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POPULATION GENETICS OF *PLASMODIUM FALCIPARUM* PARASITES EXPOSED TO ARTEMISININ TREATMENT IN PATIENTS TAKING PART IN AN EFFICACY CLINICAL TRIAL IN KISUMU COUNTY, WESTERN KENYA.

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Population genetics of *Plasmodium falciparum* parasites exposed to artemisinin treatment in patients taking part in an efficacy clinical trial in Kisumu county, western Kenya.

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DECLARATION

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

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DEDICATION

This thesis is dedicated:

To my husband, Nelson Kibichii Bore, my companion and friend whose love and support has been consistent every step of the way.

To my dad and mum, who taught me that even the largest task can be accomplished if it is done one step at a time!

To my beloved brothers and sisters who stood by me when things looked bleak and believed in me.

To all my family, symbol of love and friendship.

To all my friends who encourage and support me and all the people in my life who touch my heart, I dedicate this research.

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ACD:	Acid Citrate Dextrose
ACT:	Artemisinin based combination therapy
AL:	Artemether-Lumefantrine
ASMQ:	Artesunate-Mefloquine
AMOVA:	Analysis of Molecular Variance
CBC:	Complete blood count
CE:	Capillary electrophoresis
CI	Clonally Identical
CRs:	Clearance rates
CSP:	Circum-sporozoite protein
DFHR:	Dihydrofolate reductase
DHPS:	Dihydropteroate synthetase
DHA	Dihydroartemisinin
DNA:	Deoxyribonucleic acid
EDTA:	Ethyldiaminetetraacetic acid
GLURP:	Glutamate rich protein
GWAS:	Genome-wide association studies
ITROMID:	Institute of Tropical Medicine and Infectious Disease
IQR:	Interquartile Range
MALDI-TOF:	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight

MI:	Multiple infections
MOI:	Multiplicity Of Infections
MSP-1&2:	Merozoite surface protein 1&2
NTC:	Negative template control
ORF:	Open Reading Frame
PCE:	Parasite clearance estimator
PCR:	Polymerase chain reaction
Pfcrt:	*Plasmodium falciparum* chloroquine resistance transporter
***Pfmdr1*:**	*Plasmodium falciparum* multi-drug resistance gene 1
***P. falciparum*:**	*Plasmodium falciparum*
SEA:	Southeast Asia
SERCA:	Sarco-endoplasmic reticulum Calcium ATPase
SNP:	Single nucleotide polymorphism
SSA:	Sub-Saharan Africa
SSP2:	Sporozoite surface protein 2
SP:	Sulphadoxine/Pyrimethamine
USAMRD-K:	United States Army Medical Research Directorate-Kenya
WHO:	World Health Organization
WWARN:	Worldwide Antimalarial Resistance Network

ABSTRACT

Artemether-lumefantrine (AL), adopted in Kenya for malaria treatment, remains highly efficacious. However, there are heightened concerns because resistance to Artemisinin combination therapy (ACTs) is now well documented in Southeast Asia (SEA). This resistance is associated with slow parasite clearance rates (CRs). There is now need for more genetically determined artemisinin resistance data from malarious regions that use ACT for malaria treatment. Therefore, the main objective of this study was to determine the role that population genetics plays in *Plasmodium falciparum* parasites exposed to artemisinin treatment amongst patients taking part in an efficacy clinical trial in Kisumu county, western Kenya. To accomplish this, two sets of archived samples were used. The first set was collected before the introduction of AL (pre-ACTs n= 29) and the second set from a randomized open labeled trial (post-ACTs n= 92). A panel of 12 microsatellites (MS) and 91 single nucleotide polymorphisms (SNPs) distributed across the *P. falciparum* genome were used to genotype the parasites. The 12 MS were selected because they have previously been shown to effectively differentiate *P. falciparum* strains. Similarly, the 91 SNPs have previously been shown in a SEA study to target minor allele frequency greater than 5% and are highly polymorphic therefore important in identifying similar parasites. The SNPs were also used to determine genes that may be involved in influencing parasite CRs. The 12 MS were highly polymorphic in post-ACTs parasites and showed greater genetic diversity compared to pre-ACTs parasites ($p < 0.0226$ at 95% CI). Most post-ACTs parasites achieved clearance within 42 hours of treatment with AL having a median

clearance half-life of 2.55 hours (range 1.19-5.05). Of the 91 SNPs assessed, 78 (85%) generated allele calls in all parasites and showed positive correlation with parasite clearance half-life. Based on SNP analysis, only 15 of 92 (16.3%) post-ACTs parasites analyzed were single-clone infections and genetically related. Interestingly, no SNPs in the K13-propeller gene were observed in the Kenyan parasites. Among the 78 SNPs with positive correlation of parasites clearance, 3 SNPs on chromosome 12 and 14 were associated with delayed parasite CRs. These 3 SNPS encode *Plasmodium* proteins with unknown functions. Based on results from this study, it can be recommended that advanced technologies such as use of the CRISPR-Cas9 system should be applied to validate the role these 3 SNPs have in influencing delay in parasite CRs. In conclusion, these three SNPs may serve as genetic markers associated with ACTs resistance in Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Currently, almost half of the world's population is at risk of contracting malaria (World Health Organization, 2014). It is estimated that 198 million clinical cases occurred globally in 2013 which led to 584,000 deaths. The heaviest burden (90 % of the deaths) occurring in Sub-Saharan African (SSA) countries. Also, 78% of all deaths occur in children <5 years of age (World Health Organization, 2014). It has been reported of 91 countries and territories with malaria, 40 are estimated to have achieved a reduction in incidence rate. In Kenya, malaria is the leading cause of morbidity and mortality with about 70% of the population at risk of malaria (Kenya Malaria Indicator Survey, 2010) especially in malaria endemic areas such as Lake Victoria, western Kenya and in the coastal region. Consequently, 3.5 million children <5 years are exposed to stable malaria transmission (Kenya Malaria Indicator Survey, 2010).

Anti-malarial resistance is often influenced by several factors including drug elimination half-life, drug use, parasite intensity levels, host factors, transmission intensities and pharmaco-dynamics (Ronn *et al.*, 1996; Wongsrichanalai *et al.*, 2002). Sensitive *P. falciparum* parasites are cleared within 48 hours in 95% of the patients after treatment with artemisinin when assessed using blood smears(White, 2008). Presence of parasites at 72 hours is a predictor of subsequent treatment failure (Stepniewska *et al.*, 2010). A recent study which mapped the geographic extent of resistance in SEA and Africa found median parasite clearance half-lives ranging from

1.9 hours in the Democratic Republic of Congo (DRC) to 7.0 hours at the Thailand-Cambodia border (Ashley et al., 2014). Although parasites occasionally had clearance half-life > 5 hours from previous studies conducted in DRC and Nigeria, ACTs remain highly effective in Africa with fast clearance half-lives (Maiga *et al*., 2012; Muhindo *et al*., 2014; Zwang *et al.*, 2014). Unlike parasites from SEA that showed strong association of clearance half-lives with point mutations in the recently described K13-propeller region (Ariey *et al.*, 2014), there was no association of K13 polymorphisms with parasite clearance half-lives in African parasites (Ashley 2014). Indeed, the recently described mutations in the K13-propeller region in SEA parasites have not been found in African parasites (Ashley *et al.*, 2014; Conrad *et al.*, 2014; Kamau *et al.*, 2014; Taylor *et al.*, 2014). These observations may explain the absence of ACT resistance in Africa.

Genetically determined artemisinin resistance in *P. falciparum* has been described in SEA (Miotto *et al.*, 2013; Phyo *et al.*, 2012). In western Cambodia, parasite clearance rates have been shown to be a heritable parasite-encoded trait where patients with similar parasite clearance rates are infected with clonally identical (CI) parasites. The proportion of genetically determined artemisinin resistance has increased substantially in SEA, with parasite clearance attributable to parasite genetics increasing from 30% between 2001 and 2004, to 66% between 2007 and 2010 (Phyo *et al.*, 2012). Further, a recent study discovered several distinct but apparently sympatric parasite subpopulations with extremely high levels of genetic differentiation in Cambodia (Miotto *et al.*, 2013). Three subpopulations associated with clinical resistance to artemisinin had skewed allele frequency spectra and high levels of haplotype homozygosity, which is indicative of founder effects and recent population expansion.

Interestingly, the K13-propeller alleles were shown to be strongly associated with parasite subpopulations (Ariey *et al.*, 2014).

P. falciparum population structure is correlated with the intensity of malaria transmission. Regions of low transmission intensity such as SEA results in higher rates of inbreeding and greater population structure unlike regions of high transmission intensity such as sub-Saharan African (SSA) countries (Anderson *et al.*, 2000; Dye *et al.*, 1997) and specifically here in Kenya (Mobegi *et al.*, 2012; Zhong *et al.*, 2007). It is yet to be understood whether parasites in regions of high transmission intensity which are less structured carry parasite populations or subpopulations that are genetically similar with heritable traits such as artemisinin resistance. Previous anti-malarial drug resistance originated in western Cambodia and then spread to the rest of the world(White, 2010). Parasite populations in endemic areas of SSA differ both genotypically and phenotypically in terms of virulence, drug resistance and transmissibility (Tanner *et al.*, 1999).

Studies have shown that the number of genotypes per person directly correlates with the transmission intensity (Iwagami *et al.*, 2009; Vafa *et al.*, 2008). Genome-wide association studies (GWAS) are carried out to determine the presence of genetic markers influencing a phenotypic trait throughout the parasite genome. This can also be used to identify genomic region(s) or parasite gene(s) that is responsible for causing resistance to anti-malarial drugs (Su *et al.*, 2007). The discovery that none of the previously described important K13-propeller mutations associated with artemisinin resistance found in SEA exist in African parasite populations (Ashley *et al.*, 2014; Conrad *et al.*, 2014; Kamau *et al.*, 2014) was insightful. However, it remains unknown whether parasite genetics can provide a basis for discovering

molecular markers for artemisinin resistance in *P. falciparum* from SSA given the parasite genetic plasticity. To address this question, we analyzed the genetics and population structure of parasite from western Kenya, a region of high malaria transmission. Samples used in this study were collected before (pre) and after (post) the introduction of artemether-lumefantrine (AL) as the first-line malaria treatment in Kenya. The post-ACT parasites were from an efficacy trial where parasite clearance rates were obtained.

1.2 Artemisinin resistance

Artemisinin combination therapies (ACTs) remain the mainstay treatment of uncomplicated malaria in most malaria endemic regions. Artemisinin resistance in Southeast Asia (SEA) has been confirmed (World Health Organization, 2010) and it is characterized by slow parasite clearance rates (Dondorp *et al.*, 2009; Noedl *et al.*, 2008). There is evidence of slow parasite clearance to ACTs in Southeast Asia, which is an indication that these parasites are developing resistance (Dondorp *et al.*, 2009; Dondorp *et al.*, 2010; Noedl *et al.*, 2008; Noedl *et al.*, 2009). Artemisinin, which is a fast acting drug, has a high parasite clearance rate with the sensitive *P. falciparum* which clears within 2 days(White, 2008). Resistance to artemisinin has now been shown to have spread to other neighbouring regions of Cambodia such as Thailand-Myanmar (Burma) border (Phyo *et al.*, 2012). However, it is important to note that these are slow clearance rates and not high grade resistance. A recent report showed that there was declined responsiveness of *P. falciparum* infections to ACTs in the Kenyan coast (Borrmann *et al.*, 2011). The parasite genotypes play an important part influencing parasite clearance half-life leading to drug resistance. According to artemisinin resistant parasites from western Cambodia, it was noted that parasites

having similar parasite clearance rate are infected with genetically indistinguishable parasite clones (Phyo *et al*., 2012). Therefore, parasite relatedness is an important factor that will determine the influence of parasite genetics on clearance rates. The correlation between parasite genotypes and slow clearance rates has shown high heritability (Anderson *et al*., 2010). In this regard, mapping the genes underlying resistance trait using genome wide association studies (GWAS) is warranted (Su *et al.*, 2007).

1.3 Therapeutic efficacy studies

Anti-malarial drug efficacy is more often tested to monitor clinical and parasitological failure after treatment. This is made possible by carrying out therapeutic test in accordance to a standardized efficacy protocol (World Health Organization, 2009). This is a gold standard for determining anti-malarial drug efficacy whose results are primary in making treatment policy decisions. Usually, these studies are complemented by molecular tests that distinguish cases of recrudescence from re-infection (Snounou *et al.*, 1998; World Health Organization, 2003).

Anti-malarial drugs are used for both prophylaxis and treatment. Some of the used drugs known for malaria treatment in years past include chloroquine, quinine, sulfadoxine/ pyrimethamine (SP), amodiaquine, mefloquine and recently artemisinin combination therapy (ACTs). Thus far, malarial parasites have developed resistance to all known classes of anti-malarial drugs (World Health Organization, 2012) . The emergence and spread of resistance to anti-malarial drugs began in the late 1950s with chloroquine/quinine which became widespread in 1970s and 1980s. Later, it was followed by SP after these drugs were deployed as the first-line treatments in many parts of the world. Interestingly, there are a few countries in Southeast Asia including

western Cambodia and other Mekong sub-regions such as Thailand, Myanmar and Viet Nam which are considered to be the epicentre of drug resistance (Wernsdorfer, 1994; White, 2010). It is in this region where resistance to anti-malarial drugs have been shown to originate and spread to other countries including those in the African continent (Bijl *et al.*, 2000).

It is not clear whether high or low transmission areas record a rapid spread of drug resistance (Klein *et al.*, 2008). However, some epidemiological studies have implied that the emergence and spread of resistance is more likely to occur in low transmission areas (Roper *et al.*, 2004). Due to symptomatic malaria infections, low transmission areas are prone to high drug usage which probably provides more opportunities for selection (World Health Organization, 2010). Drug resistance tends to result from mutations in the parasite genes. For example, mutations in the parasite genes that encode Dihydrofolate reductase (*DHFR*) and Dihydropteroate synthase (*DHPS*) have since been associated with resistance to Sulfadoxine-Pyrimethamine (Hastings *et al.*, 2000). Other markers that are responsible for resistance include *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*) for chloroquine (CQ) and *P. falciparum* multi-drug resistance 1 (*Pfmdr-1*) for CQ as well as other anti-malarial drugs (Okiro *et al.*, 2010). The level of drug use varies between populations living in areas of different transmission intensities. For instance, symptomatic disease in low transmission intensities leads to increased drug use unlike high- transmission areas where drug pressure is low. Therefore, the probability of parasites coming into contact with the drug might be higher in low transmission areas than in high transmission areas. This leads to increase in the chances of selecting for resistant parasites in low transmission areas (Hastings *et al.*, 2000).

The emergence of resistance against virtually any anti-malarial drug has consistently hampered efforts to control and eradicate malaria. This resistance is due to genetic diversity, which more often are involved in parasite virulence, immune responses and generation of single nucleotide polymorphisms (SNPs). Such variants are under strong selective pressure (Meyer *et al.*, 2002). Assessing resistance by carrying out *in vivo* and *in vitro* assays which measure the therapeutic and parasitological response after treatment, is therefore important. (World Health Organization, 2009). Basically, *in vivo* studies administer anti-malarial drugs to malaria patients where drug response is evaluated. In other cases, the follow up period can be prolonged which is critical for detecting low grade resistance or recurrent parasitemia that might persist (Stepniewska *et al.*, 2004).

This study aimed to use genetic markers as a basis for establishing the role parasite genetics play in influencing parasite clearance rates. This was achieved by assessing whether genetically determined artemisinin resistance in *P. falciparum* is emerging in western Kenya as observed in Southeast Asia.

1.4 Problem statement

Recently, artemisinin resistance has been confirmed in western Cambodia and has spread to the western border of Thailand (Cheeseman *et al.*, 2012; Phyo *et al.*, 2012). The anti-malarial drug resistance emergence consistently hampers efforts to control and eradicate malaria. There are concerns that the same resistant parasites have emerged or spread to Africa like in the case of sulphadoxine/pyrimethamine. This resistance is due to recombination events leading to genetic diversity that more often than not, is involved in parasite virulence & host immune responses and often results

in generation of single nucleotide polymorphisms (SNPs) in the parasite's genome. Such parasites genetic variants are under strong selective pressure (Meyer et al., 2002). Artemisinin resistance is characterized by slow parasite clearance where resistant parasites have a long parasite clearance half-life. Clearance rate is a measure used to assess parasite drug response. Furthermore, studies from western Cambodia have shown that patients with similar parasite clearance rates are infected with parasite clones that are genetically similar (Phyo *et al.*, 2012). This is an indication that parasite genetics are critical in determining the clearance rate phenotypic trait (Anderson *et al*., 2010). In general, few studies have investigated the role of parasite genetics on artemisinin resistance in Africa. Thus, in a recent study, a decline in responsiveness of *P. falciparum* to ACTs in Kenyan coast was observed (Borrmann *et al.*, 2011). However, the role of parasite genetics in relation to clearance rates has not been investigated in Kenya. Furthermore, the molecular markers responsible for delayed parasite clearance rate have not been identified (Phyo *et al.*, 2012) as well as no phenotypic assays which can reliably identify artemisinin-resistant parasites (Dondorp *et al*., 2009).

1.5 Justification

In Kenya, ACTs are the first-line treatment for uncomplicated malaria caused by *P. falciparum*, although resistance to this drug has not been identified. However, due to the emergence of resistance in SEA, and the recent report of declined responsiveness of *P. falciparum* infections to ACTs at the Kenyan coast (Borrmann *et al.*, 2011), the need for monitoring ACT resistance in Western Kenya, a high transmission zone, cannot be over emphasized. Thus, stakeholders are putting up efforts to conduct studies to track possible emergence and spread of artemisinin resistance. This study

formed an integral part to these efforts of monitoring AL efficacy and indeed possible emergence of resistance to this drug combination. Furthermore, there are currently no laboratory molecular assays that reliably identify artemisinin-resistant parasites here in Africa (Dondorp *et al.,* 2009), although K13 propeller markers are available to identify artemisinin-resistant markers in Southeast Asia (Ariey *et al.,* 2014). This could be due to genetic difference of the African parasites. It was important to investigate the role parasite genetics plays in influencing slow clearance rates (Anderson *et al.*, 2010) and possibly identify regions within the parasite genome that may become markers here in Africa for artemisinin resistance. In order to investigate these potential resistance marker(s), a genome-wide analysis on *P. falciparum* genome is important because these parasites exhibit a vast exchange of genes (gene flow) between disparate parasite populations (Takala-Harrison *et al.,* 2013). This study may provide hitherto unidentified genetic marker(s) that could potentially be used for ACTs resistance surveillance. It has been indicated that resistant genes within parasite population increases heritability due to genetic determinants that tend to influence this trait (Phyo *et al*., 2012). In this case, patients having similar parasite clearance half-lives carry same parasite genotypes. This has been supported by *in-silico* experiments that showed high heritability within parasite genotypes with slow parasite clearance (Phyo *et al.*, 2012). Information gained from this study may be useful in surveillance of artemisinin resistance. The findings are critical in advising malaria control and elimination strategies. For instance, use of genetic markers as novel drug-resistance targets that can be used in development of new anti-malarial drugs monitoring tools. In terms of study site, Lake Victoria basin of Kenya, a unique

region that is classified as a holo-endemic was studied. This is a malaria zone with asymptomatic malaria rate of 50% (Zhou *et al.,* 2011).

1.6 Research questions

1. Can parasite clearance rate be determined in patients administered AL in western Kenya
2. Do genetically determined multiple-clone infections in *P. falciparum* parasites having similar parasite clearance rate exist in western Kenya?
3. Can the same SNP marker(s) as those used in Southeast Asia and MS markers be applicable in detecting delayed parasite clearance rate and diversity respectively in Kenya?
4. Is there any correlation between parasite genotypes and parasite clearance rates?
5. Are parasites with similar clearance rates related genetically?

1.7 Study hypotheses

1.7.1 Null hypothesis:

P. falciparum parasite genetics does not influence parasite clearance rates amongst parasites circulating in Kisumu County, western Kenya.

1.7.2 Alternative hypothesis:

P. falciparum parasite genetics does influence parasite clearance rates amongst parasites circulating in Kisumu County, western Kenya.

1.8 Study Objectives

1.8.1 General objective

To determine the role that population genetics play in *Plasmodium falciparum* parasites exposed to artemisinin treatment amongst patients taking part in an efficacy clinical trial and archived isolates in Kisumu County, western Kenya.

1.8.2 Specific objectives

1. To determine parasite clearance rates using parasite clearance estimator tool.
2. To establish existence of multiple-clone infections of *P. falciparum* parasite in study subjects by using the neutral microsatellite assay.
3. To evaluate genetic polymorphism and diversity across *P. falciparum* parasite genome using SNPs and MS markers respectively.
4. To correlate parasite genotypes with clearance rates after treatment of subjects with artemisinin-based drugs using statistical methods.
5. To determine parasite genetic relatedness using phylogenetic analysis by using Bayesian methods

CHAPTER TWO

LITERATURE REVIEW

2.1 The Life cycle of *P. falciparum* parasite

Malaria is caused by members of the genus *Plasmodium*. There are four distinct species that infect humans (*P. falciparum, P. vivax, P. malariae, P. ovale)* and another species *P. knowlesi* which causes malaria in monkeys and mostly affects the forested areas of Southeast Asia (World Health Organization, 2012). *P. falciparum* has been reported as the most virulent human parasite causing severe malarial anemia and cerebral malaria; the two major diseases are associated with mortality (Perkins *et al.,* 2011; World Health Organization, 2008). The parasites are transmitted through the bites of female Anopheles mosquitoes (approximately 40 species) which are known to transmit malaria.

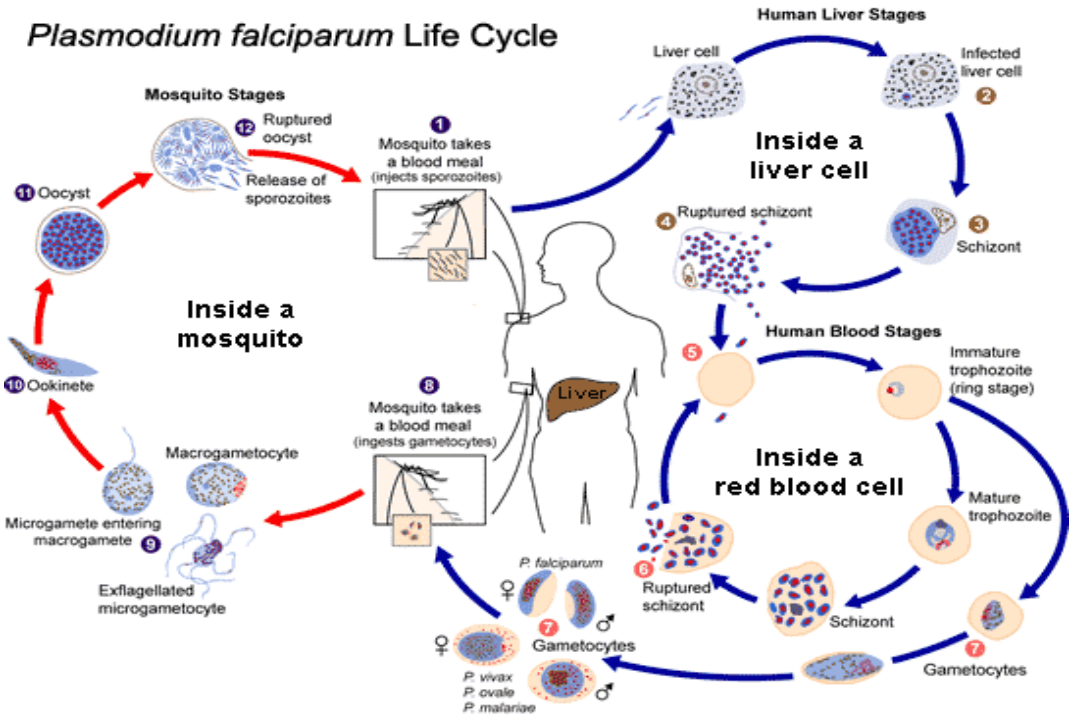


Figure 2.1: The life cycle of *P. falciparum* parasite

The cycle outlines important process in every parasite stage. This image is a public domain material taken from CDC website (https://www.cdc.gov/dpdx/malaria/).

The life cycle of *P. falciparum* comprises of three stages including:

- Pre-erythrocytic stage (which initiates infection)
- Asexual erythrocytic stage (which causes disease)
- Gametocyte stage (Infects mosquitoes that transmit parasite)

2.1.1 Pre-erythrocytic stage

In this stage, the *P. falciparum* sporozoites from an infected mosquito are released from the salivary gland of the mosquito into the human host during a mosquito blood meal. The sporozoites enter the blood stream and invade the liver cells (hepatocytes)

becoming multinucleated hepatic schizonts. The circumsporozoite protein (CS), the principal protein on the surface of sporozoites, binds to basolateral domain of the hepatocytes, and then other proteins, including sporozoite surface protein (SSP2), initiate hepatocyte invasion. The sporozoites then produce thousands of merozoites (~40,000) (Crutcher *et al.*, 1996) that are released out of the liver cells into the bloodstream (Haldar *et al.*, 2009).

2.1.2 Asexual erythrocytic stage

In this stage, once the hepatic schizont matures, it ruptures to release merozoites which invade the red blood cells (erythrocytes), multiply and rupture out of the red blood cells in a period of two days/ 48 hours for *P. falciparum.* New merozoites released invade the new red blood cells and the cycle starts again. When the parasitemia reaches a sufficient density following the high number of cycles of multiplication in the blood, the infected person develops periodic fevers, chills and other symptoms of malaria. This is the interval between infection and appearance of first clinical signs which takes about 8-15 days, duration specific to *P. falciparum* species described by (Sinden *et al.*, 2002).

2.1.3. Sporogonic stage

After several erythrocytic schizogony cycles (8-15 days for *P. falciparum*), some merozoites differentiate into sexual forms (male or female gametocytes). During a blood meal by a female *Anopheline* mosquito, the sexual forms are taken up into the mosquito's gut. Following ambient temperatures in the gut, the male gamete fertilizes the female gamete resulting to a zygote that develops into a mobile ookinete after few hours. The ookinete then goes to the stomach wall of the mosquito, grows and forms

an oocyst and after 10-14 days, the oocyst undergoes asexual multiplication, matures and burst into thousands of sporozoites which move to the mosquito's salivary glands described by (Sinden *et al.*, 2002). The parasite is poised to begin the cycle again with the mosquito's next blood meal.

2.2 Population Diversity

This revolves around study of population genetics. Population genetics entails study of distribution and change of allele frequencies. The change is influenced by evolutionary forces such as genetic drift, mutation, natural selection and migration.

Natural selection: Change of allele frequencies occurring due to differential effects of alleles on 'fitness'.

Genetic drift: Random change of allele frequencies over time among population

Mutation: this is the net change occurring in one direction where alleles are inter-converted at some rate

Migration: This also means gene flow, which is the net movement of alleles between populations occurring at some rate. This tends to introduce new allele or change the frequencies of the existing alleles.

Genetic diversity, also known as heterozygosity is measured using predominant allele at each locus to calculate the expected heterozygosity. The expected heterozygosity ranges between 0 and 1, value close to 1 indicates high genetic diversity levels in the population and 0 vice-versa.

2.3. *Plasmodium* diversity

Plasmodium falciparum parasite, a causative agent of uncomplicated malaria, is considered a successful pathogen due to its ability to generate mutants (Tuteja, 2007). The malaria parasite has 14 chromosomes numbering in ascending order of size approximately 6000-7000 genes in its genome (Gardner *et al.,* 2002). Parasite populations in endemic areas of Africa differ both genotypically and phenotypically in terms of virulence, drug resistance and transmissibility (Tanner *et al.,* 1999). Study findings show that the number of genotypes per person directly correlates with the transmission intensity (Iwagami *et al.,* 2009; Vafa *et al.,* 2008). Eventually, multiplicity of infection affects disease severity, thus worsening malaria control measures (Farnert *et al.*, 2008; Kobbe *et al.*, 2006).

Parasite population structure varies with malaria transmission intensity. Different transmission areas show dissimilar parasite population due to factors such as inbreeding. For example, low levels of parasite recombinations are mostly seen in low transmission areas and have been attributed to inbreeding which increases population structure unlike in high transmission areas (Anderson *et al.*, 2000; Dye *et al.*, 1997).

2.4. Multiplicity of Infection

In studying the parasite genetic diversity, *P. falciparum* results in complex infections due to the presence of multiple parasite clones. These mostly occur in endemic areas due to super-infection which lead to accumulation of different parasite genotypes within an infected individual (Conway *et al.,* 2001). Microsatellites, or simple sequence repeats have been used mostly to determine population structure and they have been shown to be good candidate markers in genotyping polyclonal *P. falciparum* infections (Anderson *et al.*, 2000; Greenhouse *et al.*, 2006; Su *et al.,*

1996). Since microsatellites are usually not under immune selection, their analyses are accurate methods for use especially in high transmission intensity where polyclonal infections are common (Slater *et al.,* 2005).

An effective means of studying population is by analyzing allelic variation at multiple independent loci (Imwong *et al.*, 2007). Microsatellites have been estimated to mutate at high rates. Their mode of mutation has been studied and documented to be due to strand slippage during replication (Dieringer *et al.*, 2003). Strand slippage leads to either shortening or lengthening of the DNA in genomic areas encoding these microsatellites. Thus variations in microsatellite lengths are assumed to engender new electrophoretically distinguishable alleles.

Misclassifications of genotypes can lead to underestimated levels of drug resistance. However, measuring the microsatellite product sizes using capillary electrophoresis (CE) allows for high resolution at the level of single nucleotide making the results highly reproducible. Therefore, this approach makes it possible to measure full diversity at any given loci (Greenhouse *et al.*, 2006). Multiple-clone infections (MIs) are defined as those which show more than two alleles in one or more of twelve microsatellite loci.

2.5 Heritability of *P. falciparum* parasite

Measuring the degree of parasite relatedness requires a quantitative genetic analysis that is performed by measuring heritability (H^2) (Hill *et al.*, 2004). Parasite heritability plays an important role in determining the parasite genetics/relatedness that influences drug resistance traits. Parasite relatedness is analysed at diploid stage of *Plasmodium* species (Blouin *et al.*, 1996). H^2 is dependent on factors such as existence of clonally identical parasites brought about by inbreeding and low

transmission intensities. It is easier to study heritability of parasite genotypes from low transmission areas unlike parasites from high transmission areas due to presence of single clones. Therefore, estimating H^2 of clonally identical parasites in high transmission areas can be accomplished by determining the overall parasite relatedness of the population at a particular time. Longitudinal studies such as this enables tracking of parasites to provide a better insight into the roles of factors such as immunity and drug pressure in infection dynamics (Franks *et al.*, 2001). To assess H^2, variance of parasite clearance are compared within and between clonally identical parasites recovered from more than two patients.

2.6 Artemisinin and its derivatives

Artemisinin is obtained from the *Artemisia annua* plant. Unlike other anti-malarials, this drug targets and kills various developmental stages of the plasmodia parasites (White, 1997). Characteristically, artemisinin has a poor bioavailability on its own. Therefore, it is generally used in the semi-synthetic derivative forms such as artesunate, artemether, arteether that are converted by the body to the active agent dihydroartemisinin (DHA). DHA attacks the asexual stages of parasites. Artemisinin and its derivatives that have rapid action are combined with long acting partner drugs. It also has the ability to eliminate sexual and asexual stages of *falciparum* malaria. Artemisinin is important in reducing parasitemia and transmission intensities Artemisinin which has a short half-life is used in combination with other drugs so that while it clears the parasites at the start of treatment, the second [partner] with long half-life clears the remaining parasites to avoid recrudescence states (White, 2004). Some of the commonly used partner drugs in the ACTs regimen include mefloquine, lumefantrine, piperaquine, amodiaquine, sulfadoxine/pyrimethamine and

pyronaridine. These were introduced and have been in use for more than 25 years in Southeast Asia and since 2000 in Cambodia (Anderson *et al.*, 2010). In Kenya, ACTs were recommended as first-line treatment for uncomplicated *P. falciparum* malaria in April 2004 by Kenyan ministry of Health and eventually rolled out as from July 2006 (Amin *et al.*, 2007). Some of the commonly used ACTs include Artemether-Lumefantrine (Coartem), Artesunate-Amodiaquine (Coarsucam), Artesunate-Mefloquine (Artequin) and Artesunate-Sulphadoxine/Pyrimethamine (Malosunate) (World Health Organization, 2006).

2.6.1 Mode of action of Artemisinin

The mode of action of artemisinin is uncertain. However, it is suspected that artemisinin acts on malaria parasite by perturbing redox homeostasis (Ginsburg *et al.*, 1994). The peroxide in the 1, 2, 4-trixione system has been known to contain the anti-malarial activity with the help of reactive oxygen species. When a parasitized red blood cell consumes hemoglobin, it generates oxidative stress. The heme produced directly reduces the peroxide bond in artemisinin (Meshnick, 2002). This leads to production of reactive oxygen radicals generated by high-valent iron-oxo species. These radicals then damage the parasite causing its death (Cumming *et al.*, 1997). Artemisinin is also thought to inhibit sarco-endoplasmic reticulum calcium-ATPase (SERCA)-type known as PfATPase 6 protein (Shandilya *et al.*, 2013; Woodrow *et al.*, 2006) although not validated as a target (Valderramos *et al.*, 2010).

2.7 Genome-wide association studies for analysis of parasite genetic diversity

Genome-wide association studies (GWAS) is a unique analysis tool used to determine the presence of genetic markers throughout the parasite genome. GWAS identify the genomic regions or parasite genes that are responsible for causing resistance to anti-malarial drugs (Su *et al.*, 2007). These studies distinguish the resistant gene distributed across the *P. falciparum* genome. In this case, parasite genotypes in different patients have been reported to have the same clearance half-lives and times (Takala-Harrison *et al.*, 2013). Using this tool, 91-locus genotypes with variability in African samples and that generate robust genotype data can be assessed in patients showing delayed parasite clearance These gives a broad coverage of SNPs so as to monitor artemisinin resistance closely (Takala-Harrison *et al.*, 2013). In addition to that, high heritability (H^2) has been associated with slow CRs therefore GWAS helps to show the role that parasite genotypes play on parasite clearance half-life. This generates more information on mechanism of resistance and validates markers for ACTs resistance surveillance (Anderson *et al.*, 2010).

2.8 SNP Analysis by Mass Array Technique

2.8.1 Genotyping of single infections using 91 SNPs - Agena MassArray platform

Genotyping of 91 SNPs distributed across all the 14 chromosomes of *P. falciparum* genome. (Gabriel *et al.*, 2009). The SNPs are representative from PLASMODB v.6.2 database that maximizes variability in the African *P. falciparum* samples. In these kinds of studies, the effect of parasite genotype on parasite clearance rates is assessed by correlating the 91-locus genotypes to parasite clearance half-lives.

2.8.2 Principle of SNP-Agena Mass Array

Agena SNP genotyping is based on primer-extension reactions that generate allele-specific products with distinct masses. These are then detected by MALDI-TOF (Matrix- Assisted Laser Desorption/ Ionization, Time of Flight) mass spectrometry. The assay entails initial locus-specific PCR reaction followed by single base extension with the use of mass-modified dideoxynucleotide terminator oligonucleotides. In this assay, the genotyping primer then anneals immediately upstream of the polymorphic site of interest (Gabriel *et al.,* 2009). The amplicon is mixed with matrix that absorbs UV light (laser) converting it to heat energy causing vaporization of both the sample and matrix. Charged ions generated then migrate in an electric field according to mass-to- charge ratio (m/z) values of ion until they reach the detector. Highly charged ions (small m/ z value) move faster than low charged ions. The MALDI-TOF mass spectrometry is capable of identifying the SNP allele based on distinct mass of extended primer. This assay is robust and amenable to high throughput because it utilizes a 384-well plate format (Large number of samples several SNPs analyzed at the same time).

2.9 In vivo Parasite clearance analysis

Parasite clearance time is a useful measure in assessing anti-malarial drug effect and is applicable for assessing artemisinin resistance. The World Wide Anti-malarial Resistance Network (WWARN) website (www.wwarn.org) has been instrumental in defining optimum methods and threshold values for parasite clearance times (White, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

Out of a total of 118 post-ACTs samples, 92 were sampled for use in this nested study. The patients were randomized to treatment; first arm (utilizing Artemether Lumefantrine (AL) was designed to have 9 scheduled days of drug administration while the other arm (utilizing Artesunate Mefloquine-ASMQ) had 11 scheduled subject contacts. Additionally, I used archived isolates (pre-ACTs) from the main study protocols (KEMRI-SSC 1330/WRAIR #1384; KEMRI-SSC 2518/WRAIR #1935) and adopted a retrospective longitudinal study design looking at parasite genetics before and after ACT introduction in Kenya.

3.2 Study site

This study was carried out at Malaria Drug Resistance Laboratory of the US Army Medical Research Directorate-Kenya in Kisumu County.

3.3 Study population

In this study, archived pre-ACTs isolates (n = 29) collected between 1995 and 2003 from Kisumu County, western Kenya were analyzed. These are parasites were collected at a time period before AL was adopted as the first-line treatment for uncomplicated malaria in Kenya. The post-ACTs isolates (n = 92) were collected from an *in vivo* efficacy study conducted between 2013 and 2014 in Kombewa district hospital in Kisumu **C**ounty, western Kenya.

3.3.1 Inclusion criteria

These two studies enrolled individuals irrespective of gender, aged between 6 months and 65 years presenting with uncomplicated malaria. On enrollment, the patients' demographics, (including age and gender), place of birth, place of residence, occupation, travel and malaria sickness/treatment history in the previous 2 months prior to participation in the study were recorded. Eligible criteria included: measured temperature of ≥ 37.5°C, or history of fever within 24 hours prior to presentation and mono-infection with *P. falciparum*. In addition, the study subjects had a baseline parasitemia of 200-200,000 asexual parasites/µl. The presence of malaria parasites was confirmed by microscopy and rapid diagnostic test (RDT; Parascreen®, Zephyr Biomedicals, Verna Goa, India). Whole blood sample of 2-3 ml was collected and kept in either Acid Citrate Dextrose Solution (ACD) or Ethylenediaminetetraacetic acid (EDTA) vacutainer tubes. The sample was then transported to the central laboratory within 4 to 6 hours and aliquots processed and cryopreserved in cryovials under liquid nitrogen tanks for long-term storage as previously described (Vaught, 2006). After blood draws, appropriate treatment was initiated based on policy recommended regimens available during the study period. Additionally, patients enrolled in the efficacy trial (KEMRI-SSC 2518/WRAIR #1935) were admitted at the study facility for approximately 3 days. Monitoring of parasite clearance was carried out microscopically after every 6 hours. For molecular analyses performed under this study, the archived samples were thawed prior to DNA extraction.

3.3.2 Exclusion criteria

Those who presented with signs of severe anemia, mixed Plasmodium infections or mono-infection of non-falciparum Plasmodium were excluded. Additionally, those with history of allergy or contraindications to the study treatment were excluded.

3.4 Ethical considerations

Use of pre-ACTs and post-ACTs (whole blood, FTA cards) for from voluntary study subjects was approved by the scientific steering and Ethical Review Committee of the Kenya Medical Research Institute (KEMRI), Nairobi, Kenya and the Walter Reed Army Institute of Research (WRAIR) Institutional Review Board, Silver Spring, MD. Two study protocols were used with numbers; KEMRI-SSC 1330/WRAIR #1384 for pre-ACT samples and KEMRI-SSC 2518/WRAIR #1935 for post- ACT samples. Eligible study subjects were open guided through the informed consent form after which those who consented were enrolled into the study.

3.5 Sample size determination

For molecular assays, sample size was calculated using the Raosoft® sample size calculator formula (Raosoft) taking into cosideration the incidence of malaria reduced to 15% as reported by Kenya malaria indicator survey 2010.

The sample size *n* and margin of error *E* are given by:

$$x = Z(^{c}/_{100})^{2} r\,(100-r)$$

$$E = \text{Sqrt}\left[\frac{(N-n)\,x}{n\,(N-1)}\right] = 4.74$$

Confidence level at 95%

$$n = \frac{N\,x}{((N-1)\,E2 + x)} = \mathbf{91}$$

where *N* is the populations size, *r* is the fraction of responses that you are interested in, and $Z\left(^{c}/_{100}\right)$ is the critical value for the confidence level *c*.

This will also give power to the significance of the study. This was achieved for post-ACTs isolates that will be determining the main objective of the study. However, as for pre-ACT isolates that had small sample size, calculation of unbiased expected heterozygosity (uH_E) was calculated to enable comparative studies.

3.5 Parasite clearance rates calculation

Parasite clearance rates were calculated using Worldwide Antimalarial Resistance Network (WWARN) Parasite Clearance Estimator (PCE) online tool located at http://www.wwarn.org/toolkit/data-management/parasite-clearance-estimator.

Log transformed parasite density was plotted against time in hours which was generally linear (Flegg *et al.*, 2011). This generated a slope of half-life representing the time taken for parasitemia to be reduced by half. This constant is independent of starting value of parasitemia.

The half-lives were calculated as follows:

$$T\ {}^{1}/_{2} = \log e\ (2)/k$$

$$= \frac{0.692}{\mathrm{k}}, \text{where k is the clearance rate constant}$$

3.6 Laboratory Analysis

3.6.1 Sample archive

All samples were stored in the USAMRD-K laboratories for further molecular analysis.

3.6.2 Extraction of DNA from *P. falciparum* parasite

DNA was extracted from whole blood using QIAamp DNA mini kit (Qiagen Benelux, Venlo, The Netherlands) according to the manufacturers' instruction. Briefly, QIAGEN protease (20 µl) was added into an eppendorf tube for protein digestion. Lysis buffer (200 µl) was added and mixed well. The mixture was spun and incubated on a hotplate for 10 minutes at 56^{o}C. Ethanol (200 µl) was added onto de-proteinized DNA on QIAamp spin column for purification. This was achieved by centrifugation for one minute at 8000 g in a microcentrifuge (Centrifuge 5804 R, Becton Dickinson, New Jersey, United States) and the tube containing filtrate was discarded. The spin column was then transferred into another 1.5 ml collection tube and 500 µl of wash buffer AWI added and centrifuged at 8000 g for 1 minute and the filtrate discarded. Wash Buffer AW2 (500 µl) was then added into the spin column and the preparation centrifuged for 3 minutes at 14,000 g. Old collection tube with the filtrate was then discarded followed by a dry spin (8000 g for 1 minute). The purified DNA was eluted from the spin column by addition of 200 µl elution buffer AE centrifuged at 8000 g and stored at -20^{o}C until used for PCR.

DNA was extracted from dried blood spots (three 3 mm diameter punches) using QIAamp DNA mini kit (Qiagen Benelux, Venlo, The Netherlands) according to the manufacturers' instruction. The extraction procedure is similar to extraction of DNA

from whole blood as discussed above. However, there is an added step tissue lysis using buffer ATL as dried blood spots are considered as hard tissue.

3.6.3 *Plasmodium falciparum* Genotyping

3.6.3.1 Genotyping of multiple *falciparum* infections using Twelve Unlinked Microsatellite markers

Microsatellite genotyping at one or more loci was used to identify infections containing >1 parasite clone/multiple-clone infections (MI). In this study, I analyzed the genetic diversity and population structure of 75 *P. falciparum* pre-treatment and 17 post-treatment patient isolates collected in Kombewa district hospital in Kisumu county (2013-2014) and 29 isolates from Kisumu district hospital in Kisumu county (pre-2003) using twelve polymorphic microsatellite markers bearing tri-nucleotide repeats located on various chromosomes of the parasite (Anderson *et al.*, 1999) as seen in Appendix I. The post-ACT isolates therefore totals to 92 samples used in genetic analysis. The labeled primers included: Poly α (Chr4), TA42 (Chr5), TA81 (Chr5), TA1 (Chr6), TA109 (Chr6), TA87 (6), TA40 (Chr10), 2490 (Chr10), ARAII (Chr11), PFG377 (Chr12), PFPK2 (Chr12), and TA60 (Chr13). Four loci (Poly α, ARAII, pfG377, and PfPk2) are located in open reading frames (ORFs) found in coding sequences. The other eight loci were drawn from a genomic library (Su *et al.*, 1996), and their functions are unknown. MIs were defined as those in which one or more of the twelve loci show multiple alleles. Allele scoring was determined for each parasite clone. The allele having the highest electropherogram peak among all the alleles was considered as the predominant allele. These were used in scoring the MI within individual study patients.

Hemi-nested PCR: For each microsatellite locus, the first and second primers were used in the primary reaction and the second and third primers in the secondary reaction. Primers listed third are internal to the other two and are end-labeled as described by Anderson *et al.*, 1999. This is shown in Appendix I.

Primary PCR reaction: The markers were PCR-amplified using oligonucleotide primers with unlabeled forward/reverse primers. The PCRs with all primer pairs (0.1uM working solution of each) were performed on a Thermocycler (ABI 9700 system) with 1µl of genomic DNA template, 1 Unit of ABI *Taq* polymerase, 1.2 µl of 25 mM $MgCl_2$, 1µL of 10× PCR Buffer, and 0.2 µL of 10 mM dNTPs (all supplied by Applied Biosystems) in a final reaction volume of 10µL. Cycling condition were same for all primer pairs: an initial denaturation at 94°C for 2 minutes followed by 25 cycles of denaturation at 94°C for 30 seconds, primer annealing 42°C for 30 seconds, 40°C for 30 seconds and strand extension at 65°C for 40 seconds. This was followed by a final incubation at 65°C for 2 minutes for recessed ends filling. In this assay, the PCR reactions were multiplexed using 3 primers sets (six primers in total) and were set as follows:

Multiplex 1: Polyα; PFPK2; and TA81

Multiplex 2: ARA2; TA87; and TA40

Multiplex 3: TA42; 2490; and TA1

Multiplex 4: TA60; TA109; and PFG377

The multiplex reactions in this primary PCR are enabled by the fact that the three different markers in these mixtures have flourescent labels (HEX, FAM and PET) with different absorbance and emission spectra to allow discrimination / distinction between the markers based on fluorescence.

Controls (3D7, HB3, NTC-Negative template control) were used to enable correct amplicon sizing which is dependent on downstream analyses. An NTC control was run because downstream analyses require an evaluation of background flourescence in these controls. Thus any cross contamination during PCR set-up were detected. The PCR amplicons were then stored at 4^{o}C before proceeding to secondary PCR or stored at -20^{o}C for later use.

Secondary PCR reaction: The primary PCR amplicons were used as the templates for secondary PCR. Forward and reverse primers labelled with fluorescent dyes (6-FAM, HEX, and PET) were used.The markers were run individually for each marker such that each of the four multiplexed PCR reactions produced three (3) separate PCR reaction. The reagents used to prepare master mix include : all primer pairs (10uM working solution of each), 1 Unit of ABI *Taq* polymerase, 1.5 µl of 25 mM $MgCl_2$, 1 µL of 10× PCR Buffer, and 0.3 µL of 10 mM dNTPs (all supplied by Applied Biosystems) in a final reaction volume of 15 µL. This was performed on a Thermocycler (ABI 9700 system) with 1 µl of primary amplicon as the DNA template. Cycling conditions used for this reaction was: an initial denaturation at 94°C for 2 minutes followed by 25 cycles of denaturation at 94°C for 20 seconds, primer annealing at 45°C for 30 seconds, and strand extension at 65°C for 30 seconds; and a final incubation at 65°C for 2 minutes. . Length variation of labeled PCR products

was measured on an ABI PRISM 3130/3500 Genetic analyzer (Applied Biosystems, Foster City, CA) by using Genescan GS500/600LIZ Internal size standard and analysis performed using GeneMapper software (version 4.0/4.1).

3.6.3.2 Gel electrophoresis to confirm PCR amplification

The PCR amplicons were run on ethidium bromide-stained 1.5% agarose gel prepared by mixing 2.25g of agarose powder and 150 ml 1× TAE buffer and boiling to dissolve the agarose. The agarose gel was cast pouring the solution into appropriate tanks, inserting the combs and allowing the solution to gel after adding ethidium bromide (5 µl).A 10 µl PCR product was then mixed uniformly with 2 µl of 6× loading dye (bromophenol blue) and loaded into the wells. A 5 µl PCR ladder marker (QIAGEN) was loaded into the first well and a mixture of PCR products and loading dye in the rest of the wells. The gel was installed into the gel running tank (C.B.S/scientific) filled with TBE buffer. A voltage of 90 V was set for ~30 minutes for the amplicons to evenly separate according to their molecular masses relative to their DNA size markers in the first well. Using a 312 nm UV trans-illuminator, the gel was visualized under ultraviolet light and a gel photographed using a gel documentation system (UVITEC Cambridge/MITSUBISHI Electric).

3.6.3.3 Fragment analyses of PCR amplicons by Capillary electrophoresis (CE)

For high resolution size calling, flourescent-labelled primers were used in the secondary PCR and the amplicons loaded on an ABI automated sequencer (3130 Genetic analyzer). CE can be multiplexed using different fluorescent labelled primers and are amenable to high throughput analyses or can be individually run. A mix which incorporates 9.0 µl HiDi (highly deionized) formamide and 0.5 µl internal size

standard (GeneScan LIZ500) per reaction was prepared and loaded together with 1ul of secondary amplicon on a 96 well plate. The samples were then denatured at 95°C for 5 minutes and chilled on ice to preserve the DNA fragments as single strands prior to loading on the sequencer. The size standard used represented smaller fragments less than 500 base pairs. The capillary electrophoresis system used Performance optimized polymer (POP-7) for resolution and fragment analysis purpose.

The CE output was exported to GeneMapper software version 4.0 (Applied Biosystems, USA) for fragment analysis. This generated estimates of DNA fragments relative to the size standards. DNA fragment size calculated in base pairs and peak heights (calculated in Relative Fluorescence Units - RFU) were displayed. This data collection software facilitated proper data integrity. GeneMapper software v4.0/4.1 used microsatellite analysis parameters to quantify samples containing multiple alleles per locus. Multiple allele per locus were scored if electrophoretic peaks corresponding to minor alleles were ≥33% the height of that for the predominant allele in the isolate. This ensures all stutter peaks are eliminated.

3.6.4 Scoring of microsatellite allelic length variation and Assessment of Multiple-clone infections

The samples and controls were evaluated for background fluorescence or artifacts from either PCR or CE. Companion peaks or stutter peaks (Anderson et al., 1999) were considered to be artifacts of the PCR process therefore they were disregarded in the analyses. This was especially carried out in evaluating multiple clone infections where height and spacing of companion peaks determined which peaks were included in the analyses.

Multiple-clone infections (MIs) were defined as those in which one or more of the twelve loci showed multiple alleles. GeneMapper v 4.0/4.1 (Applied Biosystems, Foster City, CA, USA) was used to automate measurement of allele length and to quantify peak heights. Multiple alleles per locus were scored if electrophoretic peaks corresponding to minor alleles were ≥ 33% the height of the predominant allele in the isolate. Allele scoring was determined for each parasite clone where the largest among all the alleles was considered the multiplicity of infection of that sample. Infection haplotypes were constructed using predominant allele from each locus as well as the frequency of the infections containing multiple clones.

For example: Stutter (Figure 3.1) associated with a PET labeled microsatellite marker (TA40) during troubleshooting.

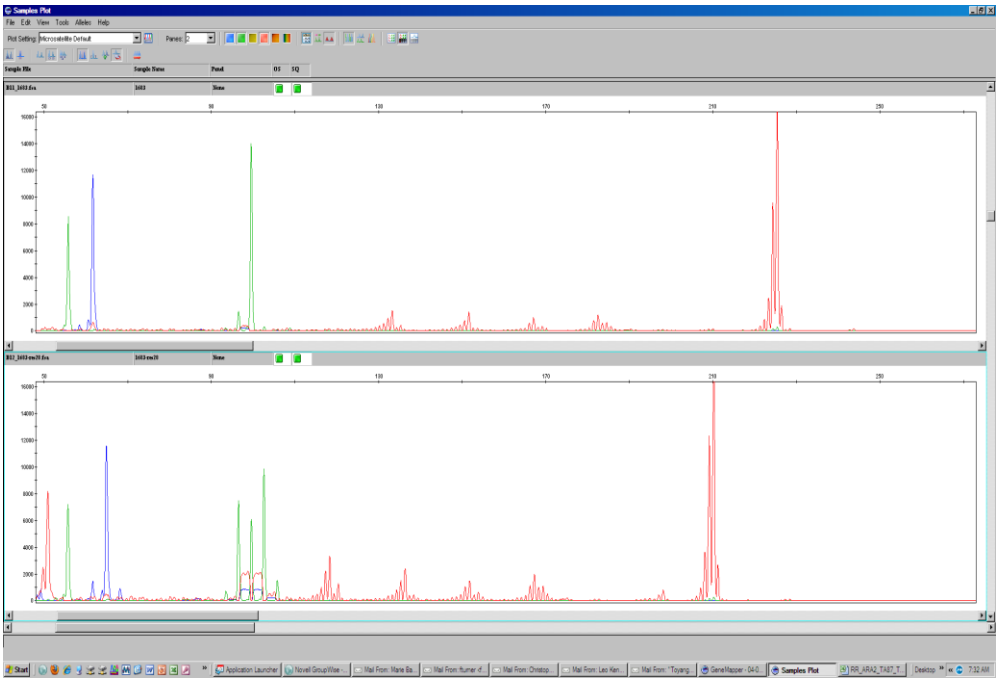


Figure 3.1: Diagram showing sample with stutter peaks

3.6.5 Estimation of genetic diversity for 1995-2003 and 2013-2014 parasite populations

Genetic diversity was measured by the number of alleles per locus and expected heterozygosity (H_E) on the basis of allele frequency data in each parasite population. In the cases of presence of multiple alleles at a locus within an infection, only the predominant allele defined by the allele with the highest peak height in electropherograms was used for allele frequency calculation. Unbiased expected heterozygosity (uH_E) is the standardized genetic diversity that was reported. This is a parameter that factors in sample size of different populations allowing comparative analysis between the two subsets of samples used. The unbiased expected heterozygosity, H_E was calculated as:

$$H_E = [n/(n-1)][1 - \sum pi^2]$$

Where *n* is the number of isolates genotyped and *pi* is the frequency of the i^{th} allele (each different allele at a locus). This was estimated using GenAlEx v2.2 (Peakall *et al.*, 2012) and confirmed using GENEPOP software (Raymond *et al.*, 2008).

The levels of diversity in different populations were compare using unpaired t-test and estimated effective population size (Ne) using observed heterozygosity and number of effective alleles. Population subdivision was estimated using Wright's *F* statistics (F_{ST}). Interpretation of the F_{ST} values at each locus was based on three categories defined earlier as no differentiation (0), low genetic differentiation (0-0.05), moderate differentiation (0.05-0.15) and great differentiation (0.15-0.25) (Balloux *et al.*, 2002; Cockerham, 1984) .

Fixation index was calculated as shown below:

$$F = \frac{H_E - H_O}{H_E}$$

Shannon diversity index (I) is a quantification measure of abundance and evenness of species present, which gives more information on the composition of a population. It is calculated:

$$I = \sum p_i \ln p_i$$

3.6.6 Construction of Multi-locus Microsatellite haplotypes for 1995-2003 and 2013-2014 parasite populations

The predominant alleles at each of the 12 microsatellite loci were used to construct the infection haplotypes. This was important to evaluate parasite genetic profile and relatedness among western Kenya parasites in circulation.

3.6.7 SNP Genotyping workflow using Agena platform

The assays were carried out in-country (USAMRD-K) and at reference laboratories in Ifakara Health Institute, Tanzania due to its capacity to support Agena platform. Ninety one single nucleotide polymorphisms (SNPs) distributed across all the 14 chromosomes of *P. falciparum* genome previously described by Phyo et al.,2012 were analyzed using PCR-based single-base extension on Sequenom MassARRAY platform (Agena Biosciences, San Diego, CA, USA) following manufacturer recommendations detailed in section 2.7.2. The multiplexed primers used in the

analysis as well as the pooled primers (into 4 vials) of the 91 SNPs can be found in the Appendix II & III. Controls used include 3D7 and DD2 and the genotyping primers are adopted from (Phyo *et al.*, 2012). Figure 3.2 below shows the step by step process from primer design to DNA amplification and SNP detection.

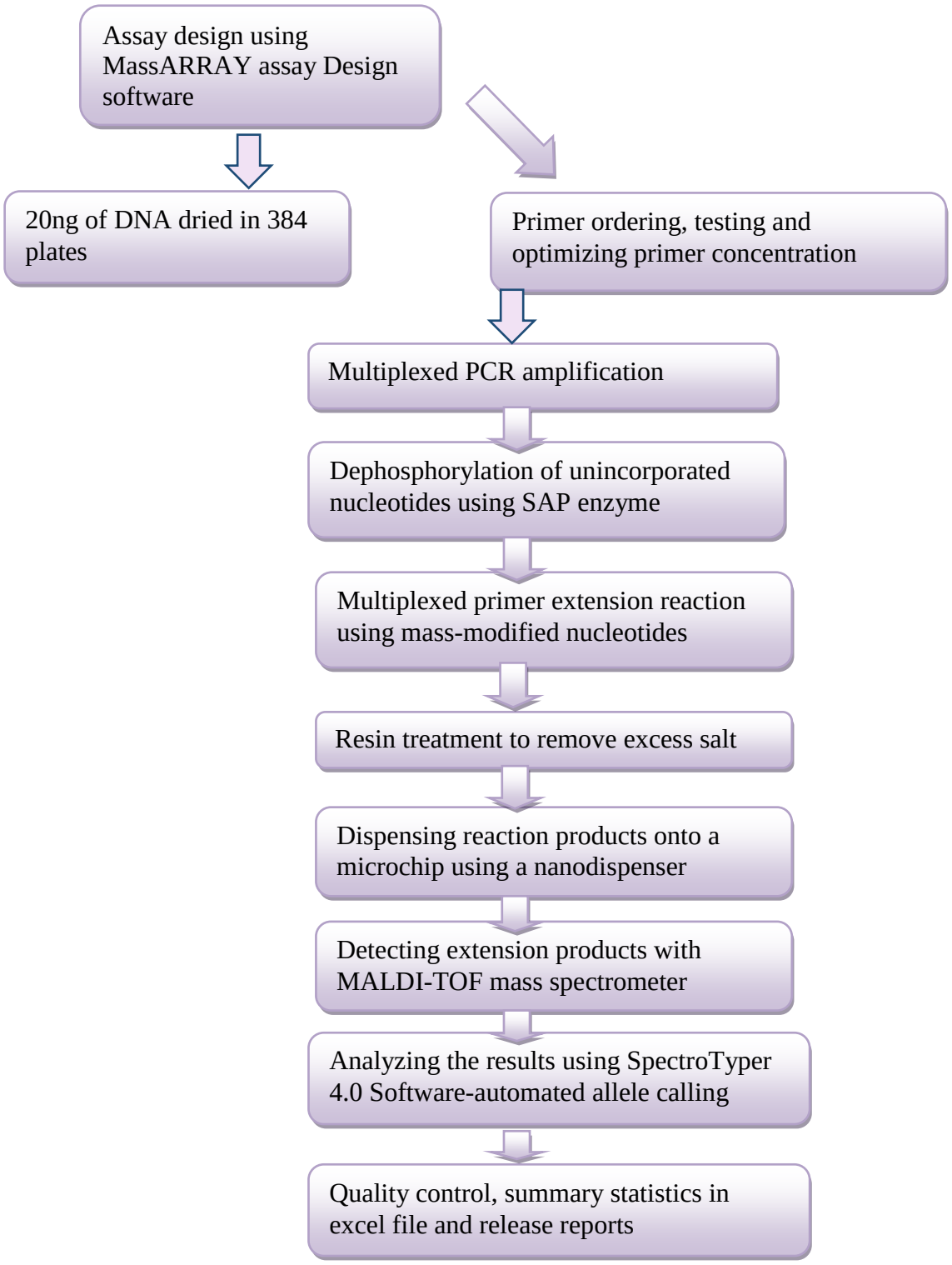


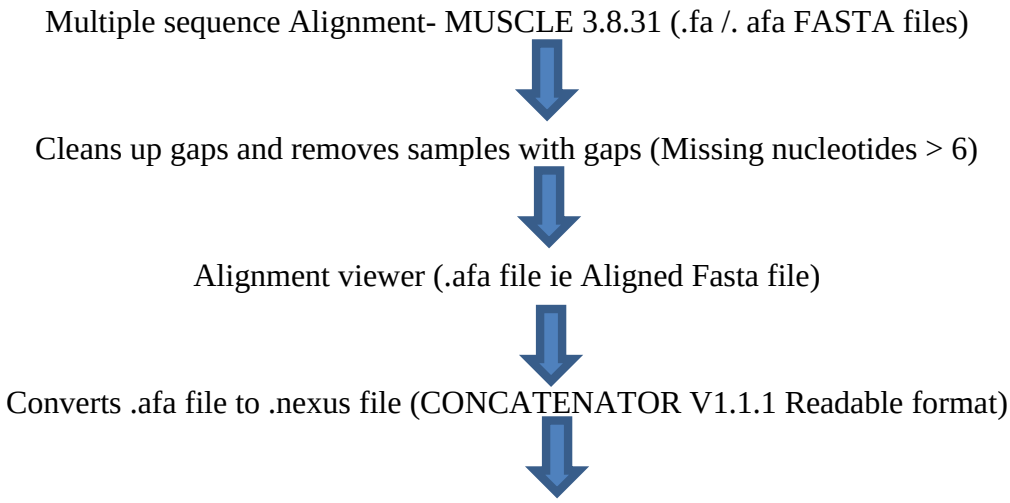
Figure 3.2: SNP genotyping workflow by MassARRAY technique

3.6.8 Measurement of parasite relatedness

Relatedness is generally estimated for diploid species (Blouin et al., 1996). However, the relatedness in blood-stage parasites between gametes of the single clones (using 78 assayed SNPs) was measured and analyzed by carrying out multiple sequence alignment using Muscle v5.8 available at (www.ebi.ac.uk/Tools/msa/muscle/). A phylogenetic tree was built using MrBayes available at (www.mrbayes.sourceforge.net/download.php) (Ronquist, 2012) and viewed using Figtree software v 1.4.3 available at (http:/tree.bio.ed.ac.uk/software/figtree) (Rambaut, 2014). Parasites that differed at less than 5% of loci were assumed to be identical-by-descent (IBD) and were referred to as being clonally identical (CI). Correlation between genetic variability was assessed as well as clearance rates; a phenotype used to determine whether clones with similar phenotypic characteristics harbor similar genotypic features. We constructed median-joining networks in NETWORK version 4.6.1.1 (http://www.fluxus-engineering.com/sharenet.htm) (Bandelt *et al.*, 1999) to visualize the relationships among *P. falciparum* isolates from pre- and post- ACT era.

Parasite relatedness of the single clones genotyped using SNPs was analyzed by carrying out multiple sequence alignment using Muscle 5.8; the phylogenetic tree was built using MrBayes and viewed using Figtree software.

The output (genotypes) was then compared among the study subjects using Bayesian midpoint tree to analyze parasite relatedness as shown in the scheme below:



3.7 Statistical analysis

3.7.1 Correlation of parasite genotypes with clearance rates

Multiple-clone infections was determined using microsatellite markers and its results used to compare parasite genotypes and drug clearance rates using Students paired t-test. Genotype calls generated were analyzed using MassARRAY SpectroTyper software 4.0v. This interprets the change in mass and converts to the nucleotide or SNP variants. SNPs present within the single infections were analyzed and recorded. The parasite genotypes were correlated with drug CRs of each study subject using Spearman rank correlation test. The allele frequencies were analyzed and compared within each other using one way ANOVA. To investigate the influence of genetics on clearance rates, correlation of the two parameters was carried out using GraphPad prism (GraphPad Software, Inc., San Diego, CA, USA).

Bonferroni's multiple comparison tests is a simple method for correcting for multiple comparisons. For example: the 3 SNPs will be tested to compute p values for each comparison. This is to test for statistical significance for the correlation determined between SNPs and clearance rates.

3.7.2 Determination of genetic diversity and population structure

To determine parasite genetic diversity and population structure, 12 MS were used to calculate allele frequencies, heterozygosity values, molecular variance, haplotypes, principal coordinates and fixation indices using GenAlEx software. Predominant alleles were used in calculation of allele frequencies. For H_E analysis, samples were grouped region-wise. For this comparison, the mean H_E values were compared by using a Mann-Whitney U statistics implemented in the statistical package GraphPad Prism (San Diego, CA, USA). A p value of ≤ 0.05 was considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 Microsatellite analysis

Allelic data from 92 post-ACTs and 29 pre-ACTs isolates was obtained having data in more than 10 loci. These were included in the subsequent analysis. That is, 121 of 146 (82.9%) parasites isolates were included; 29 of 42 (69%) were pre-ACTs and 92 of 104 (88.5%) were post-ACTs. For pre-ACTs parasites, 19 out of 29 (66%) were polyclonal whereas for post-ACTs, 74 of 92 (80%) were polyclonal.

Table 4.1: Genetic diversity of pre- and post-ACTs parasites at the 12 MS loci.

		pre-ACTs			post-ACTs		
		n = 29			*n* = 92		
MARKER	CHR	N	Na	H_E	N	Na	H_E
Polyα	4	27	16	0.942	89	33	0.967
TA42	5	28	11	0.805	91	24	0.870
TA81	5	28	10	0.857	91	32	0.942
TA1	6	29	12	0.903	86	31	0.954
TA87	6	27	15	0.908	90	34	0.953
TA109	6	28	18	0.951	88	35	0.953
TA40	10	25	18	0.947	85	32	0.944
2490	10	28	13	0.883	88	24	0.922
ARAII	11	29	15	0.900	91	27	0.943
PfPk2	12	29	13	0.895	91	30	0.953
PfG377	12	29	9	0.806	88	15	0.734
TA60	13	26	8	0.830	83	28	0.935
MEAN		28	13	0.886	88	29	0.922
SE		0.372	0.960	0.015	0.763	1.629	0.019

N = total number of isolates, Na = number of alleles for microsatellite loci, H_E = Expected heterozygosity, F_{ST}- Fixation index statistics; Mann Whitney (p = 0.0226; 95% CI). Normalized microsatellite data was used to generate the above data using GenAlEx software.

4.2 Parasite genetic diversity calculated from multiple-clone infections.

Table 4.1 above shows the 12 MS used were highly polymorphic both in pre-ACTs and post-ACTs parasites. TA109 was most polymorphic with 35 alleles whereas TA60 was the least polymorphic locus with 8 alleles. The diversity in pre-ACTs parasites was lower compared to post-ACTs. The mean number of alleles in all the loci for the pre-ACTs parasite samples was 13.17 (range 8-18) compared to post-ACTs which was 28.75 (range 15-35). The post-ACTs samples had significantly higher number of alleles in all the 12 MS loci compared to pre-ACTs (Table 1; $p < 0.0001$). The unbiased H_E was estimated per locus as shown in Table 4.1. The mean unbiased H_E for post-ACTs parasites was higher than for pre-ACTs parasites ($p = 0.0226$). With exception of 2 loci (TA40 and PfG377), the remaining 10 loci in post-ACTs parasite population had higher genetic diversity than pre-ACTs parasite population. However, most alleles were distributed widely across the two populations; post-ACTs parasite population had a large number of private alleles compared to pre-ACTs with 19.75 and 4.17 private alleles respectively. The Shannon diversity index for pre- and post-ACTs population was 2.308 and 2.955 respectively, further confirming higher genetic diversity in post-ACTs population.

4.3 Parasite population structure

The analysis of molecular variance (AMOVA) analysis revealed that most (97%) of the variance in allele frequencies was among individuals within the populations whereas only 3% of the variation was explained by differences between pre- and post-ACTs parasite populations. Wright's F-statistics revealed that 8 of 12 microsatellite markers detected significant population structure. The mean genetic differentiation

index (F_{ST}) among the pre-ACTs and post-ACTs parasite populations for all the 12 MS loci was 0.04287, reaching statistical significance (p = 0.001, Table 4.2).

Table 4.2: F_{ST} values across the 12 MS loci for pre- and post-ACTs parasites.

Marker Name	N	F_{ST} values
Polyα	116	0.0077
TA42	119	0.0302
TA81	119	0.0660†
TA1	115	0.0576†
TA87	117	0.0209
TA109	116	0.0292†
TA40	110	0.0400†
2490	116	0.023
ARAII	120	0.032†
PfPk2	120	0.0406†
PfG377	117	0.1285†
TA60	109	0.0852†
Total Mean	116.2	0.04287(SE .005)

N = total number of isolates, F_{ST} = Wrights F statistics. † indicates F_{ST} values with p < 0.05 (95 % CI) which 8 loci out of 12 account for population structure seen between pre- and post-ACTs populations which do not share any genetic diversity; PfG377 locus had the largest F_{ST} (0.1285) whereas Polyα had the lowest (0.0077).

Multi-locus haplotype construction of the 12 MS showed there were no matching haplotypes. This means there were no parasites that showed similar allelic data across the 12 loci but unique profiles. A total of 121 unique haplotypes were generated from 29 pre-ACTs and 92 post-ACTs parasites as seen below (Figure 4.1).

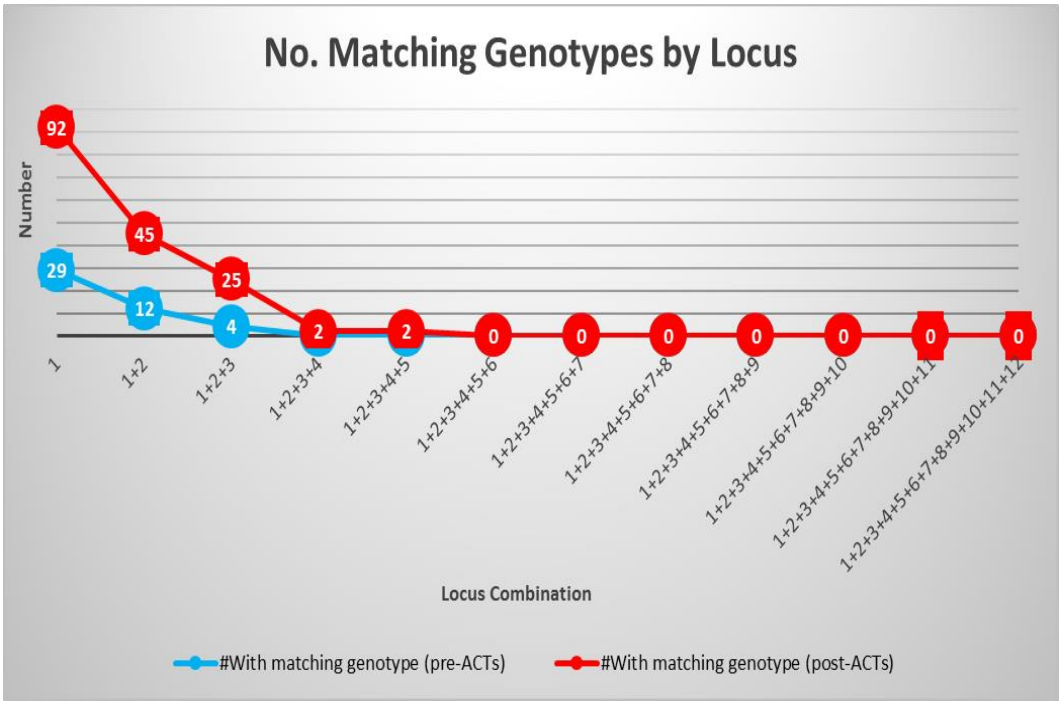


Figure 4.1: Multi-locus analysis of the 12 microsatellites showing number of matching haplotypes for both pre- and post- ACTs populations.

The highest multi-locus combination was seen in two samples pairs; one in pre-ACTs parasite population which had 8 of 12 of loci matching and the second sample in post-ACTs parasite populations which 9 of 12 loci were matching. To perform Principal Coordinate Analysis, the eigenvalues and eigenvectors were calculated, and the first two coordinates used to plot the genetic distance matrix (Figure 4.2). The percentage of variation explained by the two coordinates account for 8.87 % and 5.44 % respectively, revealing genetic difference in the two populations.

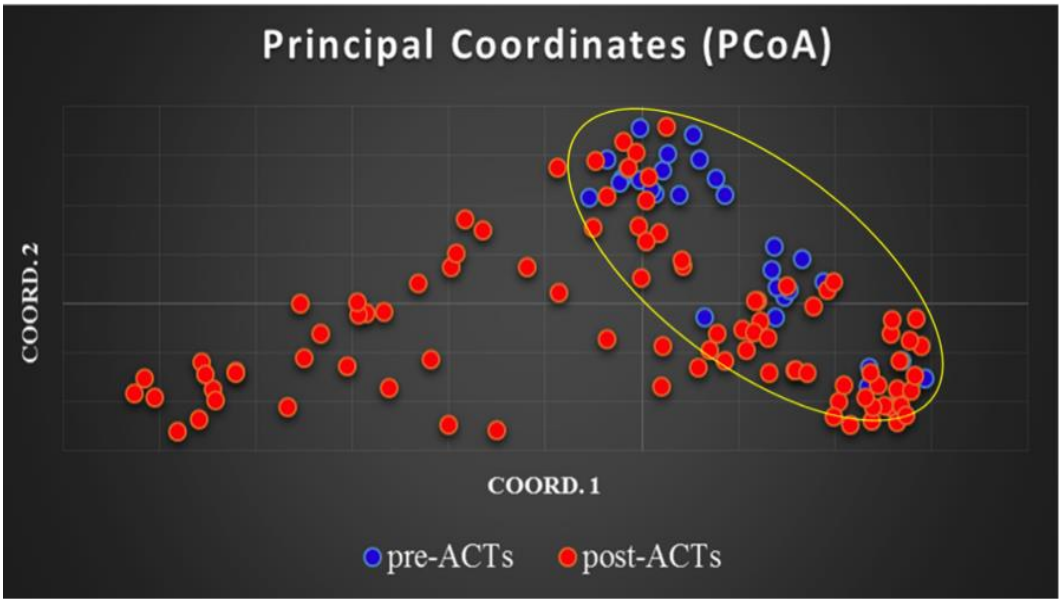


Figure 4.2: Principal Coordinate Analysis.

Microsatellite data for both pre- and post-ACTs were used to find out patterns and relationships within a multivariate dataset. This graph was plotted using genetic distance matrix; blue dots for pre- and red dots for post-ACTs.

4.4 Single Nucleotide Polymorphism Analysis

Ninety-two post-ACTs isolates comprising of seventy-five pre-treatment and seventeen post-treatment (recurrent) isolates were utilized for SNP genotyping. The post-treatment (recurrent) samples collected either on day 7, 14, 21, 28, 35 and 42 were determined to be re-infections using 91 SNP markers. Similar profile to day 0 was referred to as recrudescence while different profile to day 0 parasite was referred to as re-infection. A total of 91 SNPs found across *P. falciparum* genome were used to genotype all pre- and post- ACTs samples (Appendix II). Of the 91 SNPs genotyped, 78 (85.7%) generated robust genotype data which were used for subsequent analysis. For each SNP, the number of parasite isolates that were successfully scored are shown in Appendix IV; the mean was 68.62 parasite isolates

(range = 60-74) across all 78 SNPs. Parasites with < 5% of the 78 SNPs as mixed genotypes were considered to be single-clone infections. Of the 75 day 0 samples analyzed, 23 (30.6 %) were single-clone infections, in which 15 had robust genotype data and were used in subsequent analyses. A multi-locus combination analysis of the 78 SNPs showed no matching multi-locus haplotypes. This generated a total of 15 haplotypes in which each sample showed unique genotype combination. The highest multi-locus combination was present in 9 isolates which matched in 19 out of 78 (24.4%) loci.

4.5 Clearance slope half-life

The clearance half-life of parasite-infection of 75 post-ACTs samples was obtained. The mean parasite clearance half-life was 2.63 hours (95% confidence interval [CI], 2.44-2.81) and the median clearance half-life was 2.55 hours (interquartile range [IQR], 2.02-3.09). The parasite clearance half-life ranged from 1.19-5.05 hours as shown by the table 4.3 as well as the distribution of slope half-lives for the study subjects as seen in figure 4.3 below.

Table 4.3: Summary of slope half-life (hours) for 75 post-ACTs western Kenya parasite populations.

Statistic	Value
Median	2.55
Range*	(1.19, 5.05)
IQR**	(2.02, 3.09)

This table shows slope half-life generated after plotting hourly parasite density versus hourly time-point. *Range = (minimum, maximum); **IQR = Inter Quartile Range; (25^{th} centile, 75^{th} centile).

NB: No clearance rate data is presented for pre-ACTs parasite populations because pre-ACTs protocol did not adopt the 4 and 6 hourly dose.

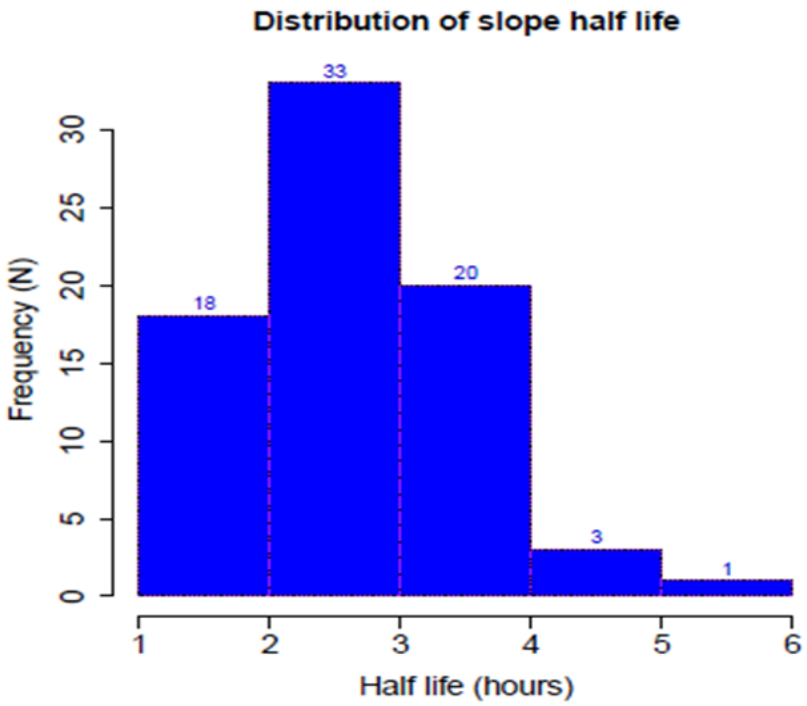


Figure 4.3: Graphical representations of the distribution of slope half-life (hours) for the post- ACTs samples (n=75).

As seen in the histogram above, most of the patients cleared parasites between 2-3 hours although we have those that cleared up to the 6th bar (represent prolonged parasite clearance which is an early warning sign of resistance).

4.6 Effect of parasite genotype on parasite clearance half-life

To determine whether parasite genotype influenced parasite clearance rates, the half-lives of parasites carrying either a wild type were compared to those carrying mutant allele at each SNP locus as seen in appendix V. Non-parametric unpaired t-test (with Welch's correction) revealed 3 SNPs in chromosome 12 and 14 (MAL12-1156125, MAL14-1199184, and MAL14-3017684) reached statistical significance. For MAL12-1156125 locus, parasites carrying mutant allele had slower clearing rates compared to wild type. Interestingly however, for MAL14-1199184 and MAL14-

3017684 loci, parasites carrying the wild type alleles had slower clearance half-lives compared to one carrying the mutant alleles (Figure 4.4). Parasites with same genotype at the 3 loci had similar clearance rate phenotype; four of the parasite infections with the slowest clearance half-lives had the same genotype where they carried mutant allele at MAL12-1156125, and wild type alleles at MAL14-3017684 and MAL14-1199184 (Figure 4.4, shown in blue). Conversely, three of the parasite infections with the fastest clearance half-lives had the same genotype; they carried wild type allele at MAL12-1156125, and mutant alleles at MAL14-1199184 and MAL14-3017684 (Figure 4.4, shown in red).

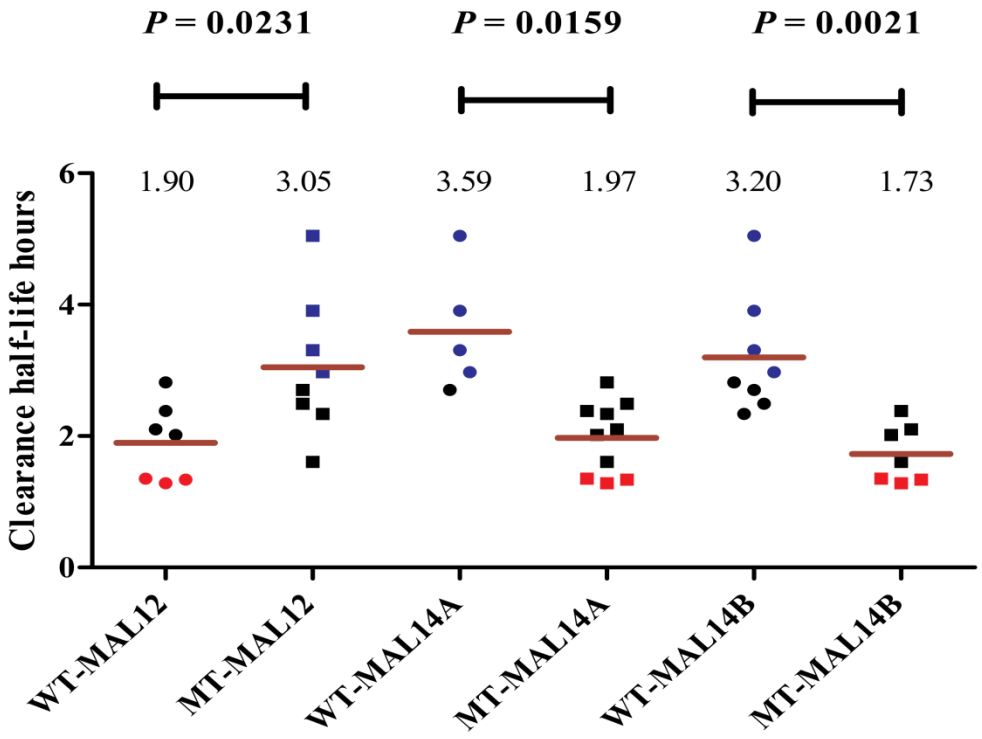


Figure 4.4: 3 SNPs out of 78 genome-wide SNPs genotyped which showed positive correlation with clearance half-life.

This is with statistical significance ($p < 0.05$; CI 95%). The SNPs are as follows: *MAL12-1156125 (MAL12), MAL14-1199184 (MAL14A) and MAL14-3017684 (MAL14B) showing their wild type (WT) and mutant (MT) states.

Further, Odds ratio analysis of the 3 SNPs (MAL12-1156125, MAL14-1199184 and MAL14-3017684) showed positive association with parasite clearance phenotype. In MAL12-1156125 SNP, mutant allele was associated with delayed parasite clearance whereas in MAL14-1199184 and MAL14-3017684 SNPs, wild type alleles were associated with delayed parasite clearance (Table 4.4).

Table 4.4: *P. falciparum* SNPs significantly associated with delayed parasite clearance rates.

Chromosome	SNP (allele)	Allele frequency	OR (95% CI)	*P* value
12	MAL12-1156125 (T/**C**)	0.474	6.00 (1.33-27.06)	0.0322
14	MAL14-1199184 (**T**/A)	0.439	6.00 (1.22-29.46)	0.0324
14	MAL 14-3017684 (**A**/T)	0.438	9.75 (2.28-41.68)	0.0016

Bonferroni's multiple comparison test showed significant correlation between MAL12-1156125 SNP mutant allele; MAL14-1199184 and MAL14-3017684 SNPs wild type alleles with delayed clearance rate as shown in Table 4.4 ($p < 0.05\%$, 95% CI). Out of the 7, no comparison matched our observations as shown in bold in Table 4.5 below.

Table 4.5: Post-test Analysis of the 3 SNPs using Bonferroni's multiple comparison tests.

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant P < 0.05	95% CI of diff
WT-MAL12 vs MT-MAL12	-1.150	2.900	No	-2.390 to 0.08981
WT-MAL12 vs WT-MAL14A	-1.690	3.768	**Yes**	-3.093 to -0.2876
WT-MAL12 vs MT-MAL14A	-0.07484	0.1982	No	-1.255 to 1.106
WT-MAL12 vs WT-MAL14B	-1.301	3.281	**Yes**	-2.541 to -0.06119
WT-MAL12 vs MT-MAL14B	0.1726	0.4214	No	-1.108 to 1.453
MT-MAL12 vs WT-MAL14A	-0.5403	1.237	No	-1.906 to 0.8254
MT-MAL12 vs MT-MAL14A	1.075	2.959	No	-0.06114 to 2.212
MT-MAL12 vs WT-MAL14B	-0.1510	0.3942	No	-1.349 to 1.047
MT-MAL12 vs MT-MAL14B	1.323	3.336	**Yes**	0.08276 to 2.562
WT-MAL14A vs MT-MAL14A	1.616	3.850	**Yes**	0.3034 to 2.928
WT-MAL14A vs WT-MAL14B	0.3893	0.8914	No	-0.9764 to 1.755
WT-MAL14A vs MT-MAL14B	1.863	4.153	**Yes**	0.4602 to 3.266
MT-MAL14A vs WT-MAL14B	-1.226	3.374	**Yes**	-2.363 to -0.08986
MT-MAL14A vs MT-MAL14B	0.2474	0.6553	No	-0.9331 to 1.428
WT-MAL14B vs MT-MAL14B	1.474	3.716	**Yes**	0.2338 to 2.713

To evaluate the relatedness of parasite genotypes to parasite clearance half-life, the single-clone infections (based on the 78 SNPs) were analyzed using the Bayesian mid-point tree. Since all the parasites in this study can be categorized as fast-clearing (compared to SEA), parasite were stratified based on the median clearance half-life of 2.55 hours which showed a significant p value. Therefore, those above the median half-life were classified as fast clearing (Figure 4.5; shown in blue) and those below the median half-life as faster clearing (shown in red) as they cleared rapidly. Amongst the 15 single-clone infections, 6 were fast clearing and 9 were faster clearing. Among the 6 fast clearing parasites, 4 with the slowest clearing rates showed higher genetic relatedness by clustering together while most of the faster clearing

parasites showed higher genetic relatedness by clustering together. For faster clearing parasites, two distinct groups were observed, each with at least 3 parasites in each cluster.

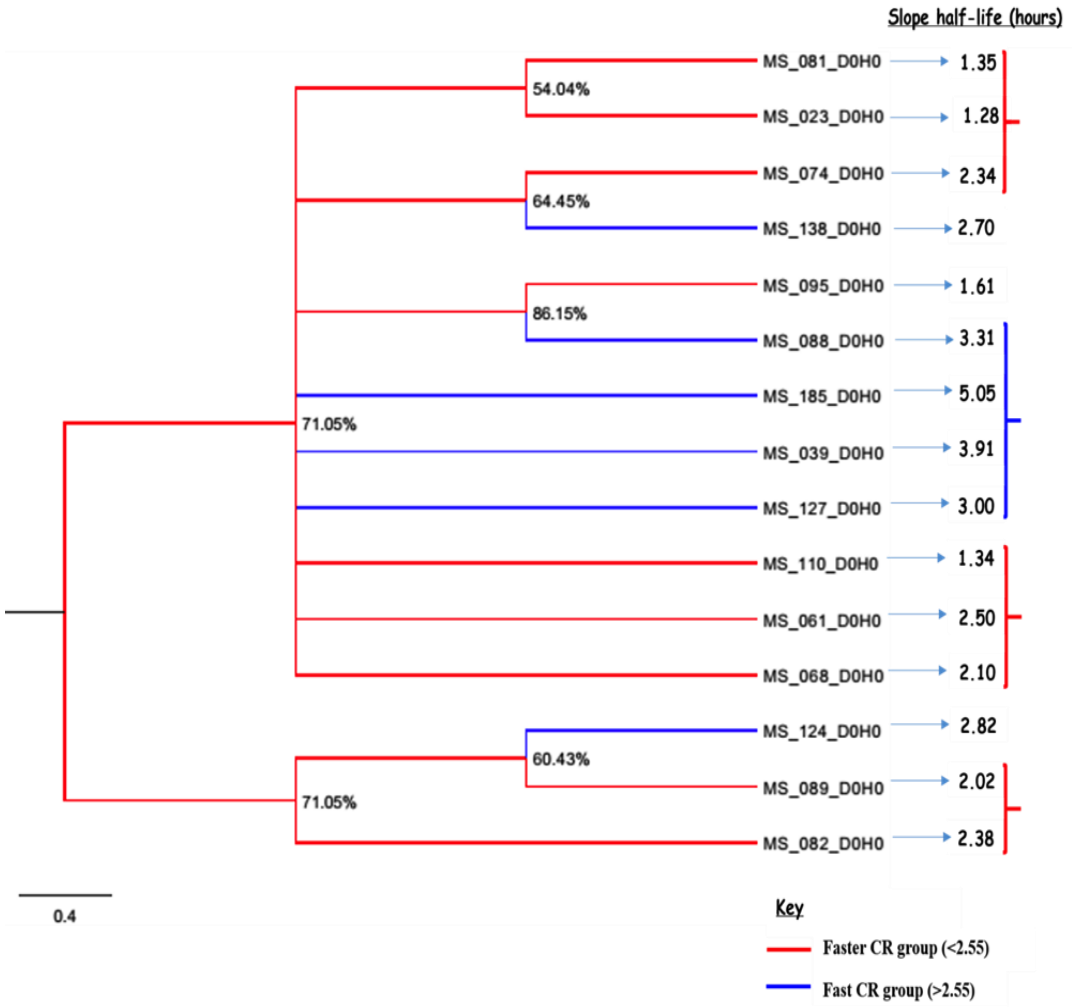


Figure 4.5: Bayesian midpoint tree showing single cloned P. falciparum haplotypes cluster in relation to clearance rates (slope half-life in hours).

This was constructed using 78 concatenated SNPs whose genetic variants at each point were used to construct parasite relatedness using MrBayes software. A 10,000,000 generations was used to run and construct the tree which gave standard deviation below 0.01 to generate higher posterior probability values.

Haplotype profiles for each of the single clones based on parasite clearance rate is shown in Figure 4.6 below.

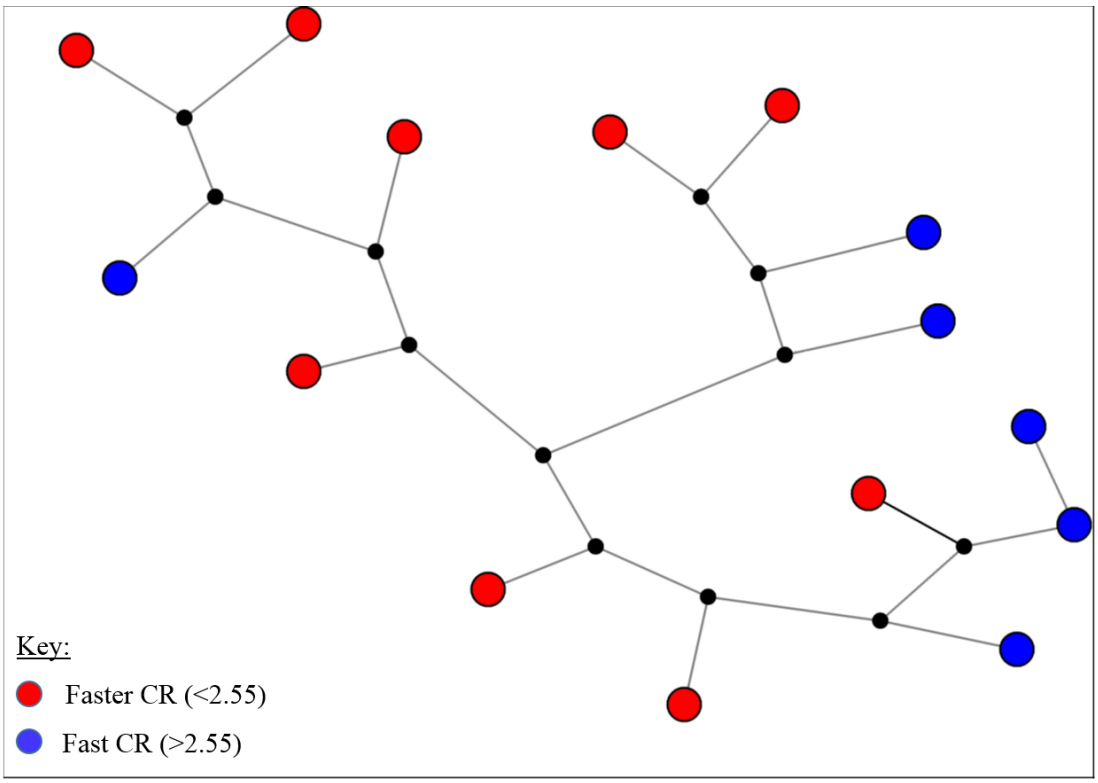


Figure 4.6: SNP-based genotypes and genetic variation seen in relation to clearance rates in 15 single clones in Western Kenya in 2013-2014.

Median –joining network diagram above shows genetic relationship of the western Kenya samples using 78 SNP haplotypes. Each circle in the network represents a unique haplotype profile with the size of the circle being proportional to the number of clones showing that particular haplotypes. The circle shown in red stands for samples with clearance rate <2.55 slope half-life (hours) while those colored in blue represent those with clearance rate >2.55 hours. The black dots are hypothetical

median vector generated by the software to connect existing haplotypes within the network with maximum parsimony.

CHAPTER FIVE

DISCUSSION

Western Kenya is a malaria endemic region with stable, high transmission throughout the year, with seasonal peaks (Bloland *et al.*, 1999; Hamel *et al.*, 2011; KEMRI, 2014). MS profile analysis applied in previous studies have shown parasites in western Kenya have high genetic diversity with high infection complexity (Ndenga, 2006; Zhong *et al.*, 2007) . In line with previous studies, our data showed presence of high genetic diversity in western Kenyan parasites with post-ACTs parasites showing significantly higher genetic diversity than pre-ACTs parasites. The post-ACTs parasites had higher number of alleles (including private alleles) in all the 12 MS loci analyzed. None of the parasite analyzed had matching haplotypes. Principal coordinate analysis revealed there was genetic difference between the pre- and post-ACTs parasite populations. Furthermore, genotyping of post-ACTs parasites with half-lives at 78 loci (distributed across the genome) revealed 3 SNPs on chromosome 12 and 14 that significantly influenced parasite clearance rates. Most importantly, I showed western Kenya parasites having genetically determined response to artemisinin treatment.

These data clearly demonstrate that pre-ACTs parasites collected between 1996 and 2003 had slightly lower genetic diversity compared to post-ACTs parasites that had high genetic diversity consistent with published work (Zhong *et al.*, 2007). To offer explanation to this finding, I considered the drug pressure exerted on these two parasite populations. Chloroquine was the treatment of choice for uncomplicated malaria infections in Kenya until 1998 (Shretta *et al.*, 2000) when sulphadoxine-pyrimethamine (SP) was introduced as the first-line treatment (Okiro *et al.*, 2010). AL

replaced SP as the first-line treatment in 2006 (Amin *et al.*, 2007) and has been widely available both in government and private clinics and hospitals. Changes in treatment regime (Chloroquine and SP replaced as first-line treatments) have been due to wide-spread treatment failure attributed to drug resistance (Ogutu *et al.*, 2005; Okiro *et al*., 2010; Shretta *et al*., 2000). In a series of studies conducted in Kisumu (which is located in western Kenya) in 1996, results showed that 85% of children under 5 years failed chloroquine treatment (*Shretta et al., 2000)* and two years after SP was introduced as the first-line treatment, estimated SP treatment failure in Kisumu was 42% (EANMAT, 2003). This indicates that post- ACTs parasite population in Kenya was under high drug pressure. This selection forced the parasite to acquire genetic variants, including those that confer drug tolerance resulting in high parasite genetic diversity within the population.

Introduction of AL in 2006 coincided with up-scaling of malaria intervention which not only increased drug availability, but also other aspects of malaria control such as increased coverage of insecticide-treated net (ITN) were on the increase (Okiro *et al*., 2010). Parasite genetic variants are associated with biological fitness cost which upon release of the pressure, and with parasite outcrossing, leads to the loss of the acquired genetic variants in a population (Miotto *et al.,* 2013). Change of drug policy in Kenya from SP to AL and scaling up of other malaria intervention strategies released SP and chloroquine drug pressure on parasite population, resulting in high genetic variability in post-ACTs parasite population (Zhong *et al.,* 2007). This was attributed to high mixed genotype infections transmission by human travel. AL remains highly efficacious in SSA and in Kenya (Agarwal *et al.,* 2013; Hamed *et al.,* 2012; Hamel *et al.*, 2011) indicating the parasite population is not under high drug pressure. This can

be interpreted to mean that selection of parasite population by AL in post-ACTs parasite is not as intense as compared to pre-ACTs selection caused by chloroquine and then SP.

Studies have shown that Kenyan parasite populations have low genetic differentiation (Gatei *et al.*, 2010; Vardo-Zalik *et al.*, 2013; Zhong *et al.*, 2007). Similarly, our study revealed low genetic differentiation with the overall mean F_{ST} of 0.0427 between pre-ACTs and post-ACTs parasite populations. Moreover, the low to moderate differentiation observed between the pre- and post-ACT era would possibly influence population structure through natural selection and genetic drift. Furthermore, principal coordinate analysis revealed that parasites from the pre-ACT clustered tightly than the post-ACT. The parasites from the pre-ACT era depicted a panmictic population with less genetic distance between them compared to the post-ACT parasites except for a few parasite populations that showed some structure.

Studies conducted in Zimbabwe and the DRC, regions of high malaria transmission, described the parasite population as having high genetic diversity (Durand *et al.*, 2003). The non-random association of alleles has significant implication for the spread of multi-locus drug resistance haplotypes with high levels of inbreeding, which increases their dispersal (Schultz *et al.*, 2010). Within the pre- and post-ACT era parasite populations, a large proportion of multiple infections was found and therefore cross fertilization and recombination between distinct parasite genomes was expected to maintain the random association among the loci. The observed genetic diversity suggests the occurrence of recombination even in areas with intense and perennial malaria transmission which may explain the phenomenon of spread of drug resistance

haplotypes across time. The data obtained in our study is consistent with the trends observed in Zimbabwe and the DRC and Kenya also (Mobegi *et al.*, 2012)..

In this study, 3 SNPs that are significantly associated with delayed parasite clearance are described. However, from post-test analysis (Bonferroni multiple comparison test) showed slight difference in mean values in some comparisons. Therefore the potential use of these SNPs as markers of resistance awaits validation. It is not unreasonable to expect the proportion of variation in parasite clearance attributable to parasite genetics to increase at a much slower rate in high transmission areas of SSA than it is SEA. However, there are some areas in SSA that have experienced reduction in malaria transmission or are low transmission areas (O'Meara *et al.*, 2008; O'Meara *et al.*, 2010) with more structured parasites (Bogreau *et al.*, 2006) and are more likely to obtain parasites with artemisinin resistant 'winning combination'.

These SNPs are non-synonymous SNPs comprising of one found on chromosome 12 and two on chromosome 14. The SNP on chromosome 12, MAL12_1156125, is located in the gene PF3D7_1228400 and the SNPs on chromosome 14, MAL14_1199184 and MAL14_3017684, are found in genes PF3D7_1430300 and PF3D7_1474000 respectively (www.plasmodb.org). PF3D7_1228400 is a gene encoding a conserved *Plasmodium* protein with unknown function (www.plasmodb.org). This gene has 28 known synonymous SNPs and its expression is present both in sexual and asexual stages. These data show that the mutant allele is associated with delayed parasite clearance. The wild type, MAL12_1156125-T is found in *P. falciparum* strains such as 3D7, 7G8 and V1_S whereas the mutant MAL12_1156125-C is found in strains such as HB3 and S. Lucia. This means that the

mutant allele is not exclusively associated with response to artemisinin drug pressure but a genotype that predisposes the parasite to be less sensitive to the drug.

PF3D7_1430300 gene encodes for a putative acid phosphatase, involved in malaria parasite metabolic pathways including drug metabolism (www.plasmodb.org). In parasite lifecycle, the gene is down regulated in ring stage but up regulated in schizonts, and has 37 synonymous SNPs. The wild type allele, MAL14_1199184-T is found in *P. falciparum* strains such as HB3 whereas the mutant allele MAL14_1199184-A is found in strains such as 3D7. PF3D7_1474000 encodes for a putative protein with unknown functions and has 44 synonymous SNPs. The wild type allele, MAL14_3017684-A is found in *P. falciparum* strains such as 3D7 and HB3. Interestingly, the presence of wild type alleles in these two SNPs appears to predispose the parasite to delayed parasite clearance to artemisinin treatment. This phenomenon has recently been described in studies that showed significant selection of wild type alleles in *P. falciparum* chloroquine resistance transporter *(Pfcrt*) gene at codon 76 and multidrug resistance 1 (*Pfmdr1*) gene at codon 86 occur in recrudescent and re-infecting parasites after AL treatment (Venkatesan *et al.,* 2014). However, since only a small number of samples were tested, additional studies are needed to determine if indeed there is any causal association between these SNPs and artemisinin sensitivity. Therefore, the three SNP markers should be validated and used as markers for artemisinin resistance.

In low transmission setting such as SEA, different patients are more often infected with CI parasites which arise due to low parasite genetic diversity and inbreeding (Anderson *et al.,* 2010; Ndenga, 2006). Similar to identical twin studies in humans, CI parasites provide an effective approach to determining if phenotypic traits have a

genetic basis (Anderson, 2010). Artemisinin resistance has been reported in SEA (Dondorp *et al.,* 2009; Noedl *et al.,* 2008; World Health Organization, 2009), and recent studies have shown the resistance phenotype can be explained by heritable genetic traits in the parasite population (Anderson *et al.,* 2010; Phyo *et al.,* 2012; Takala-Harrison *et al.,* 2013). There are no reported cases of delayed parasite clearance in SSA, and studies have reported median half-lives of less than 3 hours (Ashley *et al.,* 2014; Maiga *et al.,* 2012). Similar to the previous findings, our study revealed that the median half-life was 2.55 hours, with a range of 1.19-5.05 hours. Although western Kenya is a high transmission area with no reports of delayed parasite clearance to artemisinin treatment, bayesian midpoint tree revealed genetically related parasites which most of them clustered together based on parasite clearance.

High genetic variability in SSA parasite population might be preventing emergence of parasites that are resistance to artemisinin. Anti-malarial drug resistance may emerge de-novo when malaria parasites with spontaneously arising mutations or gene duplications conferring reduced drug susceptibility are selected for by anti-malarial drugs (White, 2004; White *et al.,* 2003). These emerging variations (mutations or gene duplication) might have relatively small phenotypic effects when acting individually. It is through the process of gene recombination that may occasionally generate a parasite possessing a 'winning combination' of alleles. This cumulatively might confer resistance to anti-malarial drugs, but must also provide a certain level of biological fitness for parasite to survive (Miotto *et al.,* 2013). However, with high genetic diversity and outcrossing, the progeny can easily loose the 'winning combination'. It is therefore likely that with proper management of combination

therapies such as AL and monitoring of drug efficacy, artemisinin resistant 'winning combination' alleles will not easily emerge in high transmission regions such as western Kenya due to high parasite genetic diversity.

Artemisinin tolerant (resistant) parasites have been reported in SEA. However, high grade resistance has not been reported (Phyo *et al.,* 2012). Despite this absence of resistance, recent studies suggest that there is evolution of the parasites towards resistance to artemisinin in SEA (Miotto *et al.,* 2013). Thus, the prevalence of K13 propeller region SNPs (C580Y, Y493H, R539T, I543T) have been validated and are associated with artemisinin resistance among the Cambodian parasite subpopulations (Ariey *et al*., 2014). These K13 propeller region SNPs are not prevalent or associated with delayed parasite clearance in African parasites (Conrad *et al.,* 2014; Kamau *et al.*, 2014; Taylor *et al.,* 2014). Identifying parasites with genetically determined response to artemisinin tolerance or identifying such subpopulations will be critical in identifying effective molecular markers for monitoring emergence and spread of artemisinin resistance in SSA. In this study, existence of parasites with genetically determined response to artemisinin treatment was demonstrated in western Kenya. From the median joining analyses using SNPs and the multi-locus analyses using MS markers, it was clear that the single-clone parasites were not clonally identical, although closely related. Second, the parasites from Western Kenya presented a median clearance rate of 2.55 hours. This rate implies that the parasites from Western Kenya are sensitive to AL compared to SEA where the median clearance rate of resistant parasites is 6 hours. Hence it is clear that by the 2013-2014, there was no resistance to AL in western Kenya.

The genetic markers used in Southeast Asia (Phyo *et al.*, 2012), were the same used to detect the influence of parasite genetics on parasite clearance rate where we found existing correlation. In addition to this, we found 3 SNPs that statistically showed the correlation, evidence of genes influencing faster (<2.55 hours) and fast clearing (>2.55 hours) parasites.

CHAPTER SIX

CONCLUSIONS, STUDY LIMITATIONS AND RECOMMENDATIONS

6.1 Conclusions

6.1.1 Determination of parasite clearance rates

Parasite clearance rate of 2.55 hour (geometric mean) was calculated and reported which suggests that Kenyan parasites are still sensitive to Artemisinin antimalarial.

6.1.2 Establishing existence of multiple-clone infections of *P. falciparum* parasite

High genetic diversity seen in western Kenya parasite populations using microsatellite markers which reported an average of 13 and 29 clones for pre-ACTs and post-ACTs respectively per patient.

6.1.3 Evaluation of genetic polymorphism and diversity across *P. falciparum* parasite genome

Three SNPs were positively and significantly correlated to delayed parasite clearance rate. This has shown the existence of parasites with genetically determined response to artemisinin treatment in Western Kenya.

6.1.4 Correlation of parasite genotypes with clearance rates

A correlation between parasite genotypes and parasite clearance rates and identified three novel SNP genetic markers in *P. falciparum* parasites that may be applied in detecting parasite CR trait in Western Kenya once validated.

6.1.5 Determination of parasite genetic in western Kenya parasite populations

Based on the SNP analyses, the study has also shown that parasites with similar clearance rates are indeed genetically related. Therefore, the null hypothesis was rejected and the alternative hypothesis stating that *P. falciparum* parasite genetics has influence on parasite clearance rates holds true.

6.2 Limitation

This study had one major limitation. Only fifteen parasite clones were used to correlate the clearance rates. This small sample size arose from the difficulty of obtaining single-clone infection in a high transmission area like Western Kenya. The small sample size lowered the power of this study and studies with large sample size are required to further elucidate these findings. Regardless of the study limitation, our data clearly demonstrate that variation in clearance rates can be explained by parasite genetics.

6.3 Recommendations

To further corroborate these findings, we recommend:

i. Investigation of parasite genetics in low transmission areas in Kenya, where malaria endemicity is similar to that found in SEA and correlate the findings to clearance rates. Therefore, surveillance of artemisinin resistant parasites should not only focus on high transmission areas in SSA but also in regions of low transmission.

ii. Based on results from this study, it can be recommended that advanced technologies such as use of the CRISPR-Cas9 system and microarray should be applied to validate the role these 3 SNPs have in influencing delay in

parasite CRs. This is to identify effective molecular marker(s) for monitoring emergence and/or spread of artemisinin resistance in SSA.

iii. Determine the function of the two unknown proteins which harbor the two SNPs and determine the role/target of the putative acid phosphatase having third discovered SNP.

iv. Besides the 91 SNPs studied here (based on SEA parasites), new additional targets should be utilized for high throughput.

v. Study other phenotype traits such as Ring Stage survival rates.

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APPENDICES

APPENDIX I: Neutral microsatellite primer sequences-12 loci MS markers, primer sequences, fluorescent dyes and size range per locus for allele scoring (Anderson et al., 1999).

Multi-plex	Locus (Anderson 1999)	Primer 5' to 3'	Fluorescent Label	size range from Anderson 1999	Size in 3D7 reference genome
1	Poly α-R	ATCAGATAATTGTTGGTA	FAM	114-201	151 bp
	Poly α-F	AAAATATAGACGAACAGA			
	Poly α-3(IR)	GAAATTATAACTCTACCA			
1	PFPK2-3R	CCTCAGACTGAAATGCAT	HEX	159-192	172 bp
	PFPK2-F	CTTTCATCGATACTACGA			
	PFPK2-R	AAAGAAGGAACAAGCAGA			
1	TA81-3F	GAAGAAATAAGGGAAGGT	PET	112-142	122 bp
	TA81-R	TTTCACACAACACAGGATT			
	TAA81-F	TGGACAAATGGGAAAGGATA			
2	ARA2-3(F)	GTACATATGAATCACCAA	FAM	63-90	73 bp
	ARA2-R	GCTTTGAGTATTATTAATA			
	ARA2-F	GAATAAACAAAGTATTGCT			
2	TA87-3F	ATGGGTTAAATGAGGTACA	HEX	90-126	100 bp
	TA87-R	ACATGTTCATATTACTCAC			
	TA87-F	AATGGCAACACCATTCAAC			
2	TA40 Rev-1	GAAATTGGCACCACCACA	PET	217	217 bp
	TA40 For	AAGGGATTGCTGCAAGGT			
	TA40 Rev-2	CATCAATAAAATCACTACTA			
3	TA42-3F	ACAAAAGGGTGGTGATTCT	FAM	182-251	201 bp
	TA42-R	GTATTATTACTACTACTAAAG			
	TA42-F	TAGAAACAGGAATGATACG			
3	2490-3R	ATGATGTGCAGATGACGA	HEX	78-93	84 bp
	2490-F	TTCTAAATAGATCCAAAG			
	2490-R	TAGAATTATTGAATGCAC			
3	TA1-3(F)	CTACATGCCTAATGAGCA	PET	159-204	186 bp
	TA1-R	TTTTATCTTCATCCCCAC			
	TA1-F	CCGTCATAAGTGCAGAGC			
4	TA60-F	CTCAAAGAAAAATAATTCA	FAM	69-99	87 bp
	TA60-R	AAAAAGGAGGATAAATACAT			
	TA60-3(IF)	TAGTAACGATGTTGACAA			
4	TA109-3F	TAGGGAACATCATAAGGAT	HEX	154-223	174 bp
	TA109-R	CCTATACCAAACATGCTAAA			
	TA109-F	GGTTAAATCAGGACAACAT			
4	PFG377-3R	TTATGTTGGTACCGTGTA	PET	89-113	98 bp
	PFG377-F	GATCTCAACGGAAATTAT			
	PFG377-R	TTATCCCTACGATTAACA			

APPENDIX II: SNP genotyping by MassARRAY technique- 91 SNPS

GENOMIC LOCATION	SNP ID	SEQUENCE	CHR	MINOR ALLELE	WILD-TYPE	MINOR ALLELE FREQ
*MAL01-354427	CombinedSNP* MAL01_354427	TACTAGGTGGTTTATTTATATAAG AATTTGCTATGTATGTTATAACAT CAGATTCATTTA[T/C]ATTATTATT TTGTTCTTCGTTTATGGTATTCTTT TTATTATTAGAAATATTATTATAAT C	1	A	G	0.43
MAL01-539149	CombinedSNP_ MAL01_539149	TCTCTTTTCAGTGGATGCCATTTGT TTTTTTAAATGTGTTAAATCATTGT GAATTTTTAT[A/G]ACTTTAATAAA TGTACGCATAAAATCTTTATATTC ATTATTTTTAAAATATTTCTTTACT	1	G	A	0.31
MAL02-217337	CombinedSNP_ MAL02_217337	TTTGTTTAGAGCATCATTTATTTTT GGAAAACATCCCTCCTTCAAAGGA TCATACGATTC[A/T]TTTCCTTTCAT ACAAAAAAATAAAAAAAATTAAA TAAATAAATATACACATATATATA AA	2	T	A	0.29
MAL02-322398	CombinedSNP_ MAL02_322398	TTACACATAACAGAAAAAACATTC ATTTGAACCCTTCAGTTTGGTTAA ACAATATCCAGG[A/G]GATGTTTTA TCTTTACATTCATTCCAATGGAGTT CACAAAATTTAATACATCCTTCAT GT	2	G	A	0.26
*MAL02-660994	CombinedSNP* MAL02_660994	TTTTTAAATAATATGTCCAAATGT GTTTTATACCAAAAAAAAGAAGCA TATCAAGAATCA[T/C]CAGAACAA AACTTCCATGGTCTTATAAATCAT AAAAATGAAGACAATGAAAAATG TCATA	2	G	A	0.17
*MAL01-354427	CombinedSNP* MAL01_354427	TACTAGGTGGTTTATTTATATAAG AATTTGCTATGTATGTTATAACAT CAGATTCATTTA[T/C]ATTATTATT TTGTTCTTCGTTTATGGTATTCTTT TTATTATTAGAAATATTATTATAAT C	1	A	G	0.43
*MAL03-249686	CombinedSNP* MAL03_249686	ATACAAAATAAAGAAGAGGAAAT TATAAAATATGTTAATGAACTACA AAA[T/C]AGTGATGTGCAAAAAGA AAAAAAAGAGTTGAAAACAATAA ATGAAGTTTT	3	G	A	0.49
MAL03-439621	CombinedSNP_ MAL03_439621	AATTTATTATTTAATTATTATAACA AAGAAAGTATTGGACATAAAAAA AA[A/G]AAAGCGAGTTTTGTAAAA GCACAATATGCTAATAATAGGATA CCATCTAA	3	G	A	0.25
*MAL03-704420	CombinedSNP* MAL03_704420	ATTAATGTGTTTTTAATATTTGAAC AATATTTTTCAAACATTTCATTTGC [T/C]CGTGTTAATGTTATGATTTTC ATTTGAGCTTTATTTAATAAATCGT TTAA	3	G	A	0.17
MAL03-748204	CombinedSNP_ MAL03_748204	CAATATGATAGAACTGTAAGAAAT TCATATGGAGATATTATTCAATTTT T[A/G]TATGGAGAAGATGGTATGG CAGGTGAATATATAGAAGATCAA ATTATAGA	3	A	G	0.44
MAL03-822470	CombinedSNP_ MAL03_822470	AAATATGGTAAATATAAAAATAAT AATAATAATAACTCTTTTTATAAC ATGAGAGATGAT[A/G]CAGAATTG ACAGACATTGAAGAAAATATATCA AGTAAAAAAAAAAAAAACAAATT TATAG	3	G	A	0.39
*MAL04-268436	CombinedSNP* MAL04_268436	TCTCATATTTATTATTCCTATTCAT AAGTAATAAACCTTTGTTGTATTTT CGAAACACAT[T/C]TTTATGTCTCC	4	A	G	0.29

		TAAGATATACCACATTTTGGACAT TATTATTATTATCATTATTATCATT				
MAL03-439621	CombinedSNP_MAL03_439621	AATTTATTATTTAATTATTATAACA AAGAAAGTATTGGACATAAAAAA AA[A/G]AAAGCGAGTTTTGTAAAA GCACAATATGCTAATAATAGGATA CCATCTAA	3	G	A	0.25
MAL04-307321	CombinedSNP_MAL04_307321	GATGATCATATATGGAATTATAAT TTAATGGGAATCAAATTTAATAAT AATCAAAAATAT[A/G]CACCACAT CTTGATATACCACAACCATTTTAT GCAGATATACATAGACCAAATCAC TTTC	4	A	G	0.16
MAL04-393114	CombinedSNP_MAL04_393114	ATTGATAACCACCAAGGTAACGAA CATTTCAACATGAATAATATTGAA AGTAAGAACAAT[A/C]TTGATGGT ATTCGTAATATGAACCATTTCAAG AGTGTGATTCCTTCAGATATGACA ACAA	4	C	A	0.45
MAL04-443828	CombinedSNP_MAL04_443828	CTTGCTATAACTATTAGATTTTGA GATGAAATTATTGATAAGTTCTTT AA[A/G]GAACCCACAGCACTTATT AAGCGTGCCGTTAATGAACTACCT AATAACAT	4	G	A	0.28
MAL04-469608	CombinedSNP_MAL04_469608	CCATGATTTTCGGCAACGGGCGTG ACCCTACTGCCGCTTTCGCCTATAT C[A/G]ACTACAAGGGCTTTTGAAA AACAAGATAAAAAGGAAATATAT AAATGAAT	4	G	A	0.44
*MAL04-933828	CombinedSNP*MAL04_933828	GGATAACTCTAAATATGTGGAATG TGGGAATGGAGACAAAGAAACGA ATGATTATAATAC[T/G]CGTCGAAA AAAGAAGGATGAGAAATTTAGTG TTAGTAGTAATGCTTTATATGATA TGAGT	4	A	C	0.35
MAL04-994352	CombinedSNP_MAL04_994352	ATTTCCTTCATTTTTATTAATATCA AACATATTATTTAAATGATTGGAT TTTTTATTATT[A/T]TTTTTTTTCTTT ATATTTTCCTCATTACGTTCCTCAA TTTCAATAACATTTTCTTCTTTT	4	A	T	0.42
MAL04-1101563	CombinedSNP_MAL04_1101563	AATTTTTTAATTTTAATTCAAGTGG ATATCATAGTTGTATGAGAAAAGA ATTTTCGAATC[A/C]TGCATATAAA TGTTTTGCAGGGAAAGGTTTTTCA AGTATGTTTAATCTGGACAAAAAA AA	4	C	A	0.29
MAL04-1140617	CombinedSNP_MAL04_1140617	GATAAAAATTATTCAAGGAATATA TTAAATAATGAGAATTCTGAAGAT ATATTTGAATCG[A/G]TTAAAAGG GTAGAATCGTATAATTATGAAAAT AATATGGTCAATGAATGTAATGTA ATCC	4	G	A	0.46
MAL05-118209	CombinedSNP_MAL05_118209	CTTCTCTTTTCTTAACCATATTAAC ATAGAAATTCATATTCCTTAGTTT AACTTTTTTAT[A/G]TTTTGTTCCCT TCCATTTTTCTCTTTTGCAATTTTT CAATGATTTTTCTTTTATATGCTT	5	G	A	0.28
*MAL05-385372	CombinedSNP*MAL05_385372	AAAAAATTATTCGATGATCTAAAA GATCTTGAAGAACTTTTTGGTGAT AG[T/C]GATGAATTAAAAGAATTA GAACATGAATATGATCATATTAAT ATAAACGA	5	A	G	0.11
MAL05-720978	CombinedSNP_MAL05_720978	TTATCTTCTGTATCATATATATAAA AATAATTTTGCTCATCTAAAACCC A[A/G]AGTCTATTTTTTCGATCAAA GGCTAGAGATTTTAACTTAAAAAT ACCATA	5	G	A	0.31
*MAL06-154776	CombinedSNP*MAL06_154776	TCCAACACATCCTTTTTTTTCTCAT GTTCAATATTTTCTTTACGATGTAC TGCGGACATA[T/C]TAAATCGTTTA ATATTTTTCCTTTTAACATTCTCAT	6	A	G	0.3

		CATAATTATTCAACACATTAGCAC				
MAL06-410691	CombinedSNP_MAL06_410691	ATACATGTATAATAAAAAATGTTGTGGTCATCTTCAACTAAAAAATGTTGAAGAATTGTC[A/G]AAGTTTGATTGTTTATTAATAAATATGGGAATAAAATTTCGAATTATTTATATATTTAAA	6	A	G	0.27
*MAL06-573730	CombinedSNP*MAL06_573730	AGAAAAACGCTCAAGGAAATTTTACACTCAGCTTAGATGATGAAGCCATC[T/C]TATCCTCTTATAATAATCCAGAAAAGAATAATACTGTATGCAAAAATAAT	6	A	G	0.2
*MAL06-593821	CombinedSNP*MAL06_593821	TCATTCTTTAATTCTTTTATTTTTCTCAAAACATTGTGCATACTCTTAAA[T/C]CCATAAGATAAGATTACCCTAAACACACAATTATTATTATTAAATAAAGA	6	A	G	0.22
MAL06-937750	CombinedSNP_MAL06_937750	ATAGGTTCTCCAATACCTATAGGAGGATTTTGTATAATGCATGTAGCAAT[A/G]CCCCCAACACTATCTCCTTTATTTTTTAGTTTCAAAATATAAGAACAAAT	6	A	G	0.39
MAL06-1184507	CombinedSNP_MAL06_1184507	AAAAAAAAAAAAACGCAAATTAATAGTGATAATTATGTGTTGACTATCAAAAATTTGATCG[A/G]TGAAAATATAGAGAACAAATCTGTAGAAAAAAAAAGGAAAAGAAATTATTTCAAGAAATA	6	G	A	0.46
MAL06-1206499	CombinedSNP_MAL06_1206499	CGTAGTGAAAGTGAAATAGATGATATAGATGAATTAGATGAAGAAGAATATGATTGGACG[A/G]AGCATGTATATAATTATAAGCCTACCACTTATTTATTACCTGGGTTGGGAGGAAGTACAT	6	A	G	0.12
MAL07-476303	CombinedSNP_MAL07_476303	CATGTCGGCACACGGAGGAAGATCCCCCTCAAACATTTTTGCTCCTTCCATGTTTTTCAT[C/G]TGCCCTGTTGAAATATCCATTTTTTCATTAATTTCCTCCTGAGAGGTTATATAATTACTA	7	G	T	0.3
MAL07-511352	CombinedSNP_MAL07_511352	GTTTATATTTGTATTAATTTTAATTTATTTACTTACCATTTTGATTGAAAATGATATGTA[A/G]GGCAAATTAAATTTCAACTTATTCATGTTTACAAAAAATAAGACAGGTATATATGAAAAG	7	G	A	0.42
*MAL07-543849	CombinedSNP*MAL07_543849	TTTTATTTTTTTTTTTCTCCATATCTATTGTAGAATCATTTTTATAATTATCTAACGCAT[T/C]TGTTTTTTTATCATAACTGGAATCTTTATTCATTTCATTTTTTGTCATTTTGTAACAAGGT	7	G	T	0.31
MAL07-759749	CombinedSNP_MAL07_759749	AAGTATCGAAAGAAGGATTAAGGAGAAACACAGAAATATAGAAGATGAAGACTCAGAGGA[A/G]AGTGACAGCAAATGGAGGAGAAGAAAAAGGGAACGAAGCAGAGATAAATATAAAGAAAGA	7	G	A	0.33
MAL07-1122023	CombinedSNP_MAL07_1122023	ATGATATATTATTTTATTGTATTAATTATATCATTATGTTTAAAGGAAAGGAAATATTTT[A/G]AATTGTTGAAAAAAAAGATAAAAATGGATATTATACAATGGCTTCATTATGGTTTTGTAA	7	A	C	0.44
*MAL07-1289206	CombinedSNP*MAL07_1289206	TTTAATAACTTGTTTTTTTTTATTATTGAATTGTGCTTTTTCTACATGAATTATGCAATT[T/C]TGTCTAAAGAGAAAATTATCAAAATATTTGATGGCCATGTCTACACTTTGTGTATATACA	7	G	A	0.15
*MAL07-1311707	CombinedSNP*MAL07_131170	TTAATAAATAATAATTTTAAACATGATGGAGTTGGTAATATAAATATG	7	A	T	0.08

	7	TATGATGGAAGG[T/C]TTAATAAA AATGAAGAGCAAAATTATTTTACC ACACCCTTGATGGAACATAAAAAT ATAA				
MAL07-1323949	CombinedSNP_MAL07_1323949	GGTTGATGAAGCCATGAATAAAA ATAAAATTGTTTTAGATTCATTAG AAAAAATGAAAAC[A/C]TGTGAAG TATGTTTTGATTGTAATGAACTAA AATCGTTAATTGAAATTATTCAGA AAAAG	7	A	G	0.31
MAL08-417613	CombinedSNP_MAL08_417613	AAAAGATAAACTGCCTAATAAATA TGCATGGTTTCTTTCGGATGGTAC ATCGATTTGCAT[A/G]TAGGCAGG CATACATACATCATCGTTTAAAAT CATATATTCTTCTGGATGTAATTCA ATG	8	G	A	0.41
MAL08-477431	CombinedSNP_MAL08_477431	AAATATAAATGTACCACAAACACA TGATAAACAAAAGTGTAAGGAAA ATTTTTCAAATTC[A/T]TTTGTTAAT GAAATATATGAAAAAGAAGAAAA AGAAACGCAAAAAATGAAAGAAT ATTTA	8	T	T	0.43
*MAL08-502163	CombinedSNP*MAL08_502163	TTTAATTTTTACACCTATGCTCTTC CTTATATATTGAGTTAAAATTTCC GTTTTCTTTAC[T/C]TCGCAAGAAT ATACTTGTGAAGCATTAAATATAC AAAAAGGAATAGATTCATCACTTA TT	8	G	A	0.12
MAL08-1057901	CombinedSNP_MAL08_1057901	AAGAATCTTTCTATTATGGGTTTGT GTAAAGAGAAAATAGTGTTAACA GAAAAGGCAACA[A/C]TATTTAAT AACATTGAATTGTTGTATTCATATT ATCTTATAGTTCAGAACGAAGCTT TAG	8	C	A	0.34
MAL08-1101130	CombinedSNP_MAL08_1101130	CATGAACACAATTTAAAAAAAATA TTAGTACCCAGACATTGCATACCG CC[A/G]CAGGATTATCAAAATGAA GAATTGATAGCAAAAGATGGACA AATTAATAA	8	G	G	0.21
MAL08-1292195	CombinedSNP_MAL08_1292195	ATTTTGAGATTTCTGTAATTATATT ATCAAGGTCTATCATAGTAGAAGA AGGAGAGGGGT[A/G]GTCTATGTT AGTTGATCTTGTTAAATCAGAATT ATTTTTGACTCCTTCAAGTATTTCA TA	8	G	T	0.39
MAL08-1311981	CombinedSNP_MAL08_1311981	AAATCTACTCCTGAAATACTTAAA AAAGTATGGGAAAGTGATATAGA TAAAAATTTACCA[C/G]GCTTGATG GAGAAAACAACCAAAAATACTTG TGTTAGAGTTGCACATGATTATGA AAAGG	8	C	A	0.37
*MAL09-492618	CombinedSNP*MAL09_492618	TATATATAACACTATTCAAAATTG TCCAATTGATTATAGAAAGGAATT GCTAAGAAATAT[T/C]TATCTTACA GGAGGTTCGAGTATTATAAGAGGC TTTAGACAAAGATTGGAGAATGAA CTC	9	A	G	0.24
*MAL09-631767	CombinedSNP*MAL09_631767	AGATCTTTCTTTATGACCAATGCA TATGATTTAACAACCGTCTTTTCTT TATCAACAGTT[T/C]TGTCTTTTTTA CCATCTCCACTTTTTGGATATATTA GTGGCAAATTAGGTTCTGTTTATG	9	A	G	0.22
MAL09-692487	CombinedSNP_MAL09_692487	ATTTTTATTATTACTATTAATATTC TTGTAATTGTTGAAATGTGTTATAT TGGGCATACA[A/C]ACATTTTTATA ATTTTCCGAAGTCTTTACATTAACT TTTTCTATAACAAAATTCCACGGC	9	A	C	0.28
MAL09-771381	CombinedSNP_MAL09_771381	AAACGACACAAATGCTAAATGCTT TTGTTCCTTTTGATTTGGATCCTCC ACCTTTGTATG[A/C]TGAAAATCCT	9	A	C	0.5

		GAAACAGTTAGGTAAAATATTATA TCCAAAATGGAATAAATACACCCA TA				
*MAL09-1111191 - RIGHT SEQ BUT	CombinedSNP* MAL09_1111191	ATCAAAAAAAAACATTAGTAACAT AATAAATAATAATGTAATATTAAA TAACTGTTCAAA[T/C]AAGGTGTCT AATAATGATTTCGATGATGAAAAG GACGCATATTTACAACATGAGTGG AAT	9	A	G	0.44
*MAL10-113266 - RIGHT	CombinedSNP* MAL10_113266	ATGTACATATTTTATTTTATTTTAT TTTTACATTGTTGTCATTCTTTTTC TTTTTTGCAG[T/C]ACATTTTTAAG AGGACACGAATAAAAATCATCGA ATCATTAAATAATGATAACAATGT AG	10	G	A	0.48
MAL10-317580	CombinedSNP_ MAL10_317580	AAGGATGATGATTCTATAATAGAT TTATTAACTGTTAATGAAAATTCT AT[A/T]GATGCCCCAACACAAGAT AATGAAAATAAAATTAATAGTTTA CAAGCATT	10	T	A	0.37
MAL10-336274	CombinedSNP_ MAL10_336274	AACCATATGACACCTAAGATGAAT TTTAATATTATGGAATATCTAATG TCTGATAATTAT[A/G]TTCATATTA ATTCTCCTCAAAGTTGTTTTAATAC ATTAACCATAAAAAATGAATTACA TG	10	A	T	0.46
MAL10-404541	CombinedSNP_ MAL10_404541	AGATATGACCCTATGGGCCCTTTT GGTAACGAGCCAAATTCTGACAAT AG[A/G]CCGTTTGAATATCAAAAT AATTTCCCCTTTTAAAATTTTTTAT TCTAATT	10	G	A	0.49
MAL10-631275	CombinedSNP_ MAL10_631275	TGGCTTAGATCAATCATATTTTCAT TTACTTGTACTTCTTCCTTTTCTTT AAATAAATAA[A/T]CATCATCAGG TAGGAGGAAATTATCATCATAATT AAATTCATTAGGAGTATATGCTTT TT	10	A	T	0.22
*MAL10-663198	CombinedSNP* MAL10_663198	TACTATAACCACTATAATCCGTAG TATTAGAATTATGATGTCGTAAAT CATCTGTGCCTT[T/G]AGTAGTACC ACATGCCATTTCATCTACACCTAC ATTTTGATATTCTACAAAATGATC ATT	10	A	T	0.26
MAL10-1300115	CombinedSNP_ MAL10_1300115	TAAAAAATGTAATAATCATAATAA ATATAGAAAGATCGATGAAAATGT AAAAGGTATACA[A/T]CTTGTTGAT GGTTCAAAAAAAAATATACAAAA TGATGAATATACATGTGATGATAA CCAA	10	T	A	0.19
*MAL10-1385891	CombinedSNP* MAL10_1385891	TAATAATATAACCGTTAATGCAAT CATTCCACATGTACGTAAAGAAAT ATTTACATTTCC[T/C]ATAGAAGAA AGAATTCCAACAGGAACTTTAATT CAAGGAAAAAATTATAAAACACTT GTT	10	A	C	0.3
MAL11-185094	CombinedSNP_ MAL11_185094	GTATATCCATTTGTGGATTATAAT CCGAATCAAATAAAATAACAATAT CAGCAGTCGTTA[A/G]GTTAATACC AATACCACCAGCTCTGGTAGATAA TAAGAAAATAAAATATTTACTATT CGG	11	A	C	0.43
MAL11-255830	CombinedSNP_ MAL11_255830	CCATAAATAATTCATCTGCTTGTTC TATATGTTTAATATCAATAATTGT GTTACCTTCTA[A/G]GGTAGCTAAA ATTCTGGATGGTTCTAATAATAAC ATAGCAAATCTTAGAGATGACTGT AT	11	G	T	0.47
*MAL11-281726	CombinedSNP* MAL11_281726	ATTATGTAATTCTTTTTTAACTTTA GATTTTATACCTATACGAACAACA TTCTTAATATT[T/C]AAATCTTCAA CACATTTTCTTAAGATATTATCAA	11	A	A	0.37

		CACTTACATTGCTAGGACCTGTTACT				
*MAL11-304323	CombinedSNP*MAL11_304323	TTATAATAGTGTGCATTATTTTCCTTTTCTTTAATACTTGCCTTGCTACT[T/C]CTTTTCTTATCCTCATCTTCATTTCTTTTTTTCATATTTTCATTATTTAC	11	G	A	0.14
*MAL11-683964	CombinedSNP*MAL11_683964	AATAGCTAGTTCAATTTCATACGCATCAACAGGTAATTTCCCTGTATATTGCAAAACATC[T/C]TTTTGTGTGTGCAAAAATAAACCAGCATTTACGTCTAGTACAATTTTGCCATCAAAAATA	11	G	A	0.4
MAL11-769993	CombinedSNP_MAL11_769993	CCATGTGAATATAATATTGTCTGTTTATTTAAATCCAAAGGTTTCTTATATAACATTATA[A/C]CAGATACTTCCGTCGAACCTCTTTTTAGTTTTTTATAATTTATATCGATATTATTTATCT	11	A	A	0.15
MAL11-896199	CombinedSNP_MAL11_896199	CTATTGGTATCATTCACACTGTTTGTATAATTCACATTATTTCCATCCAC[A/G]TTTTTGAGATTCATATTTGTTGAATCAGTAGGATCTTTTATTATAGTACT	11	A	G	0.23
MAL11-1520975	CombinedSNP_MAL11_1520975	CAAAAAAATGAAAATTTACTAAACGAAAACAATATGGATAATATAAACATGCAGGAAAAA[A/T]GTGAGACGGAAGAAGATTTAGATTTAGAAAGAGATGAAGATAAACATGATGCTCCACCTG	11	A	A	0.29
MAL11-1749804	CombinedSNP_MAL11_1749804	ACAGCTCCTGAATAAACTTCACTACCTCCTATAACACAAATTTTACCTGA[A/G]CACCCTTTATATTCATCTTTGCGGAGTTTCGGAACAATCACATCCTTGAT	11	G	A	0.39
MAL11-1802198	CombinedSNP_MAL11_1802198	TTCTAGTACCTGTTCAGATAACTTTGGGAATCGATTTAATACTCTGTTGGCTATTTTTTG[A/G]GATAAAATTTCTAGTGTCTGACTTACTCTATCTAGACAATTAAAAACAGGTGCATTAATT	11	A	A	0.32
*MAL12-83177	CombinedSNP*MAL12_83177	TCTTTTTTCATTTCCTTGTTTATTTGAATATTCATCGTCATCATCATCTTTTTTTAAATA[T/C]AAATGCATAATAGCGTTTTTAAAATCTGCCTCTCTTATATGTTTCATTTTCTCTATATCG	12	G	A	0.15
*MAL12-114173	CombinedSNP*MAL12_114173	TACTTATGGAAAAATAGAAAAAGACTTTGCTTTGATTAATGCTAGATTGGAGACCCTACA[T/C]ATAAAAAAATCATTCAGGTTTTTAAAATGATAATCCATATATATAAATGAATAAGTATAT	12	A	G	0.18
*MAL12-529412	CombinedSNP*MAL12_529412	TTCCCTCAAGTTCATCATTGTATCCATCAGACGTTTTATTATCCTTTGGCCTACACCATC[T/C]ATTTTCATCAAGTGTTAAATCACCACTACAACCATCTTGCATTCTTAATCTTAATTCTGG	12	A	G	0.29
*MAL12-1116275	CombinedSNP*MAL12_1116275	ATATGTTATTTTCTATTTTTTGTGTGTCCTTTTTTGTGTTTGCACTATCTTGATTAATAT[T/C]TCTAATGTCCGTTGTGCACGTGGATGAATAAGATTGTTGAAATTTTCTTTTTTTTTTATT	12	G	A	0.48
MAL12-1131597	CombinedSNP_MAL12_1131597	CTTTTTCATGTCTATAATATTTTCTTCATCATTTATTTTATCATTTGTGGTCGAATTATC[A/G]TTTTTTATATTTTCCAAATCTTCACCATATACATATTTTTTGGATATATTACTATCCTCG	12	G	T	0.34
*MAL12-1156125	CombinedSNP*MAL12_1156125	AACAATATGAGTAGTTCAAAGAGACATATACCACATGACATGAACCCAGA[T/C]GATAATATTGAGAAATATTTTTTCAAATCTTTGTAGTGGTATAGAGGTAAT	12	G	A	0.35

*MAL12-1591944	CombinedSNP*MAL12_1591944	GACTACAAGCATTTTATCATAAAATAAATCACCCAGCTATATCGCCATTT[T/C]TAATAGGATTATTTCCTACAAATAATGCACAAAATATAAGATTCTGTATT	12	G	A	0.37
*MAL12-1761553	CombinedSNP*MAL12_1761553	ACTATTTATTATATTTATATTATCATTACTATTATTGTTTATCGCATTTTCAGGATATCC[T/C]ATCCAGTTGGTGCTATTATTTCCTTCATCACATTTATCAACATAATCATCCTTAACATAC	12	G	AT	0.29
MAL12-2046727	CombinedSNP_MAL12_2046727	TTCCAATTTATATGTATTATTTTTTAATTGATAAAAATGACCATCTTTATGATTTGTTAT[A/G]TCTAATAAGGAATGAGAATGTTGCAAAAATTTTATTATTAATTTTTTAGTAACATCTATA	12	G	AT	0.47
MAL13-146947	CombinedSNP_MAL13_146947	TTATTTTATTGGTATTACTCAACCAAGAAAAATAGCTGTAAAAAGTATTTGTAATAGATT[A/G]AACGATGAACTAAATGAAGAAATATGTGGTTATCAAATTAGGTTTGAAAAATCGTATTTT	13	G	A	0.39
*MAL13-210569	CombinedSNP*MAL13_210569	TAATAATTATTACATATCATCTTTTTTTTATTATCGGTATAAAATTCAGTATTTTCTTTT[T/C]CCTTTATCTTATTCTTTCTTTTCTCCTGCCCCTTACTATTATTTGATATTCTTCTTAACC	13	A	A	0.29
MAL13-619422	CombinedSNP_MAL13_619422	TTTTTTCAGAGCCTGCAACTTTCCACGTGAACCTACCCTAATATTTACCA[A/G]TCCTTTTTTATTAATATTATAAAGGGTATGTGATATATTCTTGTAATAAT	13	A	T	0.46
*MAL13-1233419	CombinedSNP*MAL13_1233419	TTTACATAATTTTCGACGTCATCATTTTCATTATTACTCATAATTGAATTATCCACAATG[T/C]TTGCCTCTTTTACAGCTTTACAAAGTTGGTAAAAAATATGATAATTCCTTTCACCTTCTT	13	A	A	0.34
MAL13-1966774	CombinedSNP_MAL13_1966774	CGTGTCACATTTTATTAAACCTAAATCAATTAACTGTTCAGGTTTTTTTA[A/G]TATACCACTTTCTTCATGTAATTCTTTTACAGCATTTTGTAAAAAGTCTT	13	A	T	0.29
*MAL14-990875	CombinedSNP*MAL14_990875	AGTAGTGCTCATAACAACATGCATCATAATATAAATCATCAAGAAAATTA[T/C]AGAAATCAAAACAGCTATAGCCACTTTTCTGGTCATGGTAGCTATATCGA	14	G	A	0.32
MAL14-1199184	CombinedSNP_MAL14_1199184	TACAGTATATACTTTAATATCTGTACCAAAGGTAGATAAAAATTCTTCATATATATATGT[A/T]CCATTAGTTCGAACTTTATGTATTTCTGGACAATATTCACTACTCCTATCCTTAATTTTA	14	A	A	0.38
MAL14-1458321	CombinedSNP_MAL14_1458321	TAGTTGAATATGCTGAAGAGGTAGAATTAAAGAAGAAGGTTGAAAAAACATTTATGGGAG[A/G]AAAAGTTAATATGACAGAGAATCGCAGTGTTTTACATACAGCTTTAAGAATACCCATAGA	14	G	T	0.45
MAL14-1757546	CombinedSNP_MAL14_1757546	AGAGGTACATATTAATAGATTTTTTAAATGAAAATAGTAATAAAAATAATGTAACGCATG[A/G]TGATAGTGTATTACCAAATGGTAATGAAACGGACGCGAAAAAAAATGTGGATGAGAATAA	14	A	A	0.32
MAL14-1853537	CombinedSNP_MAL14_1853537	ATATGCTCGTATATCATTCAAAATATTAAGATGCATGATTATATCTGTAATAAGATGAGT[A/G]ACATTTATAAATACACAATTCAATTTTTTTAACATTTCAACAGGACTATTATTCAGGTAT	14	A	T	0.36

MAL13-146947	CombinedSNP_MAL13_146947	TTATTTTATTGGTATTACTCAACCAAGAAAAATAGCTGTAAAAAGTATTTGTAATAGATT[A/G]AACGATGAACTAAATGAAGAAATATGTGGTTATCAAATTAGGTTTGAAAAATCGTATTTT	13	G	A	0.39
*MAL13-210569	CombinedSNP*MAL13_210569	TAATAATTATTACATATCATCTTTTTTTTATTATCGGTATAAAATTCAGTATTTTCTTTT[T/C]CCTTTATCTTATTCTTTCTTTTCTCCTGCCCCTTACTATTATTTGATATTCTTCTTAACC	13	A	A	0.29
MAL13-619422	CombinedSNP_MAL13_619422	TTTTTTCAGAGCCTGCAACTTTCCACGTGAACCTACCCTAATATTTACCA[A/G]TCCTTTTTTATTAATATTATAAAGGGTATGTGATATATTCTTGTAATAAT	13	A	T	0.46
*MAL13-1233419	CombinedSNP*MAL13_1233419	TTTACATAATTTTCGACGTCATCATTTTCATTATTACTCATAATTGAATTATCCACAATG[T/C]TTGCCTCTTTTACAGCTTTACAAAGTTGGTAAAAAATATGATAATTCCTTTCACCTTCTT	13	A	A	0.34
MAL13-1966774	CombinedSNP_MAL13_1966774	CGTGTCACATTTTATTAAACCTAAATCAATTAACTGTTCAGGTTTTTTTA[A/G]TATACCACTTTCTTCATGTAATTCTTTTACAGCATTTTGTAAAAAGTCTT	13	A	T	0.29
*MAL14-990875	CombinedSNP*MAL14_990875	AGTAGTGCTCATAACAACATGCATCATAATATAAATCATCAAGAAAATTA[T/C]AGAAATCAAAACAGCTATAGCCACTTTTCTGGTCATGGTAGCTATATCGA	14	G	A	0.32
MAL14-1199184	CombinedSNP_MAL14_1199184	TACAGTATATACTTTAATATCTGTACCAAAGGTAGATAAAAATTCTTCATATATATATGT[A/T]CCATTAGTTCGAACTTTATGTATTTCTGGACAATATTCACTACTCCTATCCTTAATTTTA	14	A	A	0.38
MAL14-1458321	CombinedSNP_MAL14_1458321	TAGTTGAATATGCTGAAGAGGTAGAATTAAAGAAGAAGGTTGAAAAAACATTTATGGGAG[A/G]AAAAGTTAATATGACAGAGAATCGCAGTGTTTTACATACAGCTTTAAGAATACCCATAGA	14	G	T	0.45
MAL14-1757546	CombinedSNP_MAL14_1757546	AGAGGTACATATTAATAGATTTTTTAAATGAAAATAGTAATAAAAATAATGTAACGCATG[A/G]TGATAGTGTATTACCAAATGGTAATGAAACGGACGCGAAAAAAAATGTGGATGAGAATAA	14	A	A	0.32
MAL14-1853537	CombinedSNP_MAL14_1853537	ATATGCTCGTATATCATTCAAAATATTAAGATGCATGATTATATCTGTAATAAGATGAGT[A/G]ACATTTATAAATACACAATTCAATTTTTTTAACATTTCAACAGGACTATTATTCAGGTAT	14	A	T	0.36
MAL13-146947	CombinedSNP_MAL13_146947	TTATTTTATTGGTATTACTCAACCAAGAAAAATAGCTGTAAAAAGTATTTGTAATAGATT[A/G]AACGATGAACTAAATGAAGAAATATGTGGTTATCAAATTAGGTTTGAAAAATCGTATTTT	13	G	A	0.39
*MAL13-210569	CombinedSNP*MAL13_210569	TAATAATTATTACATATCATCTTTTTTTTATTATCGGTATAAAATTCAGTATTTTCTTTT[T/C]CCTTTATCTTATTCTTTCTTTTCTCCTGCCCCTTACTATTATTTGATATTCTTCTTAACC	13	A	A	0.29
MAL14-3017684	CombinedSNP_MAL14_3017684	ACACATCCAGAGGAGAGTATTTCATCTTGAATAAGTCATCCGTTAAAACATTCTGAAGAG[A/T]AGATCTATCTTTCTTCTTATCCAATAAAACATTTTTCTCTCGGTTCATATCATGAAGC	14	T	A	0.37

		GG				
MAL14-3124926	CombinedSNP_MAL14_3124926	TGTGAAGAAGAAAAAGAATTAATGGGAGCAGCTGGAGGAAGATATGTCGAAGAAGAGGTA[C/G]AGGGAGCAGTTGGAGGAAGAAATGTGGATGAAGAGGTAGAGGGAGCAGTTGGAGGAAGAA	14	C	G	0.34
*MAL12-262199	CombinedSNP*MAL12_262199	TATATGAATTATATTTTATTTATTTATTTCTTTTTTAAGGATTTGGATAA[A/G]CCGGAAAGAACTTTACAAGATACCGAAATTGTTAGACCTCAAAACAAACT	12	T	C	nd
*MAL04-809686	CombinedSNP*MAL04_809686	ATATCAAAACATAACAAGTTATATCTTCAGTATGATCAAAATAAAAGAAACTCTGAAATA[A/G]AACATACAAATCACAAAGAAGATTATTCGATGTGCGACAATGTGATAACAAGCAAAATGA	4	G	C	nd

APPENDIX III: Pooled Primers for SNP Genotyping

POOL	WELL	SNP_ID	2nd-PCRP	1st-PCRP
	W1	CombinedSNP_MAL14_3124926	ACGTTGGATGCCACATTTCTTCCTCCAACTG	ACGTTGGATGGAATTAATGGGAGCAGCTGG
	W1	CombinedSNP*MAL11_304323	ACGTTGGATGATAGTGTGCATTATTTTCC	ACGTTGGATGAAAAGAAATGAAGATGAGG
	W1	CombinedSNP_MAL10_317580	ACGTTGGATGTTTCATTATCTTGTGTTGG	ACGTTGGATGGGATGATGATTCTATAATAG
	W1	CombinedSNP_MAL11_896199	ACGTTGGATGGGTATCATTCACACTGTTTG	ACGTTGGATGAAGATCCTACTGATTCAAC
	W1	CombinedSNP_MAL08_1101130	ACGTTGGATGTATTAGTACCCAGACATTGC	ACGTTGGATGTTGTCCATCTTTTGCTATC
	W1	CombinedSNP*MAL12_114173	ACGTTGGATGGATTAATGCTAGATTGGAGAC	ACGTTGGATGGGATTATCATTTTAAAAACC
	W1	CombinedSNP_MAL10_404541	ACGTTGGATGCTATGGGCCCTTTTGGTAAC	ACGTTGGATGGGGGAAATTATTTTGATATTC
	W1	CombinedSNP_MAL11_769993	ACGTTGGATGAAAAAGAGGTTCGACGGAAG	ACGTTGGATGCCATGTGAATATAATATTGTC
	W1	CombinedSNP*MAL11_683964	ACGTTGGATGGCATCAACAGGTAATTTCCC	ACGTTGGATGGTACTAGACGTAAATGCTGG
	W1	CombinedSNP_MAL14_3017684	ACGTTGGATGCATCTTGAATAAGTCATCCG	ACGTTGGATGGCTTCATGATATGAACCGAG
	W1	CombinedSNP_MAL12_2046727	ACGTTGGATGTTTGCAACATTCTCATTCC	ACGTTGGATGAATTGATAAAAATGACCATC
	W1	CombinedSNP*MAL05_385372	ACGTTGGATGCGATGATCTAAAAGATCTTG	ACGTTGGATGATATGATCATATTCATGTTC
	W1	CombinedSNP_MAL13_619422	ACGTTGGATGTGCAACTTTCCACGTGAACC	ACGTTGGATGCAAGAATATATCACATACCC
	W1	CombinedSNP_MAL08_1292195	ACGTTGGATGTGAAATACTTGAAGGAGTC	ACGTTGGATGCTATCATAGTAGAAGAAGGAG
	W1	CombinedSNP*MAL14_990875	ACGTTGGATGACCATGACCAGAAAAGTGGC	ACGTTGGATGGTGCTCATAACAACATGCATC
	W1	CombinedSNP*MAL04_268436	ACGTTGGATGGTCCAAAATGTGGTATATC	ACGTTGGATGAACCTTTGTTGTATTTTCG
	W1	CombinedSNP_MAL07_1122023	ACGTTGGATGCATTATGTTTAAAGGAAAGG	ACGTTGGATGAACCATAATGAAGCCATTG
	W1	CombinedSNP*MAL03_249686	ACGTTGGATGCTCTTTTTTTCTTTTTGCAC	ACGTTGGATGATACAAAATAAAGAAGAGG
	W1	CombinedSNP_MAL14_1458321	ACGTTGGATGAAACACTGCGATTCTCTGTC	ACGTTGGATGTGAATATGCTGAAGAGGTAG
	W1	CombinedSNP_MAL07_511352	ACGTTGGATGTTTACTTACCATTTTGATTG	ACGTTGGATGCTTTTCATATATACCTGTC
	W1	CombinedSNP_MAL04_1140617	ACGTTGGATGCATAATTATACGATTCTACCC	ACGTTGGATGAAATAATGAGAATTCTGAAG
	W1	CombinedSNP*MAL06_573730	ACGTTGGATGCAGTATTATTCTTTTCTGG	ACGTTGGATGCGCTCAAGGAAATTTTACAC
	W1	CombinedSNP_MAL03_439621	ACGTTGGATGAACAAAGAAAGTATTGGAC	ACGTTGGATGGATGGTATCCTATTATTAGC
Vial 1	W1	CombinedSNP_MAL08_477431	ACGTTGGATGTTTGCGTTTCTTTTTCTTC	ACGTTGGATGCCACAAACACATGATAAAC
	W2	CombinedSNP_MAL11_1520975	ACGTTGGATGGGTGGAGCATCATGTTTATC	ACGTTGGATGGGATAATATAAACATGCAG
	W2	CombinedSNP_MAL04_443828	ACGTTGGATGGTAGTTCATTAACGGCACGC	ACGTTGGATGAACTATTAGATTTTGAGATG
	W2	CombinedSNP*MAL12_1591944	ACGTTGGATGTAAATCACCCAGCTATATCG	ACGTTGGATGCAGAATCTTATATTTTGTGC
	W2	CombinedSNP*MAL10_663198	ACGTTGGATGGTAGATGAAATGGCATGTGG	ACGTTGGATGTAACCACTATAATCCGTAG
	W2	CombinedSNP_MAL04_469608	ACGTTGGATGCCATGATTTTCGGCAACGGG	ACGTTGGATGTCTTGTTTTTCAAAAGCCC
	W2	CombinedSNP_MAL08_1057901	ACGTTGGATGGGGTTTGTGTAAAGAGAAA	ACGTTGGATGGCTTCGTTCTGAACTATAAG
	W2	CombinedSNP*MAL13_1233419	ACGTTGGATGTACCAACTTTGTAAAGCTG	ACGTTGGATGTTTCGACGTCATCATTTTC
	W2	CombinedSNP_MAL11_255830	ACGTTGGATGGTTATTATTAGAACCATCCAG	ACGTTGGATGCATCTGCTTGTTCTATATG
	W2	CombinedSNP_MAL04_	ACGTTGGATGTCTGCATAA	ACGTTGGATGTGGAATTATAA

		307321	AATGGTTGTGG	TTTAATGGG
	W2	CombinedSNP_MAL12_1131597	ACGTTGGATGATTTTATCATTTGTGGTCG	ACGTTGGATGGTATATGGTGAAGATTTGG
	W2	CombinedSNP_MAL07_476303	ACGTTGGATGCCTCTCAGGAGGAAATTAATG	ACGTTGGATGCCCTCAAACATTTTTGCTCC
	W2	CombinedSNP_MAL03_748204	ACGTTGGATGTCACCTGCCATACCATCTTC	ACGTTGGATGCTGTAAGAAATTCATATGGAG
	W2	CombinedSNP_MAL13_146947	ACGTTGGATGCAAACCTAATTTGATAACCAC	ACGTTGGATGCTCAACCAAGAAAAATAGCTG
	W2	CombinedSNP_MAL13_1966774	ACGTTGGATGGACTTTTTACAAAATGCTG	ACGTTGGATGCGTGTCACATTTTATTAAACC
	W2	CombinedSNP_MAL11_1802198	ACGTTGGATGAGATAGAGTAAGTCAGACAC	ACGTTGGATGCCTGTTCAGATAACTTTGGG
	W2	CombinedSNP_MAL11_1749804	ACGTTGGATGTCCGAAACTCCGCAAAGATG	ACGTTGGATGCAGCTCCTGAATAAACTTCAC
	W2	CombinedSNP*MAL12_83177	ACGTTGGATGGAAACATATAAGAGAGGCAG	ACGTTGGATGATTCATCGTCATCATCATC
	W2	CombinedSNP*MAL12_529412	ACGTTGGATGGCAAGATGGTTGTAGTGGTG	ACGTTGGATGCATCATTGTATCCATCAGACG
	W2	CombinedSNP_MAL02_217337	ACGTTGGATGTTGGAAAACATCCCTCCTTC	ACGTTGGATGTTTTTTTATTTTTTTGTATG
	W2	CombinedSNP_MAL11_185094	ACGTTGGATGTACCAGAGCTGGTGGTATTG	ACGTTGGATGCCATTTGTGGATTATAATCCG
	W2	CombinedSNP*MAL06_593821	ACGTTGGATGCTCAAAACATTGTGCATAC	ACGTTGGATGTTGTGTGTTTAGGGTAATC
	W2	CombinedSNP*MAL01_354427	ACGTTGGATGGCTATGTATGTTATAACATC	ACGTTGGATGGAATACCATAAACGAAGAAC
	W2	CombinedSNP_MAL14_1853537	ACGTTGGATGAGATGCATGATTATATCTG	ACGTTGGATGCCTGAATAATAGTCCTGTTG
Vial 2	W2	CombinedSNP_MAL04_994352	ACGTTGGATGAATTGAGGAACGTAATGAGG	ACGTTGGATGCATATTATTTAAATGATTGG
	W3	CombinedSNP_MAL05_720978	ACGTTGGATGTAAAAATAATTTTGCTCATC	ACGTTGGATGAAATCTCTAGCCTTTGATCG
	W3	CombinedSNP*MAL12_1761553	ACGTTGGATGGATGAAGGAAATAATAGCACC	ACGTTGGATGTGTTTATCGCATTTTCAGG
	W3	CombinedSNP_MAL10_631275	ACGTTGGATGGATGATAATTTCCTCCTACC	ACGTTGGATGCATTTACTTGTACTTCTTCC
	W3	CombinedSNP_MAL07_759749	ACGTTGGATGTTCCCTTTTTCTTCTCCTCC	ACGTTGGATGAGGATTAAGGAGAAACACAG
	W3	CombinedSNP*MAL08_502163	ACGTTGGATGATGCTTCACAAGTATATTC	ACGTTGGATGTTTTACACCTATGCTCTTCC
	W3	CombinedSNP*MAL02_660994	ACGTTGGATGGATTTATAAGACCATGGAAG	ACGTTGGATGGTCCAAATGTGTTTTATACC
	W3	CombinedSNP_MAL06_1206499	ACGTTGGATGGATGAATTAGATGAAGAAG	ACGTTGGATGTGTACTTCCTCCCAACCCAG
	W3	CombinedSNP_MAL03_822470	ACGTTGGATGGATATATTTTCTTCAATGTC	ACGTTGGATGAACTCTTTTTATAACATGAG
	W3	CombinedSNP*MAL10_113266	ACGTTGGATGCATTGTTGTCATTCTTTTTC	ACGTTGGATGCGATGATTTTTATTCGTGTCC
	W3	CombinedSNP_MAL08_417613	ACGTTGGATGAACGATGATGTATGTATGCC	ACGTTGGATGGCATGGTTTCTTTCGGATGG
	W3	CombinedSNP_MAL07_1323949	ACGTTGGATGCGATTTTAGTTCATTACAATC	ACGTTGGATGGGTTGATGAAGCCATGAATA
	W3	CombinedSNP*MAL07_543849	ACGTTGGATGGAAATGAATAAAGATTCCAG	ACGTTGGATGCTCCATATCTATTGTAGAATC
	W3	CombinedSNP_MAL10_336274	ACGTTGGATGTTAAAACAACTTTGAGGAG	ACGTTGGATGACCATATGACACCTAAGATG
	W3	CombinedSNP_MAL06_410691	ACGTTGGATGGTTGTGGTCATCTTCAACTA	ACGTTGGATGTAATTCGAAATTTTATTCCC
	W3	CombinedSNP*MAL13_210569	ACGTTGGATGGGGCAGGAGAAAAGAAAGAA	ACGTTGGATGCATCTTTTTTTTATTATCGG
	W3	CombinedSNP*MAL09_492618	ACGTTGGATGCCAATCTTTGTCTAAAGCCTC	ACGTTGGATGGTCCAATTGATTATAGAAAGG
	W3	CombinedSNP_MAL06_1184507	ACGTTGGATGGTGATAATTATGTGTTGAC	ACGTTGGATGTTTCTACAGATTTGTTCTC
	W3	CombinedSNP*MAL11_281726	ACGTTGGATGAGGTCCTAGCAATGTAAGTG	ACGTTGGATGACCTATACGAACAACATTC
	W3	CombinedSNP_MAL01_539149	ACGTTGGATGGAATATAAAGATTTTATGCG	ACGTTGGATGCTCTTTTCAGTGGATGCCAT

	W3	CombinedSNP*MAL04_809686	ACGTTGGATGCAAGTTATATCTTCAGTATG	ACGTTGGATGTATCACATTGTCGCACATCG
	W3	CombinedSNP*MAL07_1311707	ACGTTGGATGTTAAACATGATGGAGTTGG	ACGTTGGATGTATGTTCCATCAAGGGTGTG
Vial 3	W3	CombinedSNP*MAL03_704420	ACGTTGGATGTGTGTTTTTAATATTTGAAC	ACGTTGGATGGCTCAAATGAAAATCATAAC
	W4	CombinedSNP*MAL12_1156125	ACGTTGGATGAGAGACATATACCACATGAC	ACGTTGGATGACCTCTATACCACTACAAAG
	W4	CombinedSNP*MAL04_933828	ACGTTGGATGCACTAAATTTCTCATCCTTC	ACGTTGGATGTGTGGAATGTGGGAATGGAG
	W4	CombinedSNP_MAL09_771381	ACGTTGGATGGGGTGTATTTATTCCATTTTG	ACGTTGGATGTTTGATTTGGATCCTCCACC
	W4	CombinedSNP_MAL09_692487	ACGTTGGATGGTTGAAATGTGTTATATTGGG	ACGTTGGATGGCCGTGGAATTTTGTTATAG
	W4	CombinedSNP*MAL09_631767	ACGTTGGATGAACAGAACCTAATTTGCCAC	ACGTTGGATGGCATATGATTTAACAACCGTC
	W4	CombinedSNP_MAL06_937750	ACGTTGGATGGAAACTAAAAAATAAAGGAG	ACGTTGGATGCTCCAATACCTATAGGAGGA
	W4	CombinedSNP_MAL14_1757546	ACGTTGGATGTTCGCGTCCGTTTCATTACC	ACGTTGGATGGAGGTACATATTAATAGATT
	W4	CombinedSNP*MAL06_154776	ACGTTGGATGTTTCTTTACGATGTACTGC	ACGTTGGATGTGATGAGAATGTTAAAAGG
	W4	CombinedSNP_MAL14_1199184	ACGTTGGATGGGAGTAGTGAATATTGTCCAG	ACGTTGGATGATATCTGTACCAAAGGTAG
	W4	CombinedSNP_MAL08_1311981	ACGTTGGATGCATGTGCAACTCTAACACAAG	ACGTTGGATGGTATGGGAAAGTGATATAG
	W4	CombinedSNP_MAL04_393114	ACGTTGGATGTCACACTCTTGAAATGGTTC	ACGTTGGATGTAACCACCAAGGTAACGAAC
	W4	CombinedSNP*MAL12_1116275	ACGTTGGATGTATTCATCCACGTGCACAAC	ACGTTGGATGGTGTCCTTTTTTGTGTTTGC
	W4	CombinedSNP_MAL10_1300115	ACGTTGGATGCATCACATGTATATTCATC	ACGTTGGATGGATCGATGAAAATGTAAAAGG
	W4	CombinedSNP*MAL07_672051	ACGTTGGATGCGTTTTCTTCTTTAGGAACAG	ACGTTGGATGTTTCAAACGAAAATGGGAC
	W4	CombinedSNP_MAL02_322398	ACGTTGGATGCATTTGAACCCTTCAGTTTGG	ACGTTGGATGTGTGAACTCCATTGGAATGA
	W4	CombinedSNP*MAL09_1111191	ACGTTGGATGATGCGTCCTTTTCATCATCG	ACGTTGGATGCAAAAAAAAACATTAGTAAC
	W4	CombinedSNP*MAL10_1385891	ACGTTGGATGGTTCCTGTTGGAATTCTTTC	ACGTTGGATGGCAATCATTCCACATGTACG
	W4	CombinedSNP*MAL07_1289206	ACGTTGGATGAAAGTGTAGACATGGCCATC	ACGTTGGATGTGAATTGTGCTTTTTCTAC
	W4	CombinedSNP*MAL12_262199	ACGTTGGATGTATTTCTTTTTTAAGGATTT	ACGTTGGATGTTGAGGTCTAACAATTTCGG
	W4	CombinedSNP_MAL04_1101563	ACGTTGGATGGGATATCATAGTTGTATGAG	ACGTTGGATGCTTGAAAAACCTTTCCCTGC
Vial 4	W4	CombinedSNP_MAL05_118209	ACGTTGGATGCTTCTCTTTTCTTAACCAT	ACGTTGGATGCAAAAGAGAAAAATGGAAGGG

APPENDIX IV: Successful call rates for each SNP genotyped (N=78)

SNP ID	MAJOR ALLELE	NO.	MINOR ALLELE	NO.	MI	MISSING CALLS	Call Rate (%)	MINOR ALLELE FREQ (MAF)
CombinedSNP_MAL11-1520975	T	0	A	62	1	1	84.0	0.984
CombinedSNP_MAL04-443828	A	64	G	0	0	0	85.3	0.000
CombinedSNP_MAL12-1131597	A	18	G	29	17	0	85.3	0.453
CombinedSNP_MAL07-476303	C	20	G	28	16	0	85.3	0.438
CombinedSNP_MAL03-748204	G	53	A	7	4	0	85.3	0.109
CombinedSNP_MAL13-146947	A	29	G	21	10	4	80.0	0.350
CombinedSNP_MAL13-1966774	G	24	A	28	12	0	85.3	0.438
CombinedSNP_MAL11-1802198	G	51	A	5	7	1	84.0	0.079
CombinedSNP_MAL11-1749804	G	26	A	23	8	7	76.0	0.404
CombinedSNP*MAL12-83177	T	20	C	27	12	5	78.7	0.458
CombinedSNP*MAL12-529412	T	21	C	27	15	1	84.0	0.429
CombinedSNP_MAL11-185094	G	29	A	27	7	1	84.0	0.429
CombinedSNP*MAL06-593821	G/C	43	A/T	7	13	1	84.0	0.111
CombinedSNP*MAL01-354427	T	6	C	49	9	0	85.3	0.766
CombinedSNP_MAL14-1853537	G	15	A	41	7	1	84.0	0.651
CombinedSNP_MAL04-994352	A	27	T	26	8	3	81.3	0.426
CombinedSNP_MAL07-1122023	G	44	A	11	9	0	85.3	0.172
CombinedSNP*MAL03-249686	G/C	1	A/T	60	3	0	85.3	0.938
CombinedSNP_MAL14-1458321	A	28	G	13	22	1	84.0	0.206
CombinedSNP_MAL07-511352	G	28	A	15	21	0	85.3	0.234
CombinedSNP_MAL04-1140617	A	55	G	0	9	0	85.3	0.000
CombinedSNP*MAL06-573730	C	64	G	0	0	0	85.3	0.000
CombinedSNP_MAL03-439621	A	9	G	4	49	2	82.7	0.065
CombinedSNP_MAL08-477431	A	1	T	19	43	1	84.0	0.302
CombinedSNP_MAL14-3124926	G	46	C	2	16	0	85.3	0.031
CombinedSNP*MAL11-304323	G/C	0	A/T	63	1	0	85.3	0.984
CombinedSNP_MAL10-317580	A	18	T	25	21	0	85.3	0.391
CombinedSNP_MAL11-896199	G	64	A	0	0	0	85.3	0.000
CombinedSNP_MAL08-1101130	A	30	G	15	19	0	85.3	0.234
CombinedSNP*MAL12-114173	A/T	0	G /C	33	31	0	85.3	0.516
CombinedSNP_MAL10-404541	A	63	G	1	0	0	85.3	0.016
CombinedSNP_MAL11-769993	C	6	A	48	10	0	85.3	0.750
CombinedSNP*MAL11-683964	A/T	52	C/C	1	11	0	85.3	0.016
CombinedSNP_MAL14-3017684	A	24	T	28	12	0	85.3	0.438
CombinedSNP_MAL12-2046727	A	13	G	39	12	0	85.3	0.609
CombinedSNP*MAL05-385372	G/T	29	C	18	17	0	85.3	0.281
CombinedSNP_MAL13-619422	A	58	G	0	0	6	77.3	0.000
CombinedSNP_MAL08-1292195	A	14	G	36	13	1	84.0	0.571
CombinedSNP*MAL14-990875	A/T	64	C/G	0	0	0	85.3	0.000
CombinedSNP*MAL04-268436	C	51	A/T	6	7	0	85.3	0.094
CombinedSNP_MAL05-720978	G	0	A	62	2	0	85.3	0.969
CombinedSNP*MAL12-1761553	G/C	3	A/T	53	8	0	85.3	0.828
CombinedSNP_MAL10-631275	A	43	T	7	14	0	85.3	0.109
CombinedSNP_MAL07-759749	A	30	G	22	11	1	84.0	0.349
CombinedSNP*MAL08-502163	A/T	56	G /C	1	1	6	77.3	0.017
CombinedSNP*MAL02-660994	A/C	5	T	54	5	0	85.3	0.844
CombinedSNP_MAL06-1206499	G	17	A	33	14	0	85.3	0.516
CombinedSNP_MAL08-417613	G	49	A	1	14	0	85.3	0.016
CombinedSNP_MAl07-1323949	A	15	G /C	26	23	0	85.3	0.406
CombinedSNP*MAL07-543849	G/C	26	A/T	20	18	0	85.3	0.313
CombinedSNP_MAL10-336274	A	7	G	47	8	2	82.7	0.758
CombinedSNP_MAL06-410691	A	26	G	0	38	0	85.3	0.000
CombinedSNP*MAL13-210569	C	17	A/T	27	20	0	85.3	0.422
CombinedSNP*MAL09-492618	G/C	10	A/T	41	13	0	85.3	0.641
CombinedSNP_MAL06-1184507	T/G	22	A	17	25	0	85.3	0.266
CombinedSNP*MAL11-281726	G/C	36	A/T	14	14	0	85.3	0.219
CombinedSNP_MAL01-539149	A	14	G	34	16	0	85.3	0.531
CombinedSNP*MAL04-809686	A	26	G	18	20	0	85.3	0.281
CombinedSNP*MAL07-1311707	G/C	6	A/T	50	6	2	82.7	0.806
CombinedSNP*MAL03-704420	G/C	0	A/T	63	0	1	84.0	1.000

CombinedSNP*MAL12_1156125	A/T	16	G /C	27	14	7	76.0	0.474
CombinedSNP*MAL04-933828	A/T	53	G	1	3	7	76.0	0.018
CombinedSNP_MAL09-771381	G/C	57	A/T	0	0	7	76.0	0.000
CombinedSNP_MAL09-692487	A	3	G /C	47	7	7	76.0	0.825
CombinedSNP*MAL09-631767	A/T	51	G /C	2	4	7	76.0	0.035
CombinedSNP_MAL14-1199184	G/T	15	A	25	17	7	76.0	0.439
CombinedSNP_MAL04-393114	G/C	35	A	10	12	7	76.0	0.175
CombinedSNP_MAL10-1300115	T	34	A	9	11	10	72.0	0.167
CombinedSNP*MAL07-672051	A	16	G /C	27	13	8	74.7	0.482
CombinedSNP_MAL02-322398	G	34	A	13	10	7	76.0	0.228
CombinedSNP*MAL09-1111191	G/C	43	A/T	0	14	7	76.0	0.000
CombinedSNP_MAL04-1101563	A	23	C	22	12	7	0.0	0.386

APPENDIX V: Parasite clearance rates carrying wild and mutant types compared

Genotype Analysis: H_0 we hypothesized that there is no difference in clearance half-life in isolates with the SNPs (mutant) against those without the SNP (wild-type). $p < 0.05$ you reject the hypothesis above as you will be confident that the hypothesis above is not true.

SNP	P VALUE	SNP	P VALUE
MAL14-3017684	**0.0006**	*MAL12-1591944	<3 Values
*MAL05-385372	0.2149	MAL04-469608	0.845
MAL08-1292195	0.8639	MAL08-1057901	0.5395
*MAL04-268436	0.7182	*MAL13-1233419	0.4396
MAL12-2046727	0.8639	MAL04-307321	0.8286
MAL07-759749	0.6477	MAL12-1131597	0.9546
*MAL08-502163	<3 Values	MAL07-476303	1.0000
*MAL02-660994	0.8286	MAL03-748204	0.3481
MAL06-1206499	0.8591	MAL13-146947	0.1447
MAL07-1323949	0.3884	MAL13-1966774	0.9551
*MAL07-543849	0.3969	MAL11-1802198	<3 Values
MAL10-336274	<3 Values	MAL11-1749804	0.2238
MAL06-410691	0.3710	*MAL12-83177	0.5941
*MAL13-210569	0.1292	*MAL12-529412	0.1645
*MAL09-492618	0.2238	MAL11-185094	0.5941
MAL06-1184507	0.9451	*MAL06-593821	0.8286
*MAL11-281726	0.5135	*MAL01-354427	<3 Values
MAL01-539149	0.6787	MAL14-1853537	0.1520
*MAL04-809686	0.8591	MAL04-994352	0.7756
*MAL07-1311707	<3 Values	MAL07-1122023	0.8286
***MAL12-1156125**	**0.0205**	MAL14-1458321	0.7679
*MAL04-933828	<3 values	MAL07-511352	0.4396
MAL09-692487	<3 values mutant column	MAL04-1140617	<3 values mutant column
MAL14-1199184	**0.0013**	MAL03-439621	0.4363
MAL04-393114	<3 values mutant column	MAL08-477431	0.9480
MAL10-1300115	0.1000	MAL14-3124926	0.9425
*MAL07-672051	0.3961	MAL10-317580	0.2065
MAL02-322398	1.0000	MAL08-1101130	0.5135
*MAL09-1111191	0.9480	*MAL12-114173	0.9551
MAL04-1101563	0.7441	MAL11-769993	<3 values mutant column

APPENDIX VI: Copy of KEMRI/SSC/ERC Protocol approval

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 20, 2013**

TO: **DR. BEN ANDAGALU,**
PRINCIPAL INVESTIGATOR

THRO': **DR. RASHID JUMA,**
THE DIRECTOR, CCR,
NAIROBI

Forwarded 26/3/2013

Dear Sir,

RE: **SSC PROTOCOL NO. 2518 (*INITIAL SUBMISSION*): IN VIVO AND IN VITRO EFFICACY OF ARTEMISININ COMBINATION THERAPY IN KISUMU COUNTY, WESTERN KENYA.**

This is to inform you that during the 213th meeting of the KEMRI/ERC held on 19th March 2013, the above referenced study was reviewed.

The Committee notes that the above referenced study seeks to assess the degree of artemisinin resistance in subjects presenting with uncomplicated *P. Falciparum* malaria.

The Committee concluded that due consideration has been given to the ethical issues that may arise from the conduct of the study and granted approval for implementation effective **19th March 2013**.

Please note that authorization to conduct this study will automatically expire on **18 March 2014**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **05 February 2014**.

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

You may embark on the study

Sincerely,

EAB

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

APPENDIX VII: Copy of WRAIR/ERC Protocol approval

CORRECTION

MCMR-UWZ-C 29 April 2013

MEMORANDUM FOR Director, Human Subjects Protection Branch (HSPB), Walter Reed Army Institute of Research (WRAIR), 503 Robert Grant Ave., Silver Spring, MD 20910-7500

SUBJECT: Recommendation of Approval for the Greater than Minimal Risk Human Subjects Research Protocol **WRAIR #1935**, HRPO Log #A-17502, SSC# 2518

1. I recommend approval of the protocol, **WRAIR #1935**, HRPO Log #A-17502, SSC# 2518, entitled "*In vivo* and *In vitro* Efficacy of Artemisinin Combination Therapy in Kisumu County, Western Kenya," (Protocol Version 3.3, dated 21 November 2012), submitted by Ben Andagalu, M.D., MSc, Kenya Medical Research Institute (KEMRI)/Walter Reed Project (WRP).

2. This study is funded by the Department of Emerging Infectious Diseases of the United States Army Medical Research Unit. Regulatory approval is being sought from the Kenya Pharmacy and Poisons Board (PPB) for use of the study drugs in Kenyan citizens.

3. This will be a two-arm randomized open-label trial conducted in Kisumu County, Western Kenya at the Kisumu West District Hospital, the Kisumu East District Hospital, and the Obama Children's Hospital at the Nyanza Provincial General Hospital. 118 patients (adult/child) aged between 6 months and 65 years inclusive residing within the study area, presenting with uncomplicated malaria to one of the study sites will be recruited for the study. The general objective of this study is to assess the degree of artemisinin resistance in subjects presenting with uncomplicated *P. falciparum* malaria in Western Kenya. The primary objective is to determine parasitological clearance rates by microscopy and quantitative polymerase chain reaction (PCR) assay for the first 72 hour period after first Artemisinin Combination Therapy (ACT) dose in patients with uncomplicated *P. falciparum* malaria.

Data generated by this study will provide a snapshot of the current situation regarding *P. falciparum* sensitivity to ACTs in Western Kenya. By having subjects in one of the study arms receive artesunate and then the partner drug after completion of the artemisinin phase will enable the accurate evaluation of the artemeisinin derivative without the confounding influence of the partner drug. Sequential administration of the components of an ACT drug is recognized by the World Health Organization (WHO) as one of the ways in which ACTs can be administered. There will be close follow-up of the subjects throughout the duration of the study, and as such, subjects who fail to respond adequately will receive prompt rescue treatment. Since it is largely expected that most subjects in Western Kenya will have satisfactory responses to ACTs, data from this study will provide baseline information regarding parasite characteristics when compared to data from Thailand, an area that has reported resistance to ACTs. This, in turn, will potentially enable the identification of key markers, both in the host and the parasite, that may assist in the early detection of resistance, and also to better understand the development of resistance to ACTs.

As such, the data generated from this study, both on its own and when compared to and pooled with data from similar studies that will be conducted in Ghana, Peru and Thailand, will potentially inform both local and international policy regarding ACT use for the treatment of uncomplicated *P. falciparum* malaria.

4. The WRAIR Scientific Review Committee (SRC) approved the protocol (Version 2, dated 30 May 2012) on 13 June 2012. There were no scientific changes made to the protocol between Version 2, dated 30 May 2012 and Version 3.3, dated 21 November 2012.

5. The HSPB conducted a review of the protocol (Version 2, dated 30 May 2012) and sent the Protocol Evaluation Form (PEF), dated 11 July 2012, to the Principal Investigator (PI). The PI responded to the HSPB PEF on 15 August 2012, providing both a point-by-point response and a revised protocol (Version 3, dated 1 August 2012).

The HSPB conducted a review of the updated protocol (Version 3, dated 1 August 2012) and determined that additional items needed to be addressed. The HSPB outlined additional comments in the HSPB Follow-up #1, dated 6 September 2012, and sent it to the PI. The PI responded to HSPB Follow-up #1 on 12 September 2012, providing both a point-by-point response and a revised protocol (Version 3.1, dated 12 September 2012).

The HSPB conducted a review of the updated protocol (Version 3.1, dated 12 September 2012) and determined that the protocol was ready for submission to the 10 October 2012 Institutional Review Board (IRB) meeting. The protocol was not reviewed at the 10 October 2012 IRB meeting due to time constraints, however, comments from the IRB reviewers was sent to the PI on 12 October 2012.

The PI responded to the IRB reviewer comments on 2 November 2012, providing both a point-by-point response and a revised protocol (Version 3.2, dated 26 October 2012). The HSPB point of contact (POC) reviewed the response and determined that the protocol was ready for submission to the 14 November 2012 IRB Meeting.

6. The protocol (Version 3.2, dated 26 October 2012) was reviewed at the 14 November 2012 fully convened WRAIR IRB meeting. The WRAIR IRB voted to table the protocol with stipulations. The WRAIR IRB also recommended that the protocol be submitted to the Kenya Medical Research Institute (KEMRI) Ethical Review Committee (ERC) once the stipulations have been addressed, but prior to resubmitting the protocol for review by the fully convened WRAIR IRB.

7. The KEMRI Scientific Steering Committee (SSC) approved the protocol (Version 3.3, dated 21 November 2013) on 4 February 2013. The KEMRI ERC approved the protocol (Version 3.3, dated 21 November 2012) on 19 March 2013.

8. The PI submitted the point-by-point response and revised protocol (Version 3.3, dated 21 November 2012) to address the WRAIR IRB stipulations on 6 February 2013. The HSPB reviewed the response and determined that some supporting documentation was still pending. The KEMRI ERC approval was received on 29 March 2013 and the pending supporting documentation was received on 1 April 2013. The protocol was submitted back to the WRAIR IRB for their review on 10 April 2013.

The protocol (Version 3.3, dated 21 November 2012) was reviewed at the 10 April 2013 fully convened WRAIR IRB meeting. The WRAIR IRB voted to approve the protocol as written. **The WRAIR IRB determined that this protocol met the criteria outlined in 32 CFR 219.111, 10 USC 980, and 45 CFR 46.405, as this is a greater than minimal risk study involving children that presents the prospect of direct benefit to the individual subjects.**

9. As this is a greater than minimal risk study, this protocol was submitted to the U.S. Army Medical and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protections Office (HRPO) for headquarters level review on 13 July 2012. The USAMRMC ORP HRPO provided their pre-review comments on 31 August 2012. Since the initial submission of this protocol to the USAMRMC ORP HRPO, a new USAMRMC policy, 2013-75 has been issued. In accordance with this new policy, this protocol still requires headquarters level review by the USAMRMC ORP HRPO. The USAMRMC ORP HRPO approval is currently pending for this protocol and will need to be submitted to the HSPB prior to the issuance of the WRAIR Commander Approval Authorization.

10. The following supporting documentation should be submitted to the WRAIR HSPB prior to the issuance of the WRAIR Commander Approval Authorization: USAMRMC ORP HRPO approval.

11. The following documents are included as part of this approval recommendation:
    a. Protocol, Version 3.3 dated 21 November 2012;
    b. Recruitment Script (English, Luo, and Kiswahili), Version 2, dated 1 August 2012;
    c. Adult Consent Form (English, Luo, and Kiswahili), Version 3.2, dated 21 November 2012;
    d. Parent/Guardian Consent Form (English, Luo, and Kiswahili), Version 3.2, dated 21 November 2012;
    e. Assent Form (English, Luo, and Kiswahili), Version 3.2, dated 21 November 2012; and
    f. Translation Verification Certificates for both the Luo and Kiswahili translations of all documents outlined in 11.b-e.

12. As this is a greater than minimal risk protocol, I recommend that Volunteer Registry Data Forms be required. The WRAIR expiration date is **10 April 2014**. The PI is responsible for submitting a continuing review report to the KEMRI ERC and the WRAIR IRB in time for the report to be reviewed and accepted/approved prior to the respective

expiration dates to avoid an interruption in work. A closeout report will be due to the WRAIR HSPB on **10 April 2018**. **No changes, amendments, or addenda may be made to the protocol without prior review and approval by the KEMRI ERC, the WRAIR IRB, and the USAMRMC ORP HRPO, as applicable.**

13. The point of contact for this action is Teresa R. Soderberg, M.A., RAC, CIM, at (301) 319-9438 or Teresa.R.Soderberg.civ@mail.mil.

KENT E. KESTER
COL, MC
Chair, Institutional Review Board
Walter Reed Army Institute of Research

CF:
Ben Andagalu, M.D., MSc
Jessica Cowden, MAJ, MC
Regulatory Affairs, Kenya
Thomas Logan, COL, MS
MCMR-RP