP and 98% (4/201) by AL.

It is therefore evident that from the clinical trial both AL and DP seem to be highly efficacious, however, WHO recommends the use of highly polymorphic markers such as *P. falciparum msp2* and *P. falciparum glurp* to distinguish recrudescent from new infections (81).

Reported here are the results of the *msp2* and *glurp* genotyping on the available 40 of the 49 participants who had recurrent infections post-treatment by both DP and AL.

4.1.1 Validation of the *msp2* and *glurp* genotyping PCR methods

To validate the *msp2 and glurp* PCR genotyping methods, genomic DNA from three laboratory *P. falciparum* isolates; 7G8, 3D7 and Dd2, were used. 7G8 and 3D7 represent the 3D7 allelic type while Dd2 represent the FC27 type. As shown in Figure 4-1, the 3D7/IC specific primers amplified a fragment in 7G8 and 3D7 isolates and as expected not in Dd2 isolates, while FC27 specific primers amplified a fragment in Dd2 and not in 3D7 and 7G8, thus validating the method. Since the *glurp* amplification does not require family specific amplification, the 3D7 isolate was chosen as the positive control.

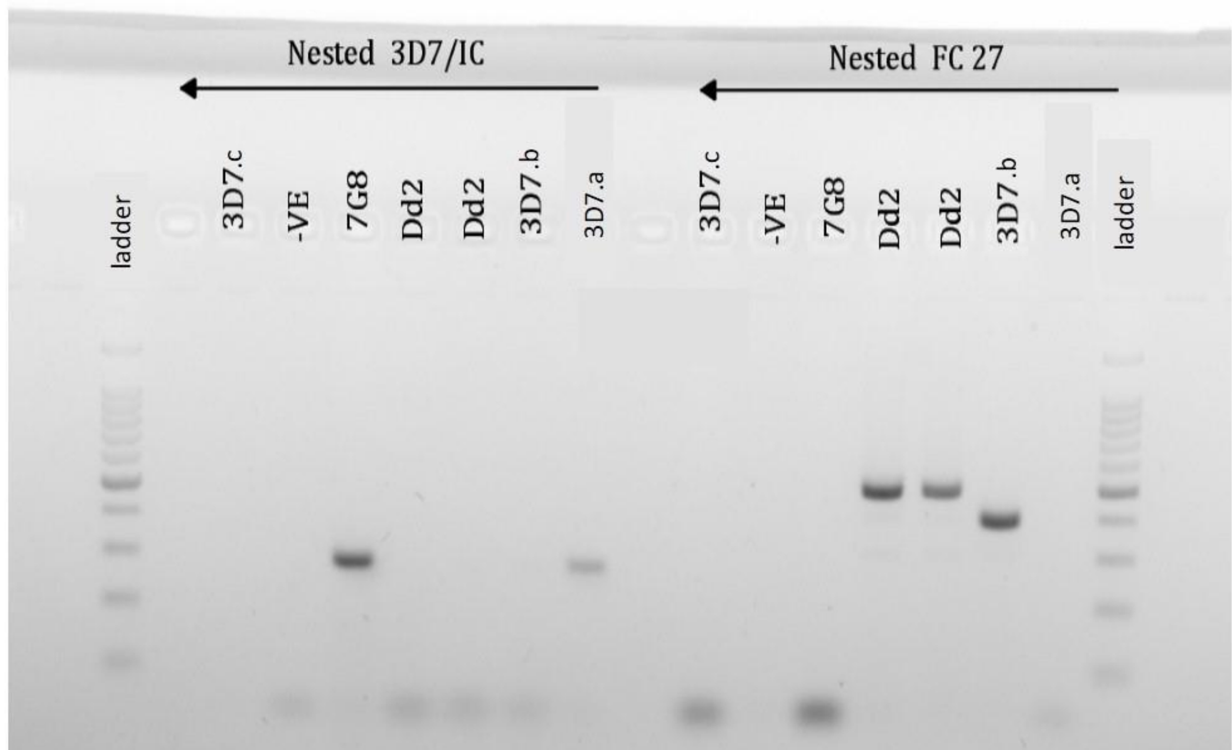

Figure 4-1: A 2% agarose gel showing 3D7 and FC27 amplifications of the Dd2, 7G8 and 3D7 run with a HyperLadder™ 100bp (Bioline, London, UK). Three different isolates were used to obtain the 3D7 DNA: the 3D7.a was obtained from parasites that were previously cultured and later diluted at a ratio of 1:100 and named 3D7.c. 3D7.b was obtained after DNA isolation of a culture of trophozoites. Both Dd2 isolates were obtained from the same culture. The negative control used was PCR clean water.

4.1.2 Genotyping the Clinical Trial Samples

The PCR cycling conditions used were as per published protocols, as shown in Table 2-2. Only nested amplicons were run on the gel with the paired samples run alongside each other for easy comparison of band sizes as shown in Figure 4-2. Of a total 49 paired microscopy-positive samples, 39 samples (Day 7(1), Day 21(7), Day 28(9), Day 42(22)), were successfully amplified at both *msp2* and *glurp* loci.

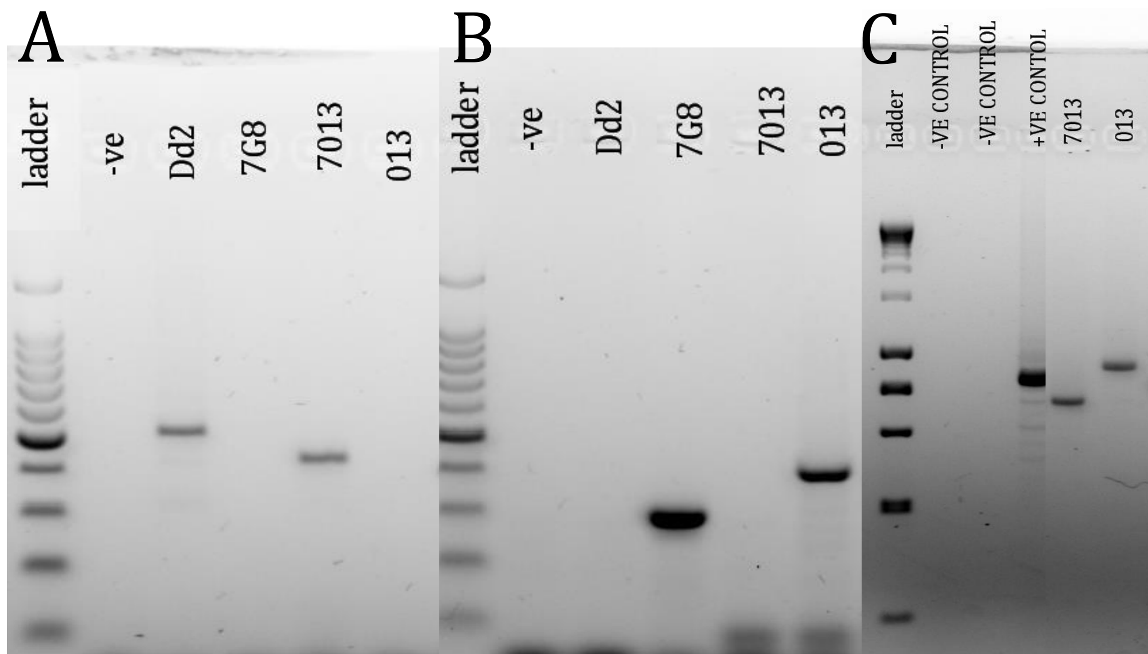

Figure 4-2: An example of a gel image showing the results of the msp2 and glurp genotyping on a single patient, obtained on day 0 and 7:FC27 amplification (A), 3D7 amplification (B), both representing the msp2 locus, and glurp amplification (C) on Sample_ID 13; Day 0 (013) and Day 7 (7013). At day 0 the patient is primarily infected with the 3D7-type clone, which is cleared by day 7 and replaced with the FC27-type clone. Due to the varying band sizes between the day 0 and 7 glurp amplification, it is confirmed that indeed these are two unique infecting clones.

4.1.3 Molecular weight determination

Gel images were visually examined to identify the samples with successfully amplified fragments. To determine the band sizes, all gel images were analyzed by a digitalized gel documentation system where band sizing and molecular weight were calculated by the Image Lab™ Software version 6.0 Bio-Rad, while using the ladder as the reference. The band detection sensitivity was set at medium band-detection sensitivity to detect the bands of interest including any faint bands, as shown in Figure 4-3. Using high band-detection sensitivity resulted in detection of background staining as bands. A .csv file containing the computed band sizes and the band intensities was saved for further analysis.

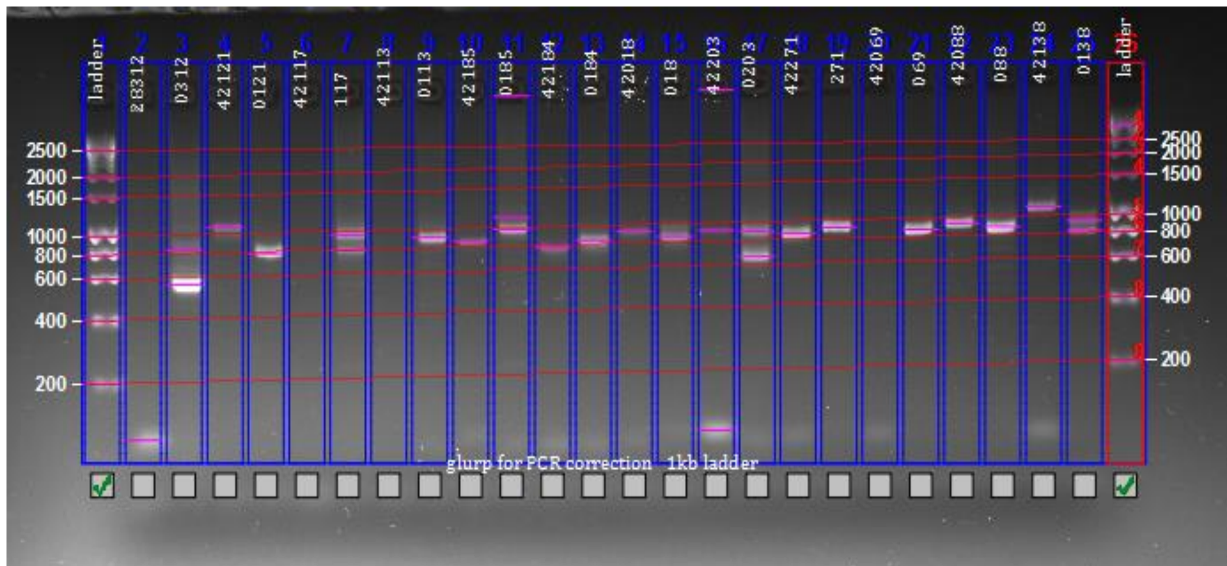

Figure 4- 3: A gel image under analysis on the Image Lab Software, showing the detected bands and band sizes in relation to the ladders at both ends.

4.1.4 Multiplicity of Infection (MOI) and Response to treatment

The MOI defined as the mean number of parasite genotypes per infection target is calculated as follows:

$$\text{MOI} = \frac{\text{mean number of alleles}}{\text{No. of patients positive at a defined locus}}$$

Table 4-1, shows the number of alleles at each locus along with the MOI for each loci in the five time-points. *msp2* recorded the highest MOI of 2.7 (Day 0) as compared to *glurp* 1.3 (Day

21), (t-test: t = 2.499, p-value = 0.0495). This marginal significance implies that the diversity of these genes is comparable and both genes would serve as adequate markers for determining the MOI in this site. The MOI by *msp2* loci is seen to decrease after treatment.

Table 4-1: Shows the total number of *msp2* (FC27 and 3D7), glurp alleles along with the MOI for each day

Time point	Number of genotypes per allelic-family/ loci				
	msp2			***glurp***	
	FC27 (%)	**3D7 (%)**	**MOI**	**RII**	**MOI**
Day 0 (n=40)	57 (47)	64 (53)	2.7	48	1.2
Day 7 (n=1)	2 (67)	1 (33)	1	1	0.5
Day 21 (n=6)	15 (68)	7 (32)	1.6	8	1.3
Day 28 (n=9)	19 (70)	8 (30)	1.6	8	1
Day 42 (n=24)	26 (54)	22 (46)	2.4	19	1.1

The mean (range) paired difference in the band sizes of identical alleles was 3.8bp (0-18bp) for FC27, 11.02bp (3.4-17.7bp) for 3D7 and 12.4bp (0-34.7bp) for *glurp*. Based on this analysis, a pre- and post-treatment sample with FC27, 3D7 and *glurp* allele size difference of 18bp, 17.7bp and 34.7bp respectively, were considered the same. A patient was considered to have a recrudescent infection if two or more alleles were similar at any loci before and after treatment or if at least one allele was similar in at least two loci. In the event there was only one data point from one loci then one similar allele was enough to call it a recrudescent infection.

msp2 genotyping confirmed a total of 12 (31%) recrudescent and 27 (69%) new infections. In

order to confirm the number of recrudescent infections identified at the *msp2* locus, further genotyping was done at the *glurp* locus. The *glurp* locus confirmed 8 of the 12 cases reported at the *msp2* locus as being recrudescent; two patients from day 28, one (day 21) and five (day 42) and 31 cases as re-infections, as shown on Table 4-2.

Under response to treatment, the adequate clinical parasitological response (ACPR), which is the absence of parasitemia irrespective of the axillary temperature on day 21, day 28 and day 42, was computed. This is compared with the PCR-corrected ACPR, which takes into account PCR data using *msp2* and *glurp* genotyping to define a new infection from recrudescent infection rather than only considering microscopy data. This translates to a PCR-corrected ACPR of 100% by DP and 99.4% by AL on day 21, 100% by DP and 98.8% by AL on day 28 and lastly, 99.5% by DP and 97.5% by AL on day 42, all shown in Table 4-2.

Table 4-2: Table showing the number of recrudescent and new infections, the PCR-corrected and uncorrected ACPR and the drug arm involved

DAY	Infection type	Drug Arm		ACPR	Drug Arm	
		DP	AL		DP	AL
21	re-infection	2	3	Uncorrected	98	97.5
	recrudescent	1	0	Corrected	100	99.4
28	re-infection	5	2	Uncorrected	98	94.4
	recrudescent	0	2	Corrected	100	98.8
42	re-infection	7	11	Uncorrected	95.5	89.5
	recrudescent	1	5	Corrected	99.5	97.5

4.1.5 Distribution of msp2 and glurp alleles

The *msp2* fragment sizes ranged from 160-650bp, with the majority of the 3D7 alleles ranging between 241 to 260bp and FC27 alleles from 281 to 320bp. The *glurp* allele sizes ranged from 600-1190bp with the most frequent alleles being between 851-930bp as shown in Appendix 5. Analysis of the proportions of the *msp2* allele families, before treatment, revealed a greater number of IC/3D7 alleles (53%) as compared to FC27 alleles (47%), as shown in Figure 4-4. After treatment the proportion of FC27 alleles are seen to be higher than the IC alleles, 63% and 37% respectively, OR=0.5, p-value=0.031 by Fisher's exact test. This means that a patient had 50% less odds of having the 3D7 allele-type of parasites after treatment as compared to having the FC27 allele type.

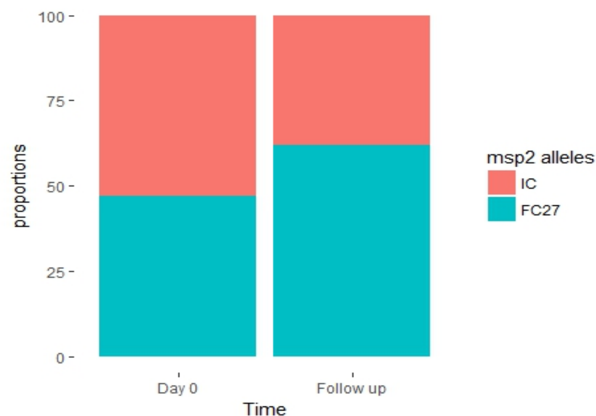

Figure 4- 4: A graph showing the proportion of msp2 alleles at baseline (before treatment) and after treatment (follow-up)

4.2 Baseline allele frequencies of the resistance related markers

Molecular genotyping of antimalarial resistance markers is an important tool in determining the emergence and spread of antimalarial drug resistance (57). In this section the pre- and post-treatment allele frequencies of three antimalarial resistance markers; Pfcrt, Pfmdr1 and *Pfk13* are presented.

Of the 312 Day 0 samples, 211(67.6%) PfCRT codon 76, 209(67.0%) PfMDR1 codon 86, 208(66.7%) PfMDR1 codon 184, 182(58.3%) PfMDR1 codon 1246 and 173(55.4%) K13 were successfully sequenced, all shown in Table 4-3.

For *Pfcrt*, 113(54%) samples carried the K76 wild type, 59(28%) had the 76T mutant and 39(18%) displayed a mixed wild type and mutant allele. Among the 211 isolates, two main haplotypes were identified, 113 (53.6%) carried the wild-type CVMNK haplotype and 58(27.5%) harbored the mutant CVIET haplotype. Only one sample was seen to harbor the rare CVMET haplotype at a frequency of 0.5%. The mixed haplotypes 39(18.4%) were mainly composed of CVIET/CVMNK, with some rare haplotypes such as CVMDT/CVIET and CVMNT/CVIET.

Sequence analysis of the three *Pfmdr1* codons displayed: 168(80%) N86 wild type, 23 (11%) 86Y mutant and 18(9%) mixed alleles, 89(43%) Y184 wild type, 85 (41%) 184F mutant and 34 (16%) mixed alleles and 162(89%) D1246 wild type, 12 (7%) 1246Y mutant and 8(4%) mixed alleles. Haplotype data in all the three *Pfmdr1* codons were obtained in 96(45%) samples. 6 haplotypes (NYD, NFD, YYD, NFY, YYY, NYY) were identified. The NFD haplotype was found most frequently in 37(38.5%) isolates, followed by NYD 30(31.4%), YYD 2(2.1%) and three rare haplotypes (NFY, YYY, NYY) each occurring in a single isolate. There were no synonymous or non-synonymous mutations observed in the propeller domain of *k13*.

Table 4-3: Baseline allele frequencies of *Pfmdr1* and *Pfcrt*

Gene (polymorphic codon)	SNP/Haplotype			
Pfcrt (72,73,74,75,76)	**SNPs**	**Frequency**	**Haplotypes**	**Frequency**
	K76*	113(54%)	CVMNK*	113(53.5%)
	76T	59(28%)	CVIET	58(27.5%)
	Mixed K/T	39(18%)	CVMET	1(0.5%)
			MIXED	39(18.4%)
Total= 211				
Pfmdr1 (codon 86, 184, 1246)	**SNPs**	**Frequency**	**Haplotypes**	**Frequency**
	N86*	168(80%)	NYD*	30(31.4%)
	86Y	23(11%)	NFD	37(38.5%)
	Mixed N/Y	18(9%)	YYD	2(2.1%)
	Y184*	89(43%)	NFY	1(1%)
	184F	85(41%)	YYY	1(1%)
	Mixed Y/F	34(16%)	NYY	1(1%)
	D1246*	162(89%)	MIXED	24(25%)
	1246Y	12(7%)		
Total =96	Mixed D/Y	8(4%)		

* represents either the wild-type allele/haplotype

MIXED *pfcrt* haplotypes identified were CVIET/CVMNK, CVMDT/CVIET, CVIEK/CVMNK or CVMNT/CVIET

MIXED *pfmdr1* haplotypes identified were NFD/YFD, NFD/NYD, NFY/NYY, YYD/NYD or NYD/NYY

4.3 Pre- and post-treatment allele and haplotype frequencies of resistance related markers

The temporal distribution of SNPs at *Pfcrt* (codon 72), *Pfmdr1* (codon 86, 184 and 1246) and *k13*-propeller domain are shown in Table 3-4.

There was a notable decrease in the mutant *Pfcrt* 76T allele from 28% (59/211) on day 0 to 9.7% (9/31) on day 42. This decline is seen to begin as soon as day 1 with the mutant 76T allele having a frequency of 21.1% (46/218). The proportion of individuals with the mixed genotype (K/T) is also seen to increase from 18% (day 0) to 29% (day 42).

The proportion of children carrying the mutant *Pfmdr1* 86Y allele is seen to slightly increase after treatment from 11% (day 0) to 12% (day 1) and thereafter is predominantly wild type. There was an overall increase in the mutant 184F allele from 41% (day 0) to 50% (day 42). The mutant 1246Y was only reported on day 0 (7%) with the follow up days showing the wild type allele.

Only one synonymous mutation at the *k13* propeller domain was reported on day 42 at codon 487 from a GTA to GTG encoding the amino acid Valine.

A p-trend test for the effect of ACT treatment showed no statistically significant difference in the frequency of *Pfmdr1* alleles except for *Pfcrt* where there was a significant decrease in the mutant allele from 28% in day 0 to 9.7% in day 42 (Odds Ratio = 0.3; [95% CI 0.13-0.63]; p=0.0285).

Haplotype analysis of the *Pfcrt* gene displayed three haplotypes. The most dominant *Pfcrt* haplotype was CVMNK and was seen to increase from 53.5% on day 0 to 61.3% on day 42, followed by the CVIET genotype and a low frequency CVMET haplotype. *Pfmdr1* haplotype analysis was limited in numbers due to availability in sequence coverage in all the three codons. However, the two main haplotypes that were dominant were the NFD and NYD, all shown in Appendix 7.

Table 4-4: Allele frequencies of mutations in *Pfcrt*, *Pfmdr1* and *k13* genes, before and after ACT treatment

Gene	Position	Wild type	Mutant	Day 0 n(%)	Day 1 n(%)	Day 2 n(%)	Day 3 n(%)	Day 7 n(%)	Day 21 n(%)	Day 28 n(%)	Day 42 n(%)	Parametric trend test slope	Parametric trend test p-value
PfCRT	codon 76	K	T	59(28)	46(21.1)	7(21.2)	0	1(33)	0	1(11)	9(9.7)		
			mixed	39(18)	49(22.5)	2(6)	0	0	2(25)	2(22)	9(29)	-0.00478	0.0285
total number				211	218	33	2	3	8	9	31		
PfMDR1	codon 86	N	Y	23(11)	14(12)	0	0	0	0	0	0		
			mixed	18(9)	10(8)	0	0	0	0	0	1(17)	0.00375	0.1682
total number				209	121	4	1	0	6	4	6		
	codon 184	Y	F	85(41)	65(56)	1(50)	1(100)	0	1(14)	2(50)	3(50)		
			mixed	34(16)	12(10)	0	0	0	1(14)	0	1(17)	-0.0031	0.4653
total number				208	116	2	1	0	7	4	6		
	codon 1246	D	Y	12(7)	0	0	0	0	0	0	0		
			mixed	8(4)	0	0	0	0	0	0	1(11)	0.0019	0.2737
total number				182	10	1	0	1	6	4	9		
K13	codon 487	V(A)	V(G)	0	0	0	0	0	0	0	1(4.5)		
			mixed	0	0	0	0	0	0	0	0	-	-
total number				168	165	7	2	1	5	4	22		

4.3.1 Paired Analysis

To determine if there were any changes in the allele frequencies after treatment, the paired samples having recurrent parasitemia at day 7, 21, 28 or 42 were compared with their respective day 0 samples. Only *Pfmdr1* codon 86 displayed a borderline significant change in the SNP frequency after treatment (McNemar's $\chi 2$= 3.78, p=0.05), with a decreased odds (OR 0.39 95% CI 0.18-0.88), shown in Table 4-5, meaning that alleles were more likely to stay the same than change after treatment.

Table 4-5: The SNP frequencies of the paired samples along with the McNemar's χ2tests for each gene

Gene	Post- and pre-treatment			McNemar's χ2 (p value)	OR (95 % CI)
	Pre	Post			
PfCRT		K76	76T	0.42(0.52)	0.75 (0.43, 1.32)
	K76	54	39		
	76T	46	61		
PfMDR1		N86	86Y	3.78(0.05)	0.39 (0.18, 0.88)
	N86	78	10		
	86Y	22	90		
		Y184	184F	1.18(0.28)	0.64 (0.36, 1.12)
	Y184	52	37		
	184F	48	63		
		D1246	1246Y	-	ND
	D1246	100	0		
	1246Y	0	100		
K13		V(A)487	487V(G)	0(1)	ND
	V(A)487	100	1		
	487V(G)	0	99		

CHAPTER FIVE

DISCUSSION

In this section we discuss the genetic diversity, the multiplicity of *P. falciparum* isolates, recrudescent and new infection cases and the ACPR in Msambweni, an area that was characterized by moderate malaria transmission in 2010 (101).

The *msp1*, *msp2* and *glurp* genes have been used to estimate the genetic diversity of *P. falciparum* populations. Molecular genotyping of such highly polymorphic genes has been adopted in antimalarial efficacy trials as well as clinical trials (102). This is because of their extensive size polymorphisms due to the intragenic repeats that vary between different alleles. In this study, two of these genes, *glurp* and *msp2*, were genotyped in order to distinguish recrudescent from new infections in pre- and post-treatment samples.

The most abundant *msp2* allele was the 3D7 allele (53%) before treatment, whereas the FC27 allele (62%) was the most dominant allele after treatment (OR=1.8, p-value=0.031, Fisher's exact). This means that it is highly likely that a patient has the FC27 allele after treatment. The FC27 allele has been shown to be more prevalent in asymptomatic infections rather than in symptomatic *P. falciparum* infected individuals (103,104), suggesting that post-treatment samples tend to be asymptomatic infections. Analysis of the clinical data confirms that indeed all the post-treatment infections were asymptomatic as no fever was reported. With regards to the baseline 3D7 abundant allele, several studies done in Southeast Asia (105–107) and Africa (108) have placed the 3D7 allele as being the most frequent in a population. In contrast, some studies have reported a higher frequency of FC27 alleles (109–111). In order to confidently determine the most prevalent allele, one would have to genotype the general population. However, we can safely conclude from our day 0 samples that the most prevalent allele is of the 3D7 type. It is therefore interesting that there is a higher odds that post-treatment infections tend to be of the FC27 allele-type, perhaps because the FC27 allele is a less virulent genotype, to some extent able to withstand drug pressure making it more transmissible.

The largest number of alleles detected in a single infection was 3 and 4 for *glurp* and *msp2*, respectively. Evidence of polyclonal infections, characterized by a single infection having more than one allele, was seen in 74% (*msp2*) and 22% (*glurp*) of the patients at baseline. The *msp2* gene recorded higher MOI values as compared to *glurp*. This could be due to the lower diversity of *glurp*, which has only one polymorphic allelic family known as RII, as compared

to *msp2* that has two highly diverse allelic families, 3D7 and FC27(112). The multiplicity of infection is seen to decline steadily from day 0 up to day 7 in both *msp2* and *glurp* loci, perhaps due to the effect of the ACT, which is known to clear parasites by day 3,therefore leading to a decrease in the number of parasites and clones per patient. The MOI values were seen to increase right from day 21 to day 42, with day 42 recording the highest number of clones per patient using the *msp2* loci. From our analysis, this is mainly due to the high number of new infections 31/39 (79%). The malaria vector is therefore continuously introducing new clones to the existing *P. falciparum* pool or some low frequency variants that were not detected by PCR at day 0 rise to a detectable frequency following the clearance of the dominant population in the infection. A study conducted in two regions of Rwanda with different malaria transmission intensities reported more polyclonal infections and high MOI in the malaria endemic area when compared to the low malaria endemic area (113). Other studies conducted in regions with declining malaria transmission such as Djibouti (114), Colombia (115) and Peru (116) have reported low levels of MOI and polyclonal infections.

Only 8 patients were seen to harbor recrudescent clone(s). With the high PCR-adjusted ACPR for day 21 (99.6%), day 28 (99.3%) and day 42 (98.2%), this study compares equally to studies done in western Kenya (117,118) where ACTs remain highly efficacious.

There was a limitation in the analysis of PCR products by gel electrophoresis due to size estimation. Capillary electrophoresis offers better resolution of allele sizes. Despite the fact that we were able to use more than one polymorphic locus and obtained a bp-range within which alleles were considered to be similar, it is possible that there was an under-estimation of recrudescent infections and an over-estimation of new infections due to the arbitrary cut-off that was set (6,119).

We describe here, polymorphisms in *Pfcrt, Pfmdr1, k13* genes isolated before and after ACT. A mutation in the *Pfcrt* gene at codon K76T has been linked to chloroquine resistance (120,121). Mutations in the *Pfmdr1* gene at position N86Y has been known to determine the level of resistance to CQ (72,122,123). In the context of this study involving the use of ACTs, the wild-type *Pfmdr1* N86 and *Pfcrt* K76 parasites have been associated with selection by lumefantrine (124). Parasite selection pressure is the increased ability of the mutant or wild-type parasite population to grow and multiply in the presence of antimalarials, in this case ACTs. Absence of drug pressure would therefore mean that the resistant parasite would

become disadvantaged relative to the sensitive parasite. Several *in vitro* studies have confirmed this notion of drug selection pressure by withdrawing drug use and the fitter susceptible parasites would grow and out-compete the resistant strains over time (6,119).

Our results demonstrate a low baseline frequency of the mutant*Pfcrt*76T (27%) and *Pfmdr1* 86Y (11%)/1246Y (7%), in *P. falciparum* isolates from Msambweni, in 2013. This biased distribution towards a high frequency of the wild-type *Pfmdr1* N86 and *Pfcrt* K76 parasites is potentially due to selection by lumefantrine. This trend has also been associated with the lack of chloroquine use that was responsible for selecting the mutant alleles leading to a resurgence of the wild type alleles (124). Our data are in line with those reporting an increase in the frequency of *Pfmdr1*-186F allele post-treatment (125) which has been associated with *in* vivo selection by AL. The increased prevalence of parasites with the *Pfcrt* K76 and *Pfmdr1* N86/184F/D1246 alleles has been reported in other studies to display higher fitness in the presence of AL and DP(126,127). Extended exposure to ACTs may select for new infections emerging from the liver, with parasites carrying the above alleles.

When the SNP frequencies were analyzed as haplotypes, the same was true, where the wild-type *Pfcrt* C72/V73/M74/N75/K76 allele was seen to be dominant over the mutant allele CVIET.

Thus far, only 4 (C580Y, R539T, Y493H and I543T) of the 13 non-synonymous mutations associated with slow parasite clearance have been confirmed to confer increased ring stage survival in drug resistant field isolates *in vitro* (60). There was no evidence of artemisinin resistance-associated mutations in the *Plasmodium falciparum* K13-propeller domain in Msambweni. This includes the major resistance associated mutation, C580Y, identified in Southeast Asia. This finding is consistent with other studies conducted in Africa where highly diverse, low frequency non-synonymous mutations (17,128,129) or no non-synonymous mutations in the *Plasmodium falciparum* K13-propeller (130) have been identified.

Following treatment with ACTs, studies have reported the selection of wild type alleles at both the *Pfcrt* and *Pfmdr1* loci(121,123,131,132). Our data confirms an increase in wild-type alleles post-treatment especially at the *Pfcrt* locus.

In this study, the frequency of the *Pfcrt* 76T mutant allele is seen to decrease from 28% in day 0 to 9.7% in day 42, along with an increase of the wild-type CVMNK haplotype. In a drug trial done in Mbita, western Kenya, a significant increase in the frequency of the wild type

CVMNK haplotype was observed as early as day 3, from 19.4% on day 0 to 90.4% on day 3(125).

Despite the limitation of few samples obtained for *Pfmdr1* haplotype analysis, our results clearly indicate a decline in the*Pfmdr1* 86Y and 1246Y mutant alleles. There was an increase in the mutant 184F allele from 41% in day 0 to 50% in day 42, with the increase seen as soon as the first day of treatment at 56% on day 1 ($p = 0.4653$). Previous studies conducted in Tanzania, Uganda and Nigeria have reported a selection of N86, 184F and 1246D *Pfmdr1* alleles in recurrent infections after treatment with ACTs (131,133–135).

In a paired analysis of the SNP frequency, the *Pfmdr1* 86Y allele showed a greater likelihood of decreasing post-treatment, therefore implying a selection of the wild-type N86 allele. The paired analysis also reveals that the pre- and post-treatment infections had a greater likelihood of maintaining the same allele post-treatment. Since most post-treatment infections were new infections rather than recrudescent infections, there appears to be a selection of the wild type alleles post-treatment. Furthermore, it is also the abundant allele circulating in the population.

Analysis of the *k13* gene showed a very low prevalence of the K13 propeller synonymous mutations. Only one sample in a patient treated with DP had a synonymous mutation at codon 487. This finding is in line with recent studies conducted in sub-Saharan Africa, that have not reported any of the polymorphisms associated with artemisinin resistance(60). The most frequent A578S mutation found in Africa has been found to be susceptible to ACT, after an *in vitro* ring stage survival assay, suggesting that the A578S is not an artemisinin-resistant mutation (136). The absence of resistance associated K13 propeller mutations concurs strongly with the efficacy of ACTs reported in this study. Moreover, the ACTs did clear the parasites by day 3, except in one patient, which still qualifies as adequate ACT efficacy where the threshold number of parasitemic patients should be <10% by day 3 as recommended by WHO. It was also observed that there was an overall low number of recrudescent infections in both treatment arms, further emphasizing the efficacy of the drugs.

CONCLUSIONS AND FUTURE RECOMMENDATIONS

The ACTs showed adequate efficacy at the study site in 2013, since no *Pfk13* mutations were observed, there were very few recrudescent infections, a high PCR-corrected ACPR was recorded for both AL and DP on days 28 and 42 and parasites were cleared by day 3, except in one patient, which is below the recommended WHO threshold of <10% parasitemic patients by day 3.

The high re-infection rate suggests a need for continued malaria prevention interventions. This study compares equally well to studies done in western Kenya (117,118) where ACTs were shown to be highly efficacious.

I recommend that current studies on the efficacy of ACTs should be done at the same site to compare the ACPR and the allele frequencies of these and additional resistant markers. The background mutations for artemisinin resistance ought to be studied in order to determine their selection by ACTs.

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APPENDICES

APPENDIX 1: ETHICAL CLEARANCE

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

KEMRI/RES/7/3/1 **March 1, 2017**

TO: **LYNETTE ISABELLA OYIER,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. BENJAMIN TSOFA,**
DIRECTOR, CGMR-C,
KILIFI

Dear Madam,

RE: KEMRI/SERU/CGMR-C/064/3403 (*RESUBMITTED INITIAL SUBMISSION*): GENOTYPING *PLASMODIUM FALCIPARUM* PARASITES FROM DRIED BLOOD SPOT SAMPLES COLLECTED FROM A STUDY TO EVALUATE THE EFFICACY OF ARTEMISININ COMBINATION THERAPY IN KENYA

Reference is made to your letter dated 15th February 2017 which the KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on February 27, 2017.

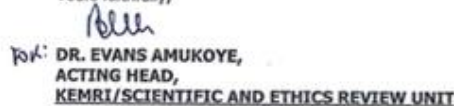
This is to inform you that the Committee determines that the issues raised at the 259th Joint Committee A, B and ERC meeting of the KEMRI Scientific Ethics Review Unit (SERU) held on January 17, 2017 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **1st March 2017.** Please note that authorization to conduct this study will automatically expire on **28th February 2018.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the SERU Secretariat by **17th January, 2018.**

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,



DR. EVANS AMUKOYE,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

APPENDIX 2: CONSENT FORM

INFORMED CONSENT AGREEMENT

Study Title: ....

I, ____(PARENT/GUARDIAN'S NAME), having full capacity to consent for my child ____________________________________ (CHILD'S NAME), do hereby consent to his/her participation in the research study entitled **'Evaluation of the Efficacy of Artemisinin Combination Therapy in Kenya: A World Bank Multi-country East Africa Public Health Laboratory Networking Project'** under the principal investigator Mr. Kimani Francis. The implications of my voluntary participation, the nature, duration and purpose; methods and means by which it is to be conducted; and the inconveniences and hazards which may reasonably be expected have been explained to me by _________________________, and are set forth in the Informed Consent Explanation, which I have signed. I have been given an opportunity to ask questions concerning this investigational study, and any such questions have been answered to my full and complete satisfaction. If there are any further questions that may arise, I may contact Mr. Kimani Francis. I understand that I may at any time during the course of this study revoke my consent and withdraw my child from the study without prejudice; however, I may be requested to have my child undergo further examinations if, in the opinion of the physician, such examinations are necessary for his/her well being.

Child's name ____
Parent's/guardian's signature:______________________ Date_______________
Parent's/guardian's Printed Name:______________________________________
Witness's Signature: ______________________________ Date: ______________
Witness's Printed Name:____

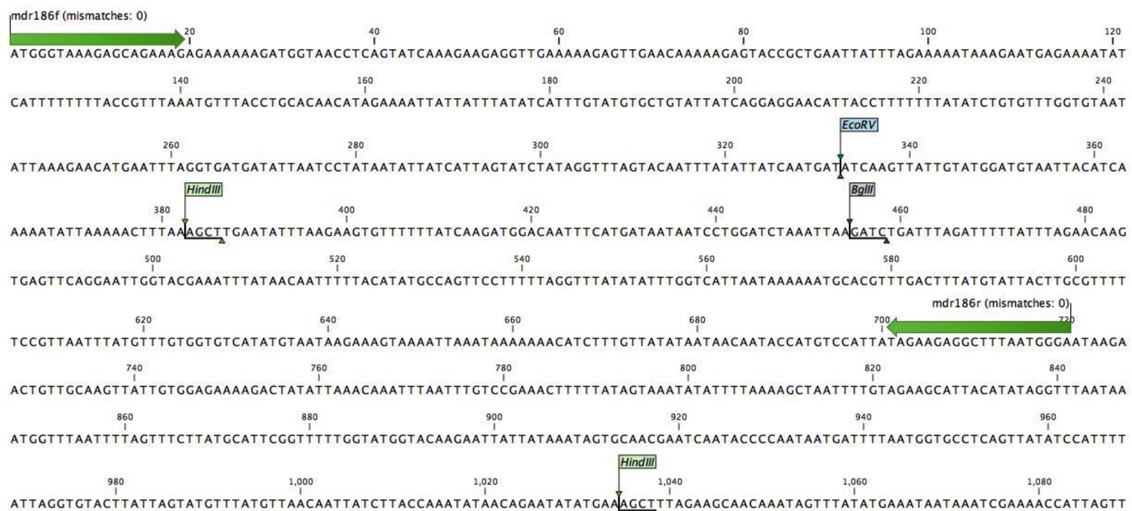
Additional consent for membrane feeding procedures:

Child's name ____
Parent's/guardian's signature:______________________ Date_______________
Parent's/guardian's Printed Name:______________________________________
Witness's Signature: ______________________________ Date: ______________
Witness's Printed Name:____

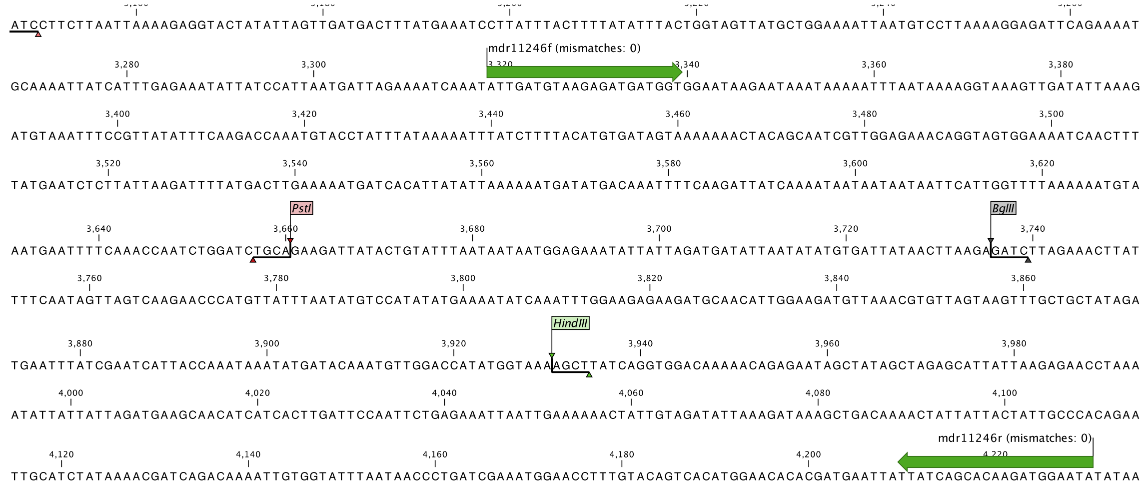
APPENDIX 3: *IN SILICO* PCR/PRIMER MAPS, USING CLC Main Workbench.

K13 Primer Map

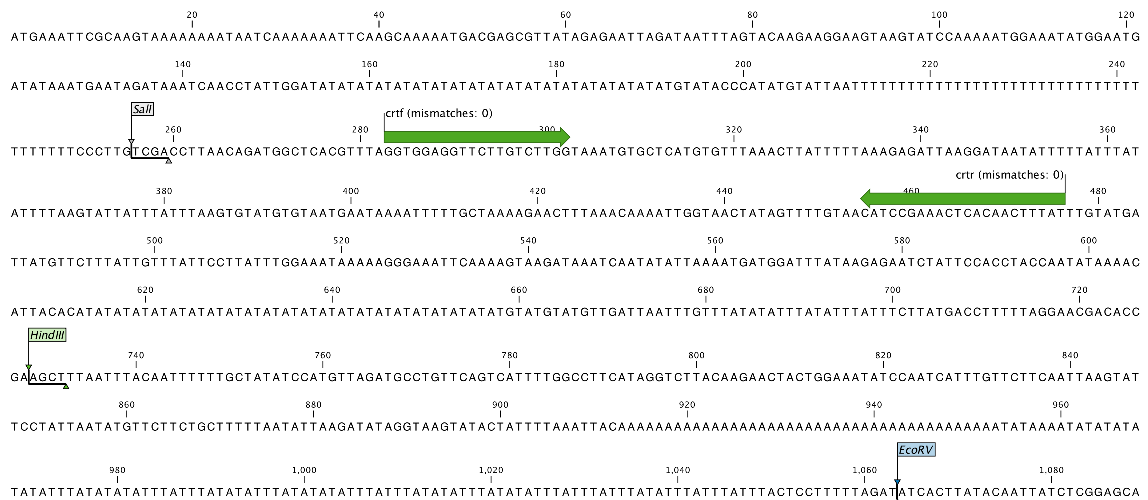
Mdr1-Codon 86 Primer Map



Mdr1-Codon 1246 Primer Map



Crt Primer Map



*Msp2*Primer Map

msp2-primary-f (mismatches: 0)

ATGAAGGTAATTAAAACATTGTCTATTATAAATTTCTTTATTTTTGTTACCTTTAATATTAAAAATGAAAGTAAATATAGCAACACATTCATAAACAATGCTTATAATATGAGTATAAGGA

GAAGTATGGCAGAAAGTAAGCCTTCTACTGGTGCTGGTGGTAGTGCTGGTGGTAGTGCTGGTGGTAGTGCTGGTGGTAGTGCTGGTGGTAGTGCTGGTGGTAGTGCTGGTTCTGGTGATGG

TAATGGTGCAGATGCTGAGGGAAGTTCAAGTACTCCCGCTACTACCACAACTACCAAAACTACCACAACTACCACAACTACTAATGATGCAGAAGCATCTACCAGTACCTCTTCAGAAAAT

CCAAATCATAAAAATGCCGAAACAAATCCAAAAGGTAAAGGAGAAGTTCAAGAACCAAATCAAGCAAATAAAGAAACTCAAAATAACTCAAATGTTCAACAAGACTCTCAAACTAAATCAA

ATGTTCCACCCACTCAAGATGCAGACACTAAAAGTCCTACTGCACAACCTGAACAAGCTGAAAATTCTGCTCCAACAGCCGAACAAACTGAATCCCCCGAATTACAATCTGCACCAGAGAA

XbaI

TAAAGGTACAGGACAACATGGACATATGCATGGTTCTAGAAATAATCATCCACAAAATACTTCTGATAGTCAAAAAGAATGTACCGATGGTAACAAAGAAAACTGTGGAGCAGCAACATCC

msp2-primary-r (mismatches: 0)

CTCTTAAATAACTCTAGTAATATTGCTTCAATAAATAAATTTGTTGTTTTAATTTCAGCAACACTTGTTTTATCTTTTGCCATATTCATATAA

APPENDIX 4: R SCRIPTS USED FOR PCR-CORRECTION

```
#Code for MSc. Bioinformatics project
#______________________________________________________________________________
#Title: Analysis of antimalarial resistance markers in children recruited into a
drug efficacy trial in Kwale, Kenya, 2013.
#Created by: Leonard Marigi Ndwiga; Admission No.: I56/80900/2015
#The scripts used were divided according to their usage on a particular objective
#______________________________________________________________________________


#_______________________________OBJECTIVE 1_____________________________

#______________________________________________________________________________
# Part 1:Cleaning the msp2 and glurp band size data, in .csv format
# Install and load the required libraries
library(dplyr)
library(data.table)
# Part 1-1: combining many .csv files into one
files = list.files(pattern = "\\.csv$")
myfiles = do.call(rbind,lapply(files, read.csv))
write.csv(myfiles, "PCR_correction.csv")

# Part 1-2: filtering the rows with values less than 150bp and greater than 800bp: to
##remove artifact bands
msp2_combined= fread("PCR_correction.csv")
msp2_filtered=msp2_combined %>% filter(`base_pairs_bp` < 800 & `base_pairs_bp`
> 150)
msp2_filtered
```

Appendix 4 cont.

```
# Part 1-3: omitting any rows with "N/A" values
msp2_filtered[msp2_filtered=="N/A"] <-NA  #the trick is to change the character
values "N/A" to what R understands tO be NA.
na.omit(msp2_filtered)

# Part 1-4: deleting columns
msp2_filtered[,c("band_no", "band_label","adj_volume","relative_front","volume",
"abs_quant","rel_quant","lane_%")] <-list(NULL)
colnames(msp2_filtered)

# Part 1-5: deleting rows
msp2_filtered=msp2_filtered[!(msp2_filtered$lane=="ladder"),]
msp2_filtered=msp2_filtered[!(msp2_filtered$lane=="NEG CONTROL"),]
msp2_filtered=msp2_filtered[!(msp2_filtered$lane=="P4035"),]
msp2_filtered=msp2_filtered[!(msp2_filtered$lane=="P4220"),]
msp2_filtered=msp2_filtered[!(msp2_filtered$lane=="POS CONTROL"),]
write.csv(msp2_filtered, "Semifinal.csv")

# Part 1-6: reshaping the table
dataLeo_v1 <- fread("Semifinal.csv") #require.package ""data.table"
msp2_filtered[,c("V1")] <-list(NULL) #code that actually deletes
dataLeo_v2 <- reshape(dataLeo_v1, idvar = "lane", timevar = "band_no", direction =
"wide")  #require.package ""reshape
write.csv(dataLeo_v2, "pcr_corrected_data.csv")
#______________________________________________________________________________
```

Appendix 4 cont.

```
#________________________________________________________________________
# Part 2: Plotting a graph to show the band frequencies of msp2 and glurp alleles
# Install and load the required packages
library(ggpubr)
library(data.table)
library(dplyr)
library(ggplot2)
# load data
msp2andglurp <- fread("bandpattern.csv")
msp2andglurp <- melt(mspBandData, id.vars = "allele_family")
ggplot(msp2andglurp) + geom_col(aes(variable, value, fill = allele_family), position =
"dodge") +
 theme_pubr() +
 theme(axis.text.x=element_text(angle=90, hjust=1),
     legend.position="right") +
 xlab(label = "Allele size (bp)") +
 ylab(label = "Frequencies")
#________________________________________________________________________
```

Appendix 4 cont.

```
# Part 3: Plotting a graph to show the proportion of msp2 alleles, before and after
## treatment
# Install and load the required packages
library(ggpubr)
library(dplyr)
library(data.table)
# To read the file
snpData <- read.csv("allele_clearance.csv", header = T, stringsAsFactors = T)
snpData= melt(snpData, id.var= "Time")
alleleData=ggplot(snpData, aes(x= Time, y= value, fill= variable)) + theme_bw () +
 theme(plot.background= element_blank()
     ,panel.grid.major=element_blank()
     ,panel.grid.minor= element_blank()
     ,panel.border=element_blank()
     ) +
 geom_bar(stat="identity") + xlab(label = "Time") + ylab(label = "proportions") +
 guides(fill=guide_legend("msp2 alleles"))
tiff("e.tiff",
   width= 4, height=4.5, units= 'in', res= 300,
   compression= "lzw",
   type= "quartz") + theme_bw()
print(alleleData)
#______________________________________________________________________


# Part 4: to do a t-test for 2-independent variables i.e MOI for msp2 & glurp
# where x and y are numeric values recorded in each of the days
x=c(2.7,1,1.6,1.6,2.4)
y=c(1.2,0.5,1.3,1,1.1)
t.test(x,y)
#______________________________________________________________________
```

Appendix 4 cont.

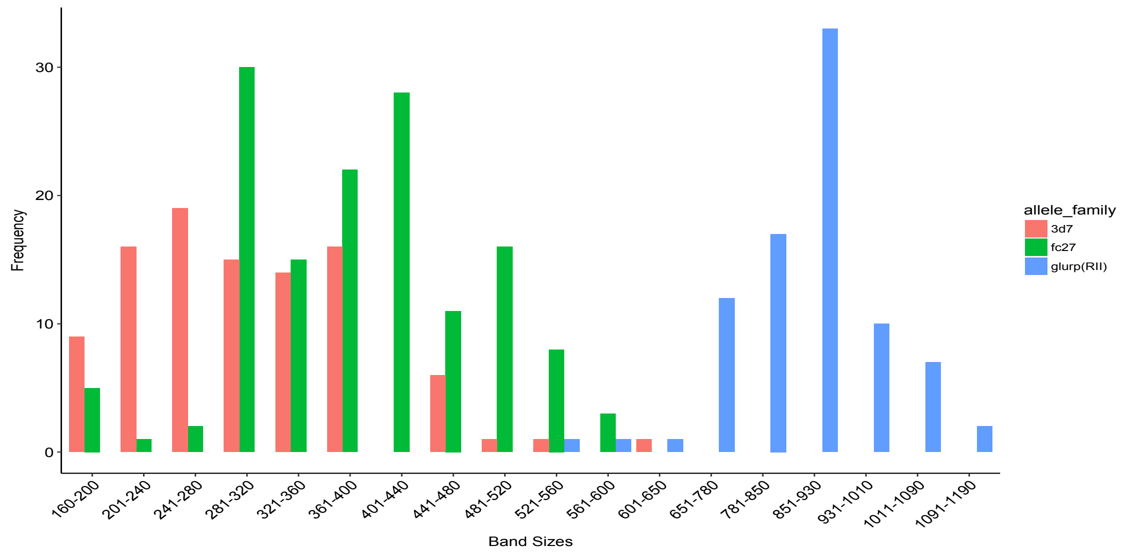
```
#______________________________OBJECTIVE 2 & 3____________________________

#______________________________________________________________________
#Having obtained all  .fasta files after sequence assembly in CLC Main Workbench 7,
##the next step was to remove any ambiguous nucleotide calls, mainly 'NNNN'.
#Part 1: Combining all the .fasta files into one file
#the script will parse the folder, under the set directory, for all files with the
##extension as ".fa"
cat *.fa > k13.fasta

#Part 1-2: removing all the 'NNNNN'
sed -e "/^[^>]/s/[^ATGCatgc]//g" k13.fasta>>nonnn.fa

#Part 1-3: removing the spaces created after removing the 'NNNNN'
awk'/^>/{print s? s"\n"$0:$0;s="";next}{s=s sprintf("%s",$0)}END{if(s)pirnt
s}'nonnn.fa>>k13.fa
```

APPENDIX 5: MOLECULAR WEIGHT CATEGORIZATION OF *MSP2* AND *GLURP* PCR PRODUCTS



APPENDIX 6: AN EXAMPLE OF A DNA AND PROTEIN MULTIPLE SEQUENCE ALIGNMENT OF THE *CRT* GENE DONE IN CLC MAIN WORKBENCH

DNA Sequence Alignment

```
MSW_49_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TAAAATTTTT GCTAAAAGAA
MSW_52_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TAAAATTTTT GCTAAAAGAA
MSW_95_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TAAAATTTTT GCTAAAAGAA
MSW_96_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TAAAATTTTT GCTAAAAGAA
MSW_98_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TAAAATTTTT GCTAAAAGAA
MSW_99_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TAAAATTTTT GCTAAAAGAA
MSW_124_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TACAATTTTT GCTAAAAGAA
MSW_166_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TACAATTTTT GCTAAAAGAA
MSW_259_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TACAATTTTT GCTAAAAGAA
MSW_266_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TACAATTTTT GCTAAAAGAA
MSW_225_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TAAAATTTTT GCTAAAAGAA
MSW_238_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AAAAATTTTT GCTAAAAGAA
MSW_23_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TAAAATTTTT GCTAAAAGAA
MSW_33_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA AAAAATTTTT GCTAAAAGAA
MSW_37_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TAAAATTTTT GCTAAAAGAA
MSW_46_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TAAAATTTTT GCTAAAAGAA
MSW_50_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATGAA TAAAATTTTT GCTAAAAGAA
MSW_107_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_111_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_112_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_114_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_116_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_117_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_121_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_127_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_144_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_147_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_155_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_156_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_15_CRT_DAY0   TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
MSW_165_CRT_DAY0  TAAGGATAAT ATTTTTATTT ATATTTTAAG TATTATTTAT TTAAGTGTAT GTGTAATTGA AACAATTTTT GCTAAAAGAA
```

Protein Sequence Alignment

```
MSW_49_CRT_DAY0   MCLNIFKEIK DNIFIYILSI IYLSVCVMNK IFAKR
MSW_52_CRT_DAY0   MCLNIFKEIK DNIFIYILSI IYLSVCVMNK IFAKR
MSW_95_CRT_DAY0   MCLNIFKEIK DNIFIYILSI IYLSVCVMNK IFAKR
MSW_96_CRT_DAY0   MCLNIFKEIK DNIFIYILSI IYLSVCVMNK IFAKR
MSW_98_CRT_DAY0   MCLNIFKEIK DNIFIYILSI IYLSVCVMNK IFAKR
MSW_99_CRT_DAY0   MCLNIFKEIK DNIFIYILSI IYLSVCVMNK IFAKR
MSW_124_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVMNT IFAKR
MSW_166_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVMNT IFAKR
MSW_259_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVMNT IFAKR
MSW_266_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVMNT IFAKR
MSW_225_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVMNK IFAKR
MSW_238_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVIEK IFAKR
MSW_23_CRT_DAY0   MCLNIFKEIK DNIFIYILSI IYLSVCVMNK IFAKR
MSW_33_CRT_DAY0   MCLNIFKEIK DNIFIYILSI IYLSVCVMKK IFAKR
MSW_37_CRT_DAY0   MCLNIFKEIK DNIFIYILSI IYLSVCVMNK IFAKR
MSW_46_CRT_DAY0   MCLNIFKEIK DNIFIYILSI IYLSVCVMNK IFAKR
MSW_50_CRT_DAY0   MCLNIFKEIK DNIFIYILSI IYLSVCVMNK IFAKR
MSW_107_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVIET IFAKR
MSW_111_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVIET IFAKR
MSW_112_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVIET IFAKR
MSW_114_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVIET IFAKR
MSW_116_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVIET IFAKR
MSW_117_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVIET IFAKR
MSW_121_CRT_DAY0  MCLNIFKEIK DNIFIYILSI IYLSVCVIET IFAKR
```

APPENDIX 7: THE FREQUENCY OF RESISTANCE-RELATED HAPLOTYPES BEFORE AND AFTER TREATMENT WITH ACT

Haplotype	Day 0 n(%)	Day 1 n(%)	Day 2 n(%)	Day 3 n(%)	Day 7 n(%)	Day 21 n(%)	Day 28 n(%)	Day 42 n(%)
*pfcrt*_CVMNK*	113(53.5)	123(56.4)	24(72.8)	2(100)	2(66.7)	6(75)	6 (66.7)	19 (61.3)
*pfcrt*_CVIET	58 (27.5)	43 (19.7)	7 (21.2)	-	-	-	1 (11.1)	3 (9.7)
*pfcrt*_CVMET	1 (0.5)	3 (1.4)	-	-	-	-	-	-
	N = 211	N =218	N = 33	N = 2	N = 3	N = 8	N = 9	N = 31
*pfmdr1*_NYD*	30 (31.4)	4 (44.4)	-	-	-	5 (83.3)	1 (50)	-
*pfmdr1*_NFD	37 (38.5)	3 (33.3)	-	-	-	-	1 (50)	1 (50)
*pfmdr1*_YYD	2(2.1)	1 (11.1)	-	-	-	-	-	-
*pfmdr1*_YFD	-	1 (11.1)	-	-	-	-	-	-
*pfmdr1*_NFY	1(1)	-	-	-	-	-	-	-
*pfmdr1*_YYY	1(1)	-	-	-	-	-	-	-
*pfmdr1*_NYY	1(1)	-	-	-	-	-	-	-
	N = 96	N = 9	-	-	-	N = 6	N = 2	N = 2

APPENDIX 8: THE FREQUENCY OF CRT AND MDR1 ALLELES PRE- AND POST-TREATMENT IN RELATION TO EACH DRUG ARM

				Day 0		Follow-up	
Gene	Position	Wild type	Mutant	AL(%)	DP(%)	AL(%)	DP(%)
PfCRT	codon 76	K	T	5 (56)	8 (42)	1 (8)	3 (17)
			mixed	1 (11)	3 (16)	4 (31)	6 (33)
total number				9	19	13	18
PfMDR1	codon 86	N	Y	0	3 (15)	0	0
			mixed	1 (11)	2 (10)	1 (20)	1 (11)
total number				9	20	5	9
	codon 184	Y	F	5 (56)	7 (35)	0	4 (40)
			mixed	1 (11)	5 (25)	1 (20)	1 (10)
total number				9	20	5	10
	codon 1246	D	Y	1 (11)	1(7)	0	0
			mixed	1 (11)	0	1 (13)	0
total number				9	14	8	8