Plasmodium falciparum Population Structure in Children with Malaria in Kisumu-Western Kenya

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Abstract

Plasmodium falciparum population structure in malaria endemic areas is characterized by extensive genotype diversity, and wide spectrum of clinical presentation; from mild, uncomplicated malaria to severe malaria and anemia. In addition individuals may harbor parasites without any clinical presentation. These observations suggest that some parasite strains are more virulent than others. In this study, we evaluated whether severity of *P. falciparum* malaria can be attributed to a single clone that outgrows others or is due to multiple clone infections and determine the prevalent clone in the study population. Blood samples (cryopreserved at -80°C) were obtained from a case control study (2006) that enrolled 60 children (<5years) with severe malarial anaemia (SMA) (defined as having blood stage asexual malaria parasites and hemoglobin (Hb) ≤6 g/dL). Each case of SMA was matched by age (±2 months) and gender to a control with uncomplicated malaria (UM) (defined as having blood stage asexual malaria parasites and Hb>6 g/dL). DNA was extracted and amplified in replicate PCR reactions that targeted *msp-1* (K1, MAD20 and RO33) and *msp-2* (FC27 and IC3D7) allelic families. Allele discrimination was achieved by use of high resolution capillary electrophoresis (CE). Genotype signatures (allele size and allele number) and multiplicity of infection (MOI) between the two groups was compared. Finally, relative abundance of parasite clones was estimated from peak height and peak area. Paired parametric t-test was used to test for statistical significance. In replicate assays, the allele numbers and sizes (up to 1 nucleotide difference) were reproducible in every case illustrating excellent assay reproducibility. MOI was not statistically different (P=0.54) between the two groups (UM=2.17, N=35 and SMA=2.0, N=35). Overall, K1 was the most prevalent allele (37%) as compared to MAD20=16%, RO33=16%, FC27=22%, IC3D7=10% (P<0.0001). For the three alleles (K1, MAD20 and FC27) evaluated, peak height and peak area equally predicted average allele abundance but the difference between the two groups was not significant (P>0.05). This study has demonstrated that (i) the number of alleles and their sizes obtained from a high resolution CE are reproducible up to 1 nucleotide, (ii) for relative quantification of allele abundance, either peak area or peak height can be used to define relative abundance, (iii) severity of *P. falciparum* malaria is not attributable to dominance of individual clones. Lastly, MOI is not predictive of disease severity.

CHAPTER ONE

1.0 Introduction

Severe malaria anaemia (SMA) is said to be the leading cause of pediatric mortality in sub-Saharan Africa. It is estimated that 190,000 to 974,000 deaths per year in sub-Saharan-Africa are attributable to SMA (Murphy and Breman, 2001). There are four *Plasmodium* species which cause malaria; *P. falciparum, P. malariae, P. ovale* and *P.vivax*. *P. falciparum* is responsible for most of the severe complications such as cerebral malaria and severe malarial anemia (Bouyou-Akotet *et al.,* 2009). In Africa, the epidemiological profile and clinical pattern of severe malaria have been shown to vary according to the intensity of exposure and age (Modiano *et al.,* 1998).

It is well known that some major antigens of the parasite such as the merozoite surface protein 1 and 2 (MSP-1 and MSP-2), are closely involved in parasite invasion of the red blood cells (erythrocytic schizogony), the stage that is associated with clinical disease. *In vitro* and mouse studies have shown that antibodies to these proteins prevent invasion of erythrocytes by merozoites by blocking the proteolytic processing of MSP-1_{42kD} to MSP-1_{19kD} (Lazarou *et al.,* 2009) therefore administering these antibodies would interfere with the asexual replication of malaria parasite. However, all attempts to reproduce these successes in humans have failed (Genton *et al.,* 2002; Ogutu *et al.,* 2009). Clearly, malaria infection in human is complex and other factors such as host immune status, genetic background and parasite determinants play a role. Among the parasite factors, virulence of certain malaria strains is considered a major determinant in malaria severity. This study intended to evaluate whether *P. falciparum* strains in patient with severe malarial anemia (SMA) are different from those with uncomplicated malaria (UM).

Genotyping has been found to be the most reliable method of identifying different malaria parasite clones. The commonly used techniques include polymerase chain reaction (PCR) restriction fragment length polymorphism (PCR-RFLP) (Felger *et al.,* 1999), PCR Sequence Specific Oligonucleotide Probing (PCR-SSOP) (Alloueche *et al.,* 2000), microsatellite analysis by PCR (Anderson *et al.,* 2000) and Reverse transcriptase PCR (RT-PCR) for genotyping *P. falciparum* gametocytes (Menegon *et al.,* 2000). These techniques target the polymorphic genes in *Plasmodium* parasites such as *msp-1*, *msp-2* and the *Glutamate rich Protein* (*glurp*). Since these three markers are unlinked single copy genes with extensive polymorphism, they are attractive targets for the determination and enumeration of genetically distinct parasite populations.

A study in Gabon demonstrated that RO33 allele was predominant in children with uncomplicated malaria (Ntoumi *et al.,* 1996). Other studies in India have identified R033 and 3D7 as the allelic types that are associated with complicated malaria, namely cerebral malaria (CM) and SMA (Sahu *et al.,* 2008). A Previous study in India had shown that MAD20 and 3D7 alleles are over-represented in severe malaria cases and a high multiplicity of infections (MOI) with respect to *msp-2* alleles in severe cases as compared to mild cases (Ranjit *et al.,* 2005). In contrast, Robert *et al* (1996) reported a lower complexity of infections in severe than in mild malaria. In a study at Asembo Kenya to assess the susceptibility to subsequent infections, Branch *et al* 2001 showed a negative correlation between complexity of infection and resistance to parasitaemia where the parasitaemia with the RO genotype showed significant resistant.

The standard method for allelic discrimination has for a long time been done by PCR amplification of target marker genes followed by separation and sizing of amplicons by gel electrophoresis (Snounou and Beck, 1998). Lately, the World Health Organization (WHO) has recommended fragment size

resolution by capillary electrophoresis (CE) as it has been found to increase sensitivity and the discriminatory power (WHO, 2007).

1.2 Problem statement

Malaria is one of the biggest global health problem particularly in sub-Saharan Africa where *P. falciparum* accounts for more than 90% of the malaria burden. The clinical presentation of malaria is complex: some individuals will carry parasitemia without an associated clinical symptoms or disease, others develop mild malaria while others develop severe disease. (Greenwood *et al.,* 1991). Children below five years and pregnant women are the most vulnerable (WHO, 2005). In Sub Saharan Africa, malaria accounts for an estimated one million mortalities each year mostly in children below the age of five excluding neonatal mortality (WHO 1997).In areas of high transmission such as the Lake Victoria basin, malaria affects mainly children below five years and the resulting morbidity and mortality is as a result of SMA(Lusingu and Von Seidlein, 2008).These observations suggest diversity in the virulence of certain *P. falciparum* strains. However the existence and contribution different parasite clones in the development of severe disease has not been fully addressed.

1.3 Justification of the Study

SMA is a major cause of death in children in the malaria holoendemic regions (Bloland *et al.,* 1999) including the L. Victoria basin. However, not all malaria episodes progress to severity. While certain host factors are important in determining malaria outcome, there is growing realization that parasite factors such as ability to cytoadhere, ability to form rosettes, among others influence disease outcome. In this study, molecular methods were used to evaluate the role of strain dominance in determining malaria outcome. Results from this study intend to provide information as to whether parasites associated with uncomplicated malaria are different from those associated with severe disease. Use of high resolving CE will help in allele discrimination that cannot be achieved by the widely used gel

separation systems. Identification of virulent parasite clones will promote a targeted approach in vaccine design and ultimately reduce malaria associated childhood mortality.

1.4 Hypothesis

P. falciparum population structure in patients with severe malaria anemia is different from those with uncomplicated malaria

1.5 General Objective

To determine if malaria parasites from children with severe malaria anemia are genotypically distinct from those obtained in children with uncomplicated malaria.

1.5.1 Specific Objectives

1. To compare *P. falciparum* genotype multiplicity during uncomplicated and severe malaria in children.
2. To determine the prevalence of the different *P. falciparum* clones during uncomplicated and severe malaria in children.
3. To compare peak area and peak height of *P. falciparum* clones during uncomplicated and severe malaria in children.

1.5.2 Research Questions

1. Is severe malarial anemia a consequence of single dominant parasite clone or as a result of multiple clones in a *P. falciparim* malaria infection?
2. Which *P. falciparum* alleles are prevalent in the study population?
3. Is there a difference in the peak height and peak area values of *P. falciparum* clones detected in mild and severe malaria anemia?

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Background

Malaria is a major public health problem with an estimated 300 million cases annually and one million deaths (WHO, 2008), mostly among African children under the age of 5 years. In Kenya, the Division of Malaria Control estimates that malaria alone accounts for 30-50% of all outpatient attendance, 20% of all admissions to health facilities and causes about 20% of all deaths in children under five years (www.nmcp.or.ke., accessed on 18/09/2010). Malaria is caused by protozoan parasites belonging to the genus *plasmodium*, transmitted by the female *anopheles* mosquito. The major vector population in Sub-Saharan Africa includes the members of the *Anopheles gambiae* complex and *An. funestus* (Okara *et al.,* 2010).

There are over 100 species of *plasmodium* all of which infect red blood cells of mammals, reptiles and birds but only five: *P. falciparum, P. vivax, P. ovale P. malariae* and *P. knowlesi* infect humans. Of the five, *P. falciparum* causes the most severe form of the disease and is responsible for highest malaria associated morbidity and mortality (Kang *et al.,* 2010).

2.2 *Plasmodium falciparum* life cycle

The life cycle of malaria parasite involves the female *anopheles* mosquito and the human host (Figure 1). The female mosquito driven by olfactory cues from host derived volatile compounds lands and draws blood from the human host and simultaneously injects saliva and sporozoites into the skin (Aly *et al.,* 2009). Sporozoites spend only a few minutes in peripheral circulation before they invade hepatocytes, where they replicate asexually to form liver schizonts. This replicative stage is referred to as exoerythrocytic schizogony and involves multiple nuclear divisions without cytoplasmic division

that last upto 14 days, depending on the *Plasmodium* species. The progenies, called merozoites are then released into the blood following rapture of the host merozoite-filled hepatocyte and quickly and specifically enter erythrocytes. The initial interaction between the merozoite and RBC is random and involves reversible interactions between proteins on the merozoite surface and the host erythrocyte (Cowman and Crabb, 2006). A tight junction is then formed once the apical end of the merozoite is directly in contact with RBC which facilitates the gradual entry of merozoite into the host cell. After entering the RBC, the parasite undergoes a trophic period (rapid growth) and asexual replication to form trophozoites often called 'ring-form' due to its signet ring morphology in Giemsa-stained blood smears. The asexual multiplication of merozoites in RBCs is referred to as erythrocytic schizogony. Nuclear division lasts 48-72 hours and produces 16-32 merozoites per infected erythrocyte (Haldar *et al.,* 2007). Subsequent rapture and merozoite release is associated with characteristic fever and symptoms of malaria. The released merozoites initiate a new wave of RBC invasion thereby incessantly propagating the parasite cycle. At some stage that is not clearly known, some of the parasites differentiate into sexual erythrocytic stages through a process called gametocytogenesis (Alano and Carter, 1990). What triggers the switch from the asexual pathway to the production of the sexual stages is not fully known. Inducing stress with high levels of asexual parasitaemia, anaemia, antiparasitic immune responses, and chemotherapeutic agents have been shown to be associated with gametocytogenesis (Talman *et al.,* 2004).

When ingested by female mosquito, mature circulating forms of the sexual stages undergo a rapid process of gametogenesis resulting in the formation of male (microgametocytes) and female (macrogametocytes) gametocytes. One female gamete emerges from one female gametocyte and three mitotic division cycles give rise to eight male gametes (Lobo and Kumar, 1998). While in the mosquito's stomach, the microgametes penetrate the macrogametes generating diploid zygotes. The

zygotes in turn become motile and elongated (ookinetes) which penetrate the midgut wall of the mosquito where they develop into oocysts. Within the oocysts, zygote undergoes rapid meiotic division to form haploid sporozoites. The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands (Matuschewski, 2006). Inoculation of the sporozoites into a human host completes the parasite life cycle life cycle.

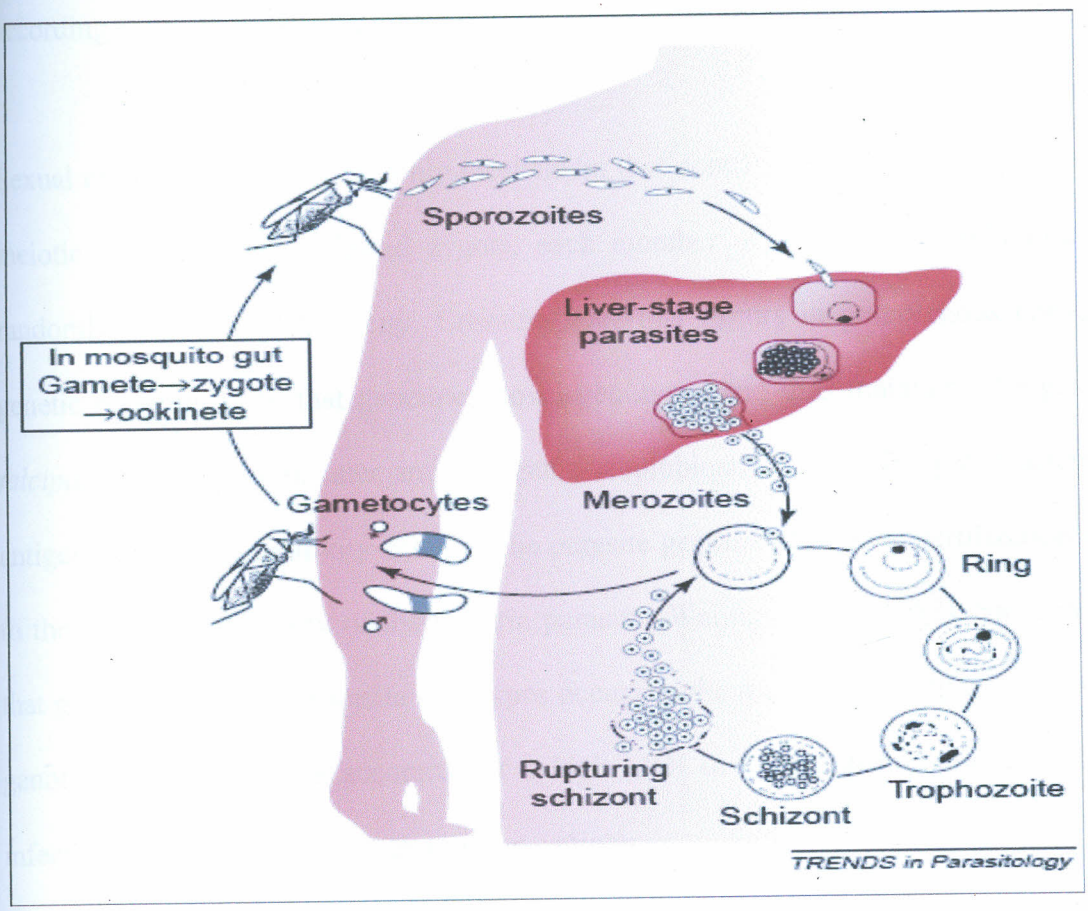

Figure 1 Life cycle of the malaria parasite. Adapted from (Good, 2005)

2.3 Genetic Recombination

Mating patterns in malaria parasite populations have been shown to be both random and non random. A study in Tanzania demonstrated random mating by examining the polymorphic *msp-1* and *msp-2* genes

in oocysts from wild-caught mosquitoes compared to same alleles from blood parasites. The study showed that most infections contained mixtures of genetically distinct clones, and 45 out of 71 oocyts were heterozygous for one or both genes indicating that random mating events probably occurred within mosquito bloodmeals between gametes belonging to different parasite clones (Babiker *et al.,* 1994). A similar study in Papua New Guinea (PNG), a region of relatively low transmission intensity showed a high degree of inbreeding, meaning that the genetic structure of *P. falciparum* will vary according to the transmission intensity (Haddad *et al.,* 1999).

Sexual reproduction is not the only mechanism that contributes to extensive parasite diversity. During meiotic division of the diploid zygote, each member of a given pair of chromosomes segregates randomly into haploid progeny. Crossing over that occurs in homologous chromosomes results in genetic recombination that generates novel genotypes and thus maintains the genetic diversity in *P. falciparum*. In addition, inter and intragenic recombination determining characters such as enzymes, antigens and drug sensitivity occur in the parasite genome after cross fertilization and this contributes to the extensive diversity of the malaria parasite (Walliker *et al.,* 1987). Since sexual recombination that gives rise to novel parasite genotypes occurs in the mosquito, a single infection leads to a distinct genotype infection. This accounts for a high MOI in high transmission settings where the rate of infection is higher than low transmission settings.

Thus, genetic diversity of the malaria parasite population is a good indicator of malaria transmission intensity. In high transmission settings, individuals infected with *P. falciparum* harbor multiple parasite genotypes as compared to low transmission areas where majority of the infections are monoclonal (Haddad *et al.,* 1999). Different genetic markers such as circumsporozoite protein (CSP), MSP-1, MSP-2, and GLURP have been used to determine the diversity of malaria parasites.

2.4 Merozoite Surface Antigens

The *msp-1, msp-2 and glurp* genes code for highly diverse surface antigens found in malaria parasites and are commonly used as markers for genotyping *P. falciparum*. These three genes are unlinked, single copy with extensive polymorphism. Therefore, the diversity of these markers indicates parasite clonal diversity. Since the parasite is haploid during the human asexual part of the parasite life cycle, these markers are stable in human host and can be used to define parasite populations within the host.

2.4.1 Merozoite Surface Protein-1 (MSP-1)

P. falciparum msp-1 gene is approximately 5,000 base pairs (bp) long, located on chromosome 9 and is 74% AT rich (Pan *et al.,* 1999). Sequence comparison has shown that *msp-1* gene is divided into 17 blocks (Figure 2). The gene has 7 variable blocks interspersed by five conserved and five semi-conserved blocks (Kiwanuka, 2009). In blocks 1, 3, 5, 12, and 17, the sequences are conserved whereas blocks 2, 4, 6, 8, 10, 14 and 16, the sequence show extensive diversity. The remaining blocks 7, 9, 11, 13, and 15 have semi conserved sequences (Raj *et al.,*2004). Each of the variable blocks is dimorphic with two alleles K1 and MAD20. However Block 2 near the N-terminal of *msp-1*gene is the most dimorphic with respect to size and sequence and has three distinct allelic families; MAD20, K1 and RO33 (Heidari *et al.,* 2007).

MSP-1 plays an important role in erythrocyte invasion by merozoite and results from the proteolytic cleavage of a 190-200 kDa precursor. Although the full length *msp-1* gene is highly polymorphic, the C-terminal epidermal growth factor (EGF)-like domains in MSP-1_{19} is relatively conserved with only six non-synonymous single nucleotide polymorphisms (SNPs) which result in expression of different amino acids. These mutations are located at position 1644 (G/C) in the first EGF domain and at

positions 1691(A/C), 1699(G/A), 1700(G/A), 1701(G/A) and 1716(C/T) of the second EGF domain (Zakeri *et al.*, 2010).

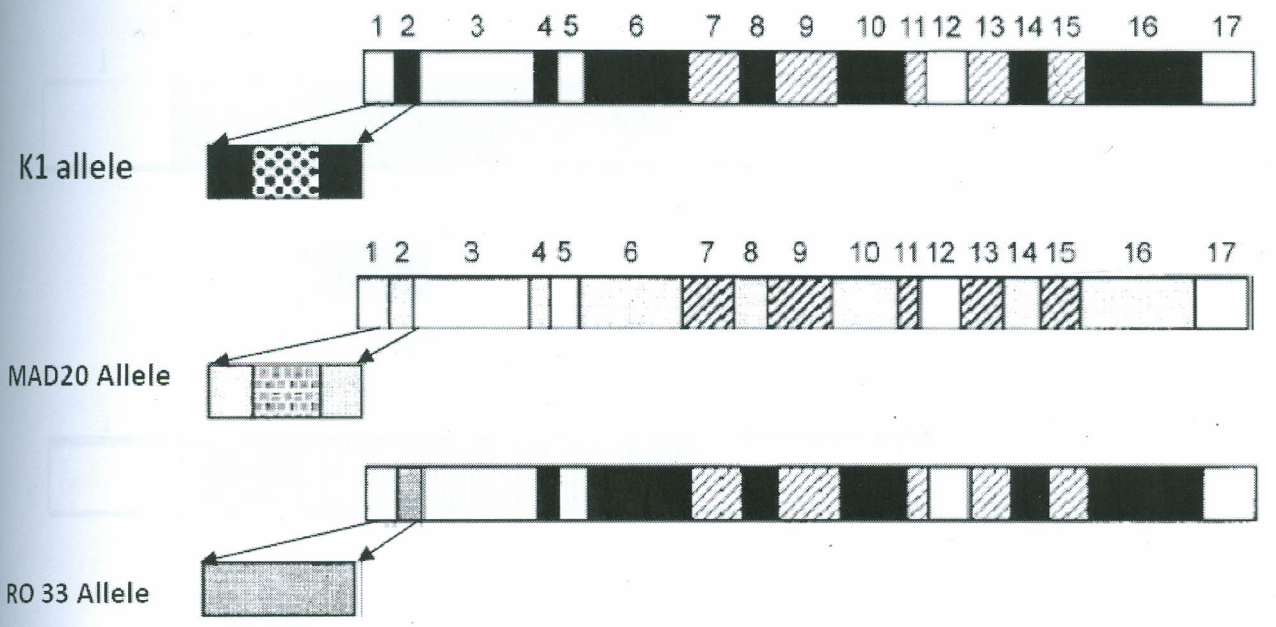

Figure 2. Schematic presentation *P. falciparum msp-1* gene.

The conserved blocks are shown as, open boxes, semi conserved blocks as hatched boxes and the polymorphic blocks as closed boxes. Allele families, K1 and MAD20 are present in all the polymorphic blocks. Block 2 has an additional allele family RO33. *Adapted from* (Da Silveira *et al.*, 1999).

.4.2 Merozoite Surface Protein-2 (MSP- 2)

The MSP-2 has also been used as a candidate antigen for malaria vaccine development. The *msp-2* gene located on chromosome 2 comprises of conserved ammino (N) and carboxyl (C) terminal domains Block1 and Block 5), highly polymorphic central repeats (Block 3) that are flanked by dimorphic domains (Block 2 and 4) (Figure 3). The polymorphic central region is composed of repeats flanked by nonrepetitive sequences. The repetitive sequences form the two allelic families: FC27 and IC3D7 (Kang *et al.*, 2010). These polymorphic regions are used as genetic markers and provide a means to

assess the composition of parasite population. Of these three loci *msp-2* has been found to be the most polymorphic (Heidari *et al.*, 2007).

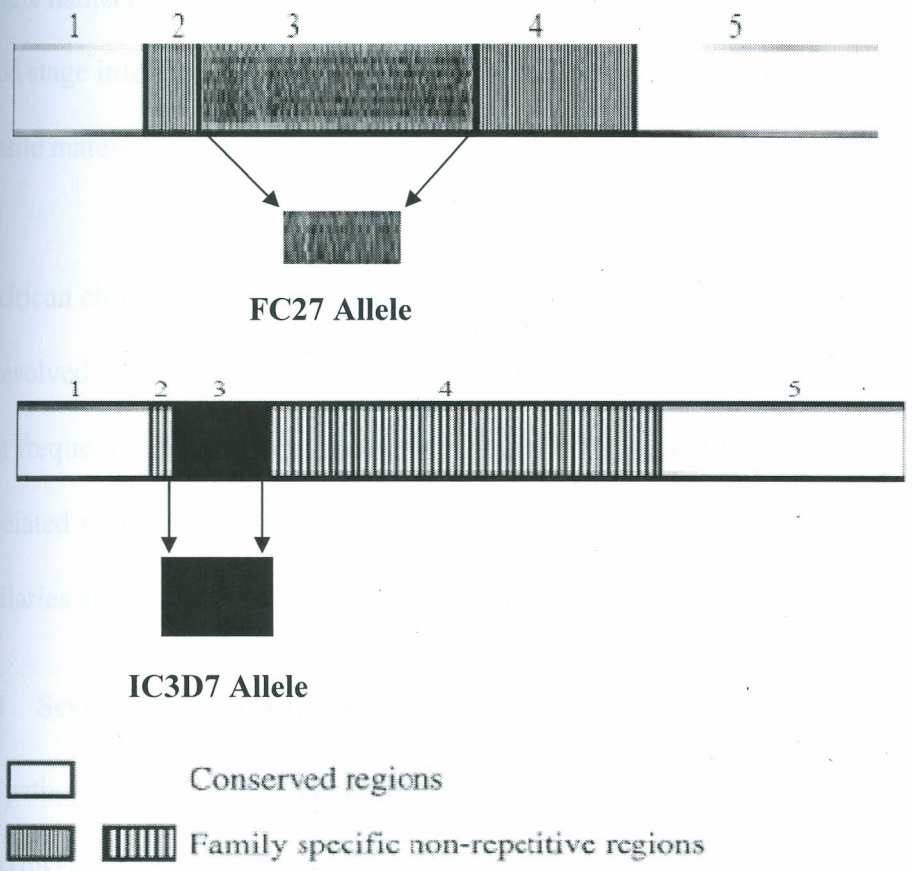

Figure 3 Schematic diagram of *P. falciparum msp-2* gene.
Adapted from (Ekala *et al.*, 2002).

2.4.3 Glutamate rich Protein (GLURP)

GLURP is a 220 kDa polypeptide that is expressed in all asexual stages of the parasite. The GLURP gene is located on chromosome 10 and consists of two tandem repeats (RI and RII) and appears only as one allelic family. The RII is most diverse and therefore normally targeted for genotyping (Hogh *et al.*, 1993). In the current study, only *msp-1* and *msp-2* were used to genotype the plasmodium parasite. Although *glurp* has a high repeats, it is less polymorphic than *msp-1* and *msp-2* (Greenhouse *et al.*, 2006).

2.5 Disease Severity

P. falciparum causes the majority of infections in Africa and is responsible for the most severe form of malaria namely severe anemia or cerebral malaria. Malaria associated pathology only occurs during the blood stage infection and is related to the rapture of infected erythrocytes and the subsequent release of parasite material and metabolites, hemozoin (malaria pigment) and cellular debris.

In African children, among whom the great majority of malaria deaths occur, *P. falciparum* malaria can be resolved into an array of complex clinical presentation ranging from mild to severe malaria. The most frequent manifestation of severe malaria is SMA and CM. The pathogenesis of severe malaria is associated with cytoadherence and rosetting phenomena that lead to sequestering of infected cell on the capillaries and microvenules of the brain and other vital organs (Chen *et al.*, 2000).

2.5.1 Severe Malaria Anaemia

The pathogenesis of SMA is complex and results from the direct destruction of parasitized red blood cells (pRBCs), indirect destruction of non-parasitized red blood cells (nRBCs) by immune mechanisms and bone marrow suppression associated with imbalances in cytokine production (Mackintosh *et al.*, 2004).

Destruction of red blood cells (RBCs) occurs as a result of parasite invasion and replication. As the parasite grows within the RBC, the erythrocyte becomes less deformable which may contribute to RBC destruction and impair the microcirculatory flow. The reduction in red cell deformability occurs not only in pRBC but also in nRBC. Normally nRBCs have to undergo considerable deformation to squeeze through the sequestered microcirculation. Increased destruction of nRBC is proposed to result from mechanisms such as bystander intravascular hemolysis or accelerated senescence arising from

lipid peroxidation, reduced red cell deformability (Dondorp *et al.,* 1999), modification by surface-bound IgG or complement (Goka et al., 2001), and adsorption of parasite derived antigens (Layez *et al.,* 2005). In a murine model of SMA, the principal cause of anaemia was due to uptake of uninfected erythrocytes by monocytes and macrophages (Evans *et al.,* 2006).

Reduced complement regulatory proteins such as Complement Receptor 1 (CD35) and Decay Accelerating Factor (CD55) in children with severe anaemia has also been reported as a major contributor to SMA (Waitumbi *et al.,*2000). Clinically, SMA is manifested as severe normocytic anaemia (Hb<5g/dL), hyperparasitaemia (>250,000/μL in areas of high transmission), hypoglycemia (<40mg/dl), renal impairment, and haemoglobinuria (WHO, 2008). In addition to removal of pRBC and nRBC, decreased erythrocyte production and/or erythropoietic response also contribute to SMA. Studies have also shown that cytokines and mediators of inflammation such as hemozoin (Rudin *et al.,* 1997) contribute to reduced erythropoiesis.

2.5.2 Cerebral Malaria

The pathogenesis of fatal cerebral malaria (CM) is not completely understood, in part because data from patients in whom a reliable clinical diagnosis is established is rare. Two theories have been put forward to explain CM: The first theory is based on the obstruction of small vessels of the brain by sequestered parasites (Maitland *et al.,* 2003), thereby causing coma, febrile illness with convulsions and other neurological impairments. Accumulation of pRBCs interferes with microcirculation leading to ischemia. In addition to parasitized RBCs, leukocytes (Grau *et al.,* 2003) and platelets (Combes *et al.,* 2006) have also been described in brain blood vessels in patients with cerebral malaria. The second theory is based on overproduction of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and Interleukin (IL)-1. TNF-α upregulates endothelial cytoadherence receptors and can cause

hypoglycemia and dyserythropoiesis which are symptoms of severe disease (Newton *et al.*, 2000). IL-1 has been shown to induce production of high levels of reactive nitrogen intermediates (nitric oxide, nitrite and nitrate) *in vivo* (Rockett *et al.*, 1992). Nitric oxide is formed enzymatically in both postsynaptic and presynaptic structures as well and therefore is a neurotransmitter (Garthwaite, 1991). Therefore high levels of cytokine-induced production of nitric oxide could be an important mediator in CM.

Heterogeneity in parasite virulence is one of the several factors that have been proposed to contribute to the wide spectrum of disease severity in *P. falciparum* malaria. A number of parasite phenotypes have been identified as possible virulence factors; these include cytoadherence (receptor mediated binding of infected erythrocytes to endothelial surfaces of blood vessels) mediated by CD36, Intercellular adhesion molecule 1 (ICAM-1) and thrombospondin (TSP), rosetting (binding of two or more uninfected erythrocytes to an infected erythrocyte) mediated by complement receptor 1, and existence of several variable antigenic gene families (*var* family, *rosettin/rif* family and the *pf60* family) (Gupta *et al.*, 1994). The existence of the wide array of clinical presentation of malaria (mild, SMA, CM and placental malaria) is an indication of possible existence of extensive diversity of the malaria parasite. The parasite uses many strategies to maintain parasite diversity, among them antigenic variation in gene families such as the *var*, *rosettin/rif* and the *pf60* family and genetic recombination in the mosquito (Deitsch and Hviid, 2004).

2.6 *Plasmodium falciparum* genotyping

2.6.1 Pyrosequencing

Pyrosequencing has been used reliably to quantify alleles in mixed malaria infections. It is a real time sequencing method that detects release of pyrophosphate during nucleotide incorporation by an enzyme cascade that generates light proportional to the amount of nucleotide incorporated. This technique allows sequencing of short stretches of nucleotides (10-20 bp) surrounding known polymorphisms without sequencing the rest of the conserved sequence. Pyrosequencing software can quantify the proportion of each alternative nucleotide at each alternative SNP site based on relative peak heights (Takala *et al.*, 2006).

2.6.2 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

PCR-RFLP is a DNA finger printing technique that uses restriction endonucleases to cut DNA into fragments on the basis of abundance of restriction site. PCR-RFLP uses restriction enzyme to digest the amplified PCR products whereafter the fragments are separated on high resolution gels. PCR-RFLP genotyping of parasites for *msp-2* has been used to distinguish individual parasite infections concurrently present in a blood sample (Felger *et al.*, 1999).

2.6.3 Sequence-specific oligonucleotide probing (SSOP)

SSOP has been described as a method of typing polymorphisms at the T-cell epitopes within the Th2R and Th3R regions of the *P. falciparum* CSP. In this technique the target loci is first amplified by PCR, the amplicons of the polymorphic region are then dot blotted against a nylon membrane and probed with oligonucleotides for the various SNPs. The probes are designed according to the published sequences of field and laboratory isolates for both Th2R and Th3R epitope regions. An autoradiogram s then examined for presence or absence of spots (Alloueche *et al.*, 2000).

2.6.4 Reverse Transcriptase PCR (RT-PCR)

RT-PCR is used for specific detection and genetic characterization of *P. falciparum* gametocytes in the blood of malaria infected individuals and uses highly polymorphic *pfg377* gene as target (Menegon *et al.*, 2000).

2.6.5 Microsatellite Marker Analysis

Multilocus genotyping has been used to study the population structure of *P. falciparum* (Anderson *et al.*, 2000), in antimalarial drug trials to define treatment outcome following antimalarial drug administration (Snounou and Beck, 1998), and in vaccine trials to assess the molecular impact of the vaccine on parasite multiplicity (Waitumbi *et al.*, 2009). This technique involves a nested PCR amplification of the loci of interest. The primary PCR uses primer pairs corresponding to the conserved sequences, spanning the polymorphic regions of block 2 of *msp-1* gene and block 3 of *msp-2* gene. Amplicons from the primary PCR are used as templates for the nested reaction. Allele specific primer sets are used to amplify the K1, MAD20, and RO33 alleles of the *msp-1* gene and the FC27 and IC3D7 alleles of *msp-2* alleles. Allelic discrimination is usually achieved by running the PCR amplicons in agarose gel electrophoresis whereby amplicons are distinguished from each other based on fragment size. The fragments are stained with DNA dyes such as ethidium bromide and visualized on an ultra violet (UV) transilluminator. Nucleic acids have a negative charge due to their phosphate backbone and on electrophoresis will migrate towards the anode. Migration along the gel is dependent on fragment size; with smaller fragments moving faster compared to larger molecular weight fragments. By using molecular ladders of known size, the size of the PCR amplicons can be determined. However, due to limited resolving power of gel electrophoresis, adequate discrimination of alleles especially in high transmission settings where the MOI is high may not occur (Gupta *et al.*, 2010).

When compared to conventional gel electrophoresis, capillary electrophoresis (CE) was found to provide better resolution of *P. falciparum* alleles (Gupta *et al.,* 2010) . The reverse primers used in capillary electrophoresis are labeled with fluorescent dyes. Tagging of fluorescent dyes to primers is achieved two ways; the preferred route to make primers for automated DNA fragment analysis is by use of phosphoramidite reagents similar to those used for oligo synthesis, but containing a protected fluorescent dye instead of a protected nucleoside that is attached at the 5' end. Alternatively, the oligonucleotide is first synthesized with a 5'-amino modification using an amino linker C6 phosphoramidite. After cleavage from the support and deprotection, the 5'- terminal group is manually conjugated to a reactive succinimidyl dye. After conjugation the labeled product is purified from unlabelled contaminants and unincorporated dyes.These differently colored fluorescent dyes enables detection of amplicons in the capillary system. For CE, PCR products are loaded onto a gel matrix inside a capillary (internal diameter; 50-100 μm) and separated by size by applying electric charge. A laser scans the gel to detect bands containing fluorescent dye. Besides the high resolution, CE has been recommended by the world health organization for allele discrimination and sizing due to its high throughput, ability to multiplex by using different fluorescent dyes for different alleles, thereby reducing cost and time (MMV and WHO, 2007). Sample loading, separation and size calling are automated. The utility of CE technique has been validated for a variety of forensic applications (Butler *et al.,* 2004), and the analysis of multiple-locus variable-number tandem repeat (Yokoyama *et al.,* 2006). A part from fragment sizing, CE also calculates the peak height and area for each amplicon. These two parameters were evaluated to add another dimension when studying the population structure of malaria parasite clones.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

The study was a retrospective case-control study where archived samples were used. These samples were obtained from young children (<5years) who were recruited as cases and controls into the study. These samples were cryopreserved at -80°C at the Kondele Basic Science facilities.

3.1.1 Sample size determination

The assays were performed on archived DNA specimens that were collected from a prospective case control study that had enrolled patients with severe malaria (cases) and their age and sex matched controls with uncomplicated malaria in 2006. The study patients were recruited from the pediatric ward of Kisumu District Hospital, Nyanza Province and samples were cryopreserved at -80°C at the Kondele Basic sciences laboratories.

A total of 120 children were enrolled, 60 with SMA and 60 with UM. This sample size was based on results of complement receptor-1(CR1) molecules on RBC from a previous study by Stoute *et al* (2003). In that study, a standard deviation of 114 CR1 molecules was obtained between cases of severe malarial anemia and controls. Assuming the real difference between the groups is 84 molecules, a sample size of 60 in each group would give 80 % power to detect that difference, $\alpha = 0.05$ (two-sided). This sample size was calculated using StatMate software (Graph Pad, San Diego CA).

3.1.2 Ethical consideration

Participation in the study was voluntary and each parent or guardian of the participants signed an informed consent before the child was enrolled in the study. Scientific and ethical approval for the study was obtained from the Ethical Review Committee of the Kenya Medical Research Institute, Nairobi (SSC No 879) and the Walter Reed Army Institute of Research, Silver Spring, Maryland, USA (WRAIR # 1145).

3.2 Study population

Two groups of children were enrolled. Group one (SMA) comprised children (age ≤ 5 yrs) admitted to the participating hospital with asexual *P. falciparum* parasitemia confirmed by a positive Giemsa-stained blood smear and anemia defined as (hemoglobin ≤ 6 g/dL). Group two (UM) were age (±2 months) and sex matched with symptomatic uncomplicated malaria. Other details of the study, including collection of blood samples have been published elsewhere (Nyakoe *et al.*, 2009).

3.3 Inclusion and exclusion criteria

For this study, patients with SMA were defined as children with asexual *P. falciparum* parasitemia as determined by either thin or thick Giemsa-stain and a hemoglobin level ≤6 g/dL as determined by the coulter counter (Beckman Coulter Ac T 5 Diff Hematology analyser, Beckman Coulter Inc, USA). Each case was matched by age (±2 months) and gender to a child with UM. Inclusion criteria for UM were asexual *P. falciparum* parasitemia, hemoglobin level >6 g/dL plus symptoms suggestive of malaria such as an axillary temperature ≥37.5°C or, in its absence, presence of any two of the following: nausea and/ or vomiting, diarrhea, irritability, or poor feeding). Subjects were excluded from participation if there was evidence of concomitant infections (i.e. pneumonia meningitis, sepsis etc), evidence of immune-comprised status (e.g. thrush and tuberculosis), and history of blood transfusion

within the preceding six months. Additional exclusion criteria applicable to all groups were the inability or unwillingness of the parent or guardian to give consent and the presence of other infection or malignancy.

3.4 Isolation and purification of DNA

DNA was isolated from EDTA blood (EDTA is anticoagulant) using QIAamp DNA Blood mini Kits (QIAGEN Inc., CA) according to the manufacturers guidelines. Briefly, 200 µL of EDTA blood was placed in a sterile 1.5 mL micro centrifuge tubes. This was followed by addition of 200 µL of lysis buffer and 20 µL QIAGEN protease enzymes in order to digest the proteins. The mixture was vortexed and incubated on a hotplate for 10 minutes at 57°C. The de-proteinized DNA preparation was then purified on QIAamp spin columns after addition of 200 µL of ethanol. This was achieved by centrifugation for one minute at 8000 rpm in a micro centrifuge (Centrifuge 5804 R, Becton Dickinson, New Jersey, United States) and the tube containing filtrate discarded. The spin column was then transferred into another 1.5 mL collection tube and 500 µL of buffer AW1 added and centrifuged at 8,000 rpm for one minute and the filtrate discarded. 500 µL of buffer AW2 was added into the spin column and the preparation centrifuged for three minutes at 14,000 rpm. Finally, the purified DNA was eluted from the spin column by addition of 200 µL elution buffer (AE) and stored at –20°C until required.

3.5 PCR amplification of *P. falciparum msp-1* and *msp-2* genes

The primers used for the amplification are shown in Table 1 . DNA was amplified in a two step nested PCR. In the primary reaction, primers targeting the entire block 2 of *msp-1* and block 3 of *msp-2* were used as described previously in (Liljander *et al.*, 2009). In the secondary reaction, allele specific

primers were used to amplify the K1, MAD-20 and the RO33 alleles of *msp-1* and FC27 and IC3D7 of *msp-2*. In the nested reaction, the allelic primers had fluorescent labels. Reverse primers were labeled with different fluorescent dyes at the 5′ end(Table 1); K1 with NED (yellow), MAD20 with PET (red), RO33 with VIC (green), FC27 with 6-FAM (blue) and ICD37 with VIC (green).All primer sets were from applied Biosystems.

Table 1 Details of the primers used in the primary and nested PCR for *msp-1* and *msp-2*

	Primer	Primer Sequence	Dye (color)
Primary PCR	MSP-1F	5'-CTAGAAGCTTTAGAAGATGCAGTATTG-3'	
	MSP-1R	5'-CTTAAATAGTATTCTAATTCAAGTGGATCA-3'	
	MSP-2F	5'-ATGAAGGTAATTAAAACATTGTCTATTATA-3'	
	MSP-2R	5'-CTTTGTTACCATCGGTACATTCTT-3'	
Nested PCR	**MSP-1**		
	K-1F	5'-AAATGAAGAAGAAATTACTACAAAAGGTGC-3'	
	K-1R	5'-GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3'	NED (yellow)
	MAD20F	5'-AAATGAAGGAACAAGTGGAACAGCTGTTAC-3'	
	MAD20R	5'-ATCTGAAGGATTTGTACGTCTTGAATTAAC-3'	PET (red)
	RO33F	5'-TAAAGGATGGAGCAAATACTCAAGTTGTTG-3'	
	RO33R	5'-CATCTGAAGGATTTGCAGCACCTGGAGATC-3'	VIC (green)
	MSP-2		
	FC27F	5'-AATACTAAGAGTGTAGGTGCARATGCTCCA-3'	
	FC27R	5'-TTTTATTTGGTGCATTGCCAGAACTTGAAC -3'	6FAM (blue)
	ICF	5'-AGAAGTATGGCAGAAAGTAAKCCTYCTACT-3'	
	ICR	5'-GATTGTAATTCGGGGGATTCAGTTTGTTCG-3'	VIC (green)

(Liljander *et al.*, 2009)

3.5.1 Primary PCR

The final concentration of the master mix consisted of 1× PCR buffer, 2 mM $MgCl_2$, 125 µM dNTP and 0.02 units/µL of AmpliTaq® DNA polymerase (Applied Biosystems), and 250 nM each of the flanking primer pairs (Table 1). 3 µL of extracted DNA was used as the template and the final volume of the reaction adjusted to 25 µL. The cycle conditions were initial denaturation at 95 °C for 5 min followed by 25 cycles of annealing at 58 °C for 2 min, extension at 72 °C for 2 min, denaturation at 94 °C for 1 min with a final round of amplification at 58 °C for 2 min and 72 °C for 5 min. These reactions were carried out in a Peltier Thermal Cycler (MJ Research Inc., Watertown, MA, USA). For positive controls, samples previously confirmed to amplify K1, MAD20, RO33, FC27 and IC3D7 alleles were included in each of the runs. Negative control runs did not have target DNA.

3.5.2 Nested Reaction

The final concentration of the master mix consisted of 1× PCR buffer, 1 mM $MgCl_2$, 125 µM dNTP and 0.02 units/ µL of AmpliTaq® DNA polymerase, and 125 nM of the respective *msp-1* allelic type-specific primers (K1, MAD20, and RO33 types) as shown in Table 1 . For *msp-2* allelic amplification, the final concentration of the master mix consisted of 1× PCR buffer, 1 mM $MgCl_2$, 125 µM dNTP and 0.02 units/µL of AmpliTaq® DNA polymerase, and 125 nM of the FC27 allelic primers. However, for IC3D7 allele amplification, 0.05 units/ µL of AmpliTaq® DNA polymerase was used and the concentration of the primer pair was raised to 300 nM. 1 µL product from each primary reaction was used as a template for amplification. For the IC3D7 nested reaction, 2 µL product from each primary reaction was used as a template.

The cycle conditions for the amplification of the *msp-1* allelic types were as follows: initial denaturation at 95 °C for 5 min followed by 30 cycles of annealing at 61°C for 2 min, extension at 72 °C for 2 min, denaturation at 94 °C for 1 min, and a final step at 61 °C for 2 min and 72 °C for 5 min.

For the *msp-2* allelic types, the cycle conditions were: initial denaturation at 95 °C for 5 min, followed by 30 cycles of annealing at 58 °C for 1 min, extension at 72 °C for 2 min, denaturation at 94 °C for 1 min, and a final round at 58 °C for 1 min and 5 min at 72 °C. All amplifications were performed in 0.2 mL PCR amplification tubes (GeNunc®) with a total reaction volume of 25 µL per tube and were carried out in a Peltier Thermal Cycler (MJ Research Inc., Watertown, MA, USA). The PCR products were wrapped in aluminium foil to avoid quenching of the fluorescent dyes and stored at -20 °C for capillary electrophoresis.

3.6 Capillary electrophoresis and fragment analysis of *msp-1* and *msp-2* alleles

CE was performed in a 3130 Genetic Analyzer (Applied Biosystem) as recommended by the manufacturer. All reagents including the polymer were from Applied Biosystem. CE generates a size estimate for DNA fragments relative to a size standard. Both the sample and the size standard DNA are co-loaded into the capillary electrophoresis system. After run completion, DNA fragments are displayed as peaks, with fragment size calculated in base pair (bp), a height calculated in relative fluorescence units (rfu) and a calculated area underneath the associated peak. Stutter peaks were eliminated by setting a background cut-off 300 rfu as previously described (Liljander *et al.*, 2009)

A reaction mix (950 µL), enough for 96 samples in a 96 well plate was prepared by adding 50 µL of LIZ standard (GeneScan-500LIZ® was used for smaller fragments less than 500 bp such as K1, MAD20, RO33 and FC27 whereas GeneScan-1200LIZ® between 500 - 1200 bp fragments such as IC3D7) in 900 µL of Hi-di (Highly deionized) formamide. The mix was pulse vortexed and centrifuged at 8000 rpm (Microfuge®22R Centrifuge, Beckman Coulter). 9 µL of the mix was then dispensed into the 96 well plates (MicroAmp™ Applied Biosystems). 1 µL of amplified product from the nested reaction was then added to the 96-well plate, vortexed and then briefly centrifuged at 3000 rpm (Sorvall RT6000B centrifuge) to ensure uniform mixing, eliminate bubbles and sediment particles.

Samples were denatured at 95°C for 5 min and then chilled in ice to maintain the single strands prior to loading in the Genetic Analyzer. The samples were then loaded in the 36 cm capillary unit containing performance-optimized polymer, (POP-7^{TM}) for resolution and fragment sizing. CE output was exported into GeneMapper® Software version 4.0 (Applied Biosystems) for fragment analysis.

3.7 Determination of Multiplicity of infection (MOI)

Each PCR fragment for each locus was classified as clonal infection with one parasite genotype (clone). The detection of more than one PCR fragments for either *msp-1* or *msp-2* loci (an infection with more than one parasite genotype) was defined as having multiple *P. falciparum* clones. The number of distinguishable alleles for K1, MAD20 and RO33 for *msp-1* and FC27 and IC3D7 for *msp-2* genes was determined for each parasite isolate and the largest of these numbers was considered the MOI of that sample (Waitumbi *et al.*, 2009).

3.8 Relative clonal abundance

Alongside the fragment sizes generated from capillary output, the genetic analyzer also calculates peak height and peak area of the associated allele. For this, the relative abundance was estimated in percentage by dividing peak signal of each fragment by the total signal of all the fragments in the same allele family for each SMA case and UM control as was described previously (Ford and Schall, 2011). Peak heights and areas were compared between SMA cases and UM malaria and their suitability in estimating relative allelic abundance was evaluated.

3.9 Data analysis

Data generated from CE was exported to excel sheets for computational analysis with Graphpad prism (GraphPad Software Inc. San Diego CA). Student's paired t-test was used to compare MOI in

children with UM and those with SMA. *P* values less than 0.05 were considered significant. One way ANOVA was done to compare allele frequencies for both *msp-1* and *msp-2* allelic families

CHAPTER FOUR

4.0 Results

4.1 Subjects enrolled.

The study enrolled 57 cases and 44 controls whose clinical and anthropometric characteristics are shown in Table 2. Cases were enrolled followed by controls matched by age and gender, and due to this kind of enrolment, more cases than controls were enrolled to take care of the outliers. The mean age for the cases was 16.67±9.21 and 17.02±9.60 months in the controls. The mean Hb level in cases was 4.5±1.0 g/dL and 8.80±1.40 g/dL in the controls.

Table 2 Anthropometric and clinical characteristics of patients enrolled in a malarial anemia case control study.

Characteristics	*Severe Malaria*	*Uncomplicated Malaria*	*P- value*
Sample size (N)	57	44	ND
Age (months)	16.67±9.21	17.02±9.5	ND
Parasitemia (parasites/µl, Mean ± SD)	97,100+114,000	80700±106,000	0.661
Hb level (g/dl, mean ± SD)	4.5+1.0	8.9+1.3	<0.001

P = Paired t test performed with the 44 paired samples. ND = not done.

4.2 Capillary Electrophoresis output

Following the nested PCR amplification, the products were run in a genetic analyzer 3130 (Applied Biosystems). The genetic analyzer software converts the banding pattern into electropherograms (Figures 4 and 5). The position of the peak along the x-axis corresponds to the size of allele(s) (Figure 4). The corresponding allele peak height represents the amplitude measured in relative fluorescent units (rfu), while the area under the generated peak is referred to as peak area (

Figure 5). Threshold rfu was set at 300 in order to remove background fluorescence. These data was then exported to excel sheets for statistical analysis.

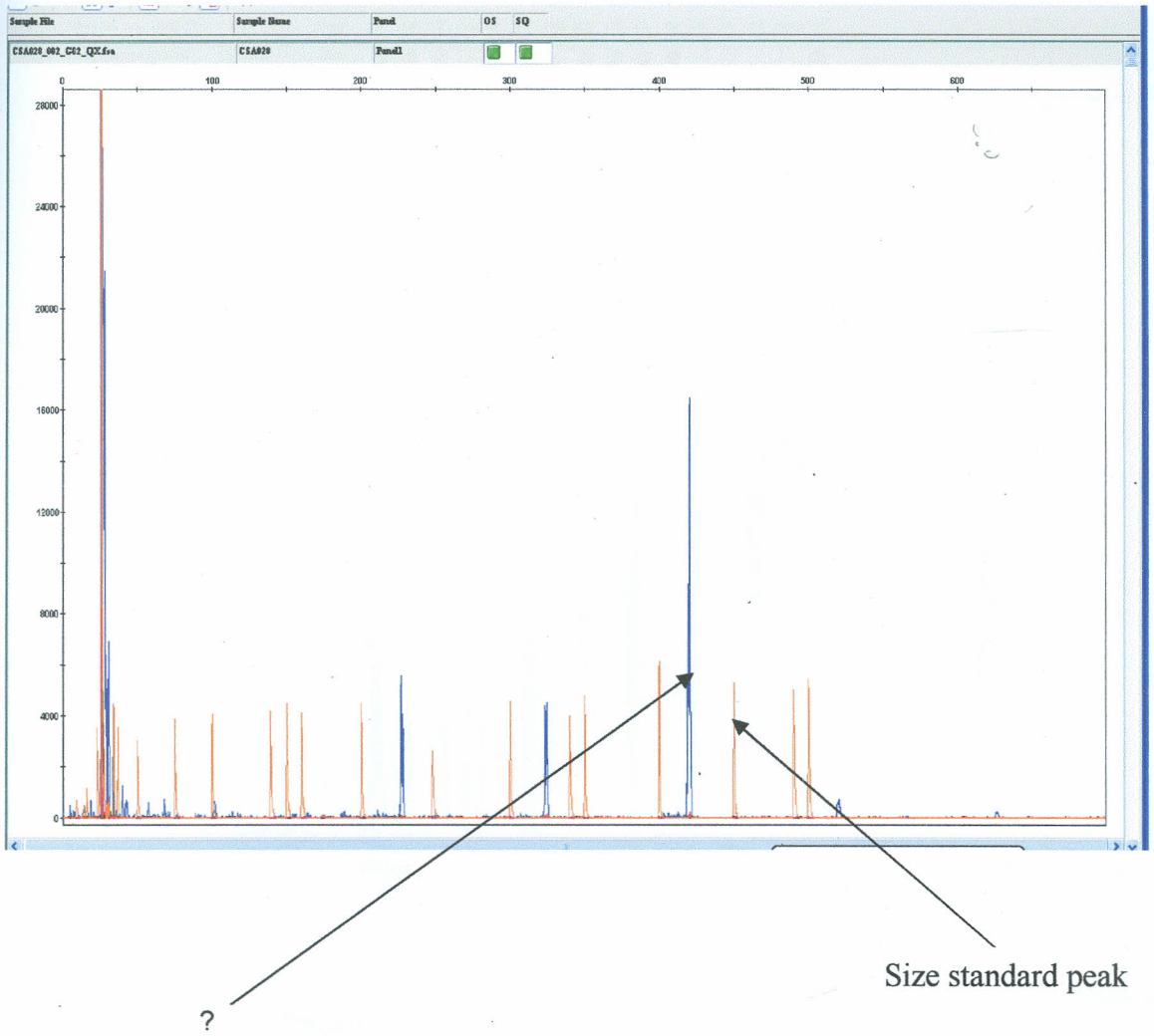

Figure 4. Electropherogram showing PCR amplicons and molecular size markers following CE.

Fig 4 shows raw data output prior to analysis by the genemapper software. In this case, the blue colored tracings shows three amplicons of different allele sizes whereas the orange peaks show the size standard (LIZ 500) used to calibrate the allele sizes.

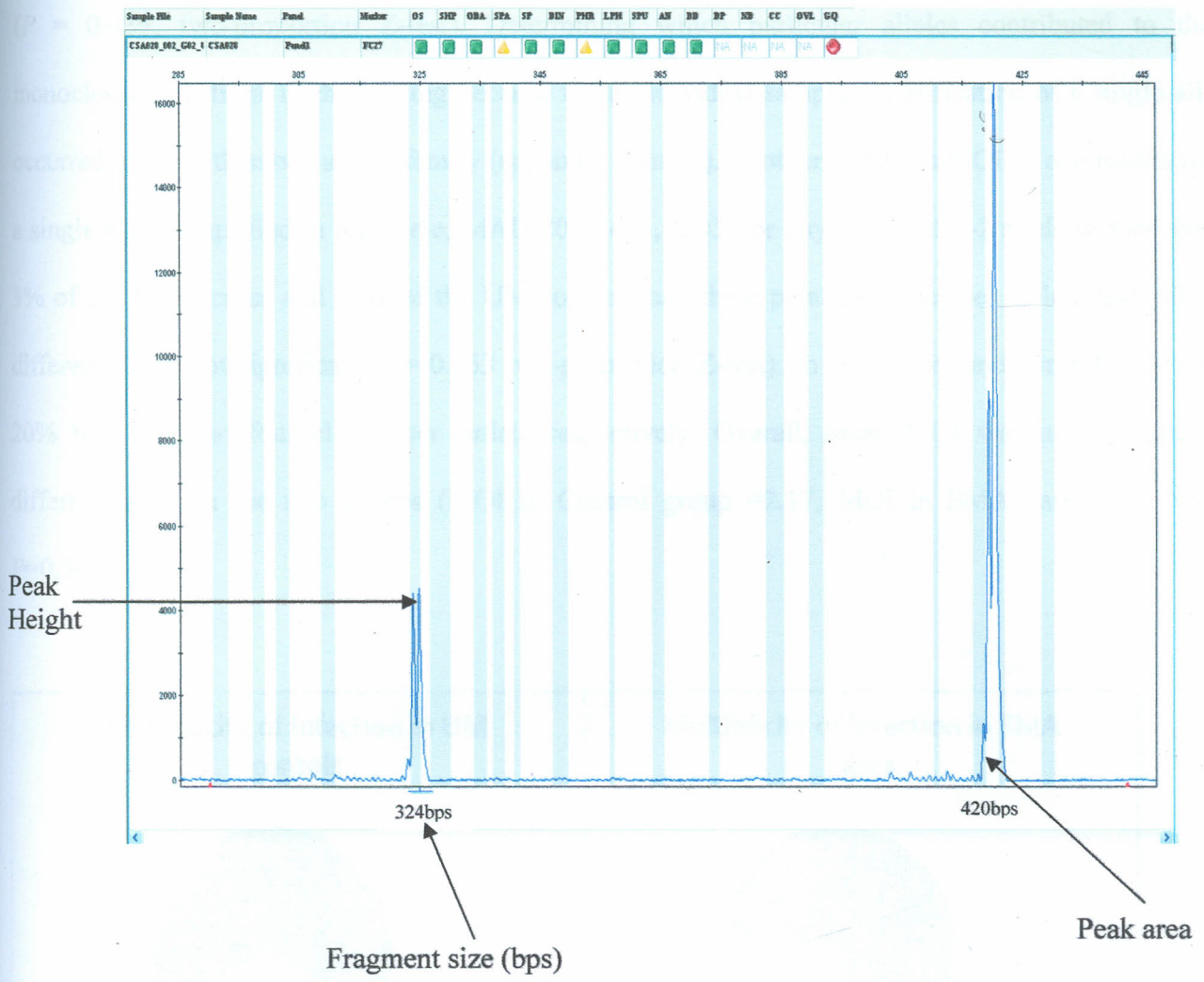

Figure 5. Electropherogram showing allele peak height and peak area.

Following analysis by the genemapper software each peak is assigned the fragment size (bps) displayed on the X-axis, the peak height (rfu) on the Y- axis and the calculated peak area.

4.3 Multiplicity of infection (MOI)

To determine MOI, the number of distinct *msp-1* and *msp-2* alleles per isolate was identified and the highest number was considered the MOI of that isolate (Waitumbi *et al.,* 2009). The mean MOI between the SMA cases and UM controls was then compared. In Figure 6, 43% of the SMA cases and 34% of the UM controls had monoclonal infections but this difference was not statistically significant

(P = 0.439, two-proportion Z-test). Determining which particular alleles contributed to these monoclonal infections is challenging because in an individual sample, amplification of a single allele occurred in more than one allelic family (appendix 1 and 2). In other words an MOI of one results from a single allele amplified in K1 allele, MAD 20 allele , RO33 or any other MSP-2 allele or vice versa. 3% of the SMA cases and 12% of the UM controls had three parasite clones per isolate and yet this difference was not significant P = 0.153 two-proportion Z-test). In both cases and controls, 34% and 20% had two and four clones per isolate respectively. Overall, mean MOI was not significantly different between the two groups (MOI in Control group =2.17, MOI in SMA cases=2.0, N=35, P=0.54).

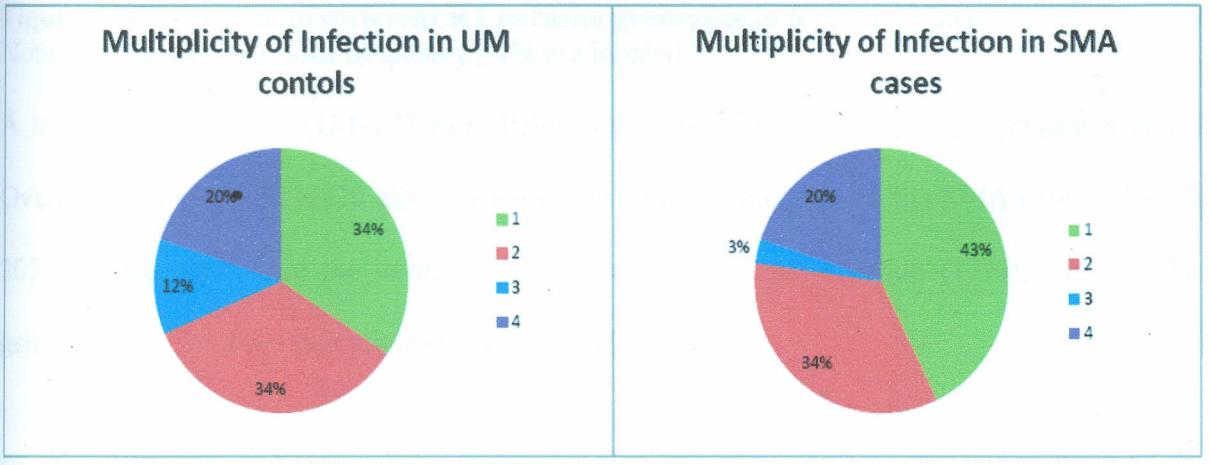

Figure 6. MOI in SMA cases and UM controls.

4.4 *P. falciparum* allele frequency in SMA and UM groups

Figure 7-11 summarizes the allele frequencies in SMA cases and UM controls. K1 allele was the most polymorphic with 119 alleles (159-319 bp) being detected (57 in SMA and 62 in UM, Table 3). For all *msp-1* and *msp-2* alleles, the number of alleles amplified in cases and controls did not differ significantly ($P > 0.05$).

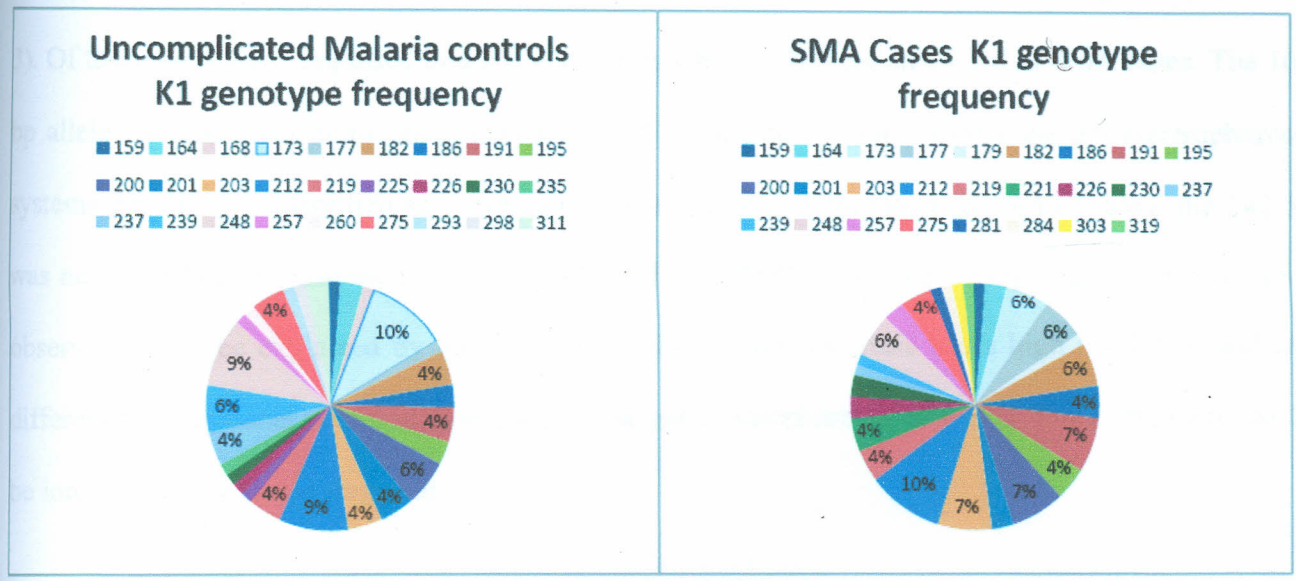

Figure 7 Distribution of different K1 parasite genotypes in SMA and UM.
Note: Only genotypes with frequency ≥4% are labeled.

A total of 50 MAD20 (131-222 bp) alleles were amplified in both groups (Figure 8 and Table 3). Overall, 44% of the MAD20 alleles were in the controls and 56% in the SMA cases. The 170 bp and 205 bp fragments were the dominant genotypes in the control group (17%), whereas the 213 bp genotype was the dominant allele in SMA cases (26%).

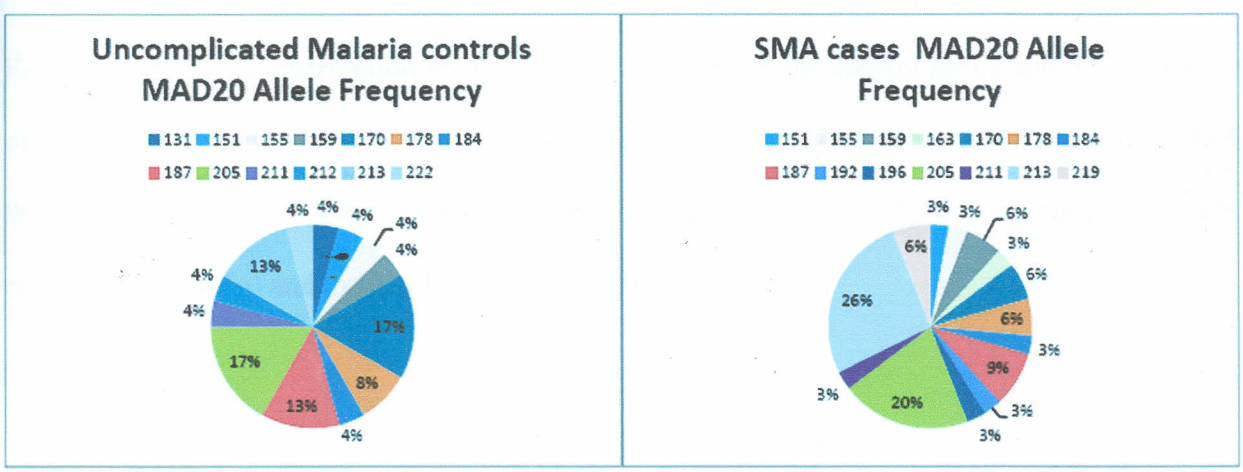

Figure 8 Distribution of MAD20 alleles in SMA cases and UM controls.

A total of 50 (155-161 bp) RO33 alleles were amplified in both cases and controls (Figure 9 and Table 3). Of these 44% were amplified in the UM controls while 56% were amplified in SMA cases. The 160 bp allele was the most dominant genotype (>80%) in the two groups. Under the gel electrophoresis systems the RO33 is identified as a monomorphic allele of 142 bp. However, in this study, the 142 bp was not identified, but instead four other alleles (155 bp, 158bp, 160 and 161bp) were amplified. This observation can be explained by the fact that capillary electrophoresis has a high resolution and can differentiate fragments upto one base pair, unlike gel electrophoresis where the three fragments could be interpreted as a single fragment.

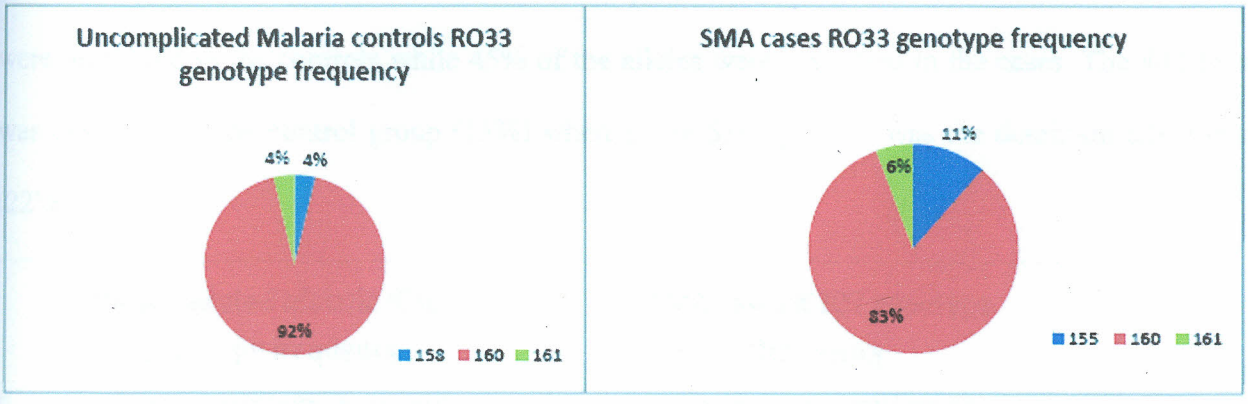

Figure 9 Distribution of the RO33 allele in SMA cases and UM group.

Figure 10 shows the distribution of different FC27 alleles in the control group and SMA cases. A total of 70 alleles were amplified in both groups (Table 3). Of these, 46% were in the controls while 54% of the alleles amplified in SMA cases (P=0.346). The 420 bp FC27 allele was the dominant allele in the control group (19%) and the 337 bp dominant in the cases (23%).

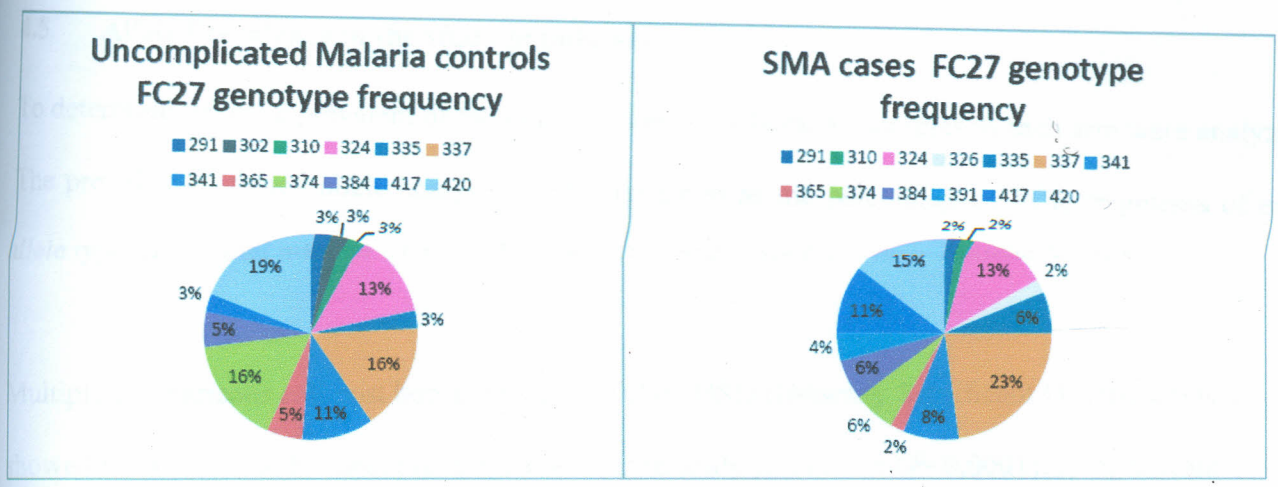

Figure 10 Distribution of FC27 alleles in SMA cases and UM controls.

For the IC3D7 loci, a total of 31 clones were amplified (Table 3). Of these clones 55% of the alleles were amplified in the controls while 45% of the alleles were amplified in the cases. The 492 bp allele was dominant in the control group (15%) whereas the 516 bp allele was the dominant allele in cases (22%).

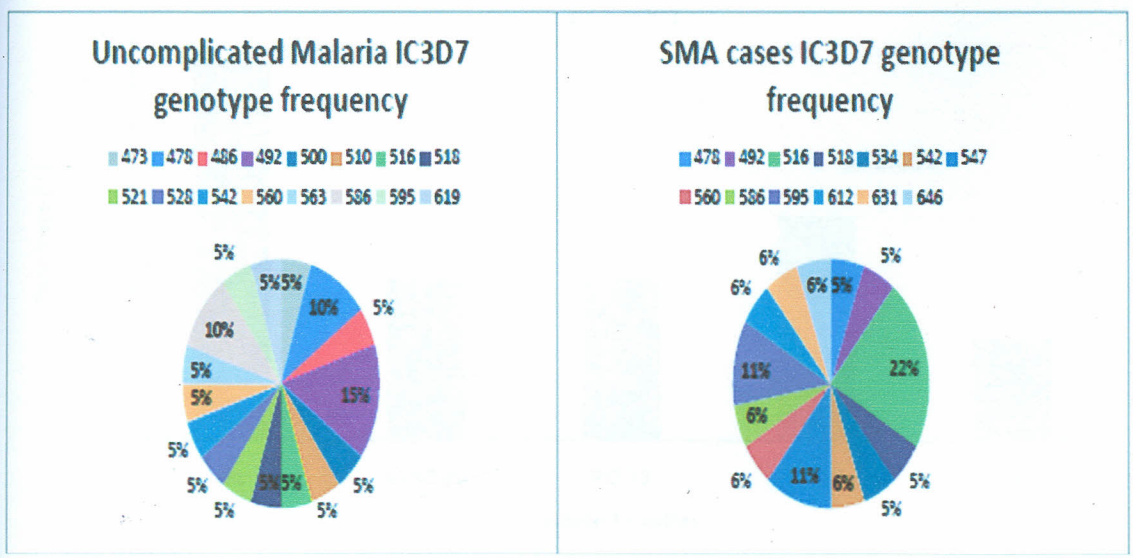

Figure 11 Distribution of the IC3D7 alleles in SMA cases and UM controls.

4.5 Allele Prevalence in the study population

To determine the most prevalent of the *msp-1* and *msp-2* alleles, 35 samples in each arm were analyzed. The prevalence of each allele analyzed was determined as the percentage of PCR fragments of each allele type in the total number of amplified bands for both *msp-1* and *msp-2* allelic families.

Multiple comparisons and post hoc analysis by Tukey HSD (Honestly Significant Difference) test showed K1 allele was the most prevalent allele in the study population ($P<0.0001$). A significant difference was also observed between FC27 and IC3D7 allele ($P=0.002$) in *msp-2* (Figure 12). Comparing the frequency of the *msp-1* and *msp-2* genotypes in the SMA cases and the uncomplicated malaria controls did not yield any statistical difference in the number of alleles between the SMA and UM controls ($P>0.05$) (Table 3).

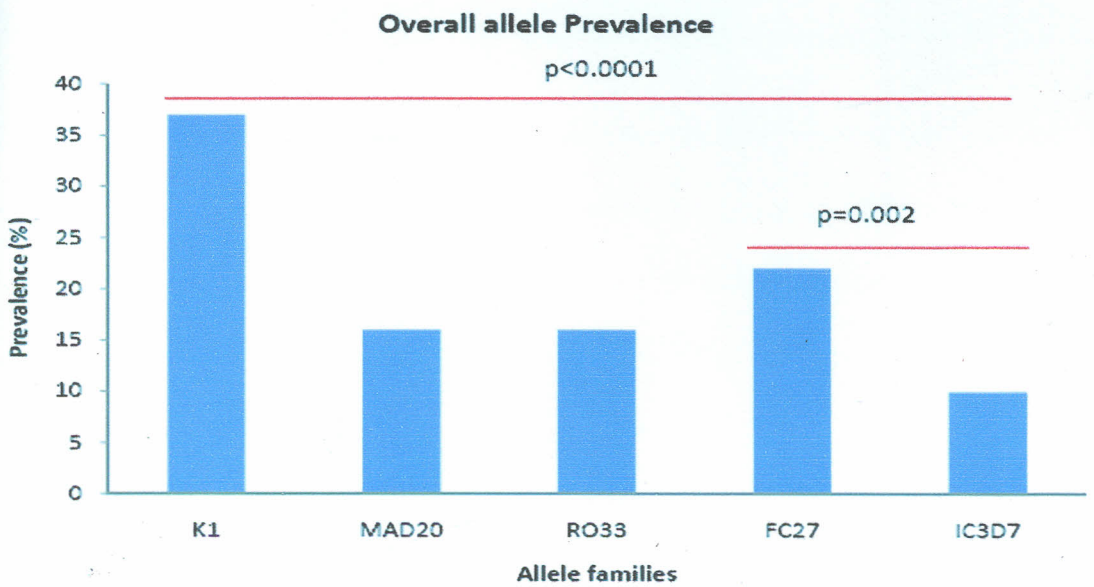

Figure 12 Graph showing the prevalence of different *msp-1* and *msp-2* alleles within the study population.

Table 3 Table showing comparison of allele frequencies between SMA cases and UM controls.

Allele Family	No. of Alleles		
	SMA Cases	**UM Controls**	***P*-value**
K1	57	62	0.720
MAD20	28	22	0.475
RO33	28	22	0.110
FC27	38	32	0.362
IC3D7	14	17	0.447

4.6 Comparison of peak height and peak area in SMA and UM groups

Besides the size and number of fragments obtained from the genetic analyzer genemapper also calculates the fragment amplitude (peak height) and area under the peak (peak area) Figure 5). The data showed high reproducibility for the allele size and number (Table 4).

Table 4 Sample data showing replicate samples (K1 allele) with reproducible allele size and number but variable peak height and area.

	Allele 1			Allele 2			Allele 3		
Sample Name	**Size (bp)**	**Peak Heigh (rfu)**	**Peak Area**	**Size (bp)**	**Peak Height (rfu)**	**Peak Area**	**Size (bp)**	**Peak Height (rfu)**	**Peak Area**
CSA_027_K1_1	257	2182	13033	275	2674	16498			
CSA_027_K1_2	257	2259	13706	275	2621	16686			
CSA012_K1_1	219	797	4733	298	380	2381			
CSA012_K1_2	219	1097	6764	298	431	2935			
CSA120_K1_1	230	8066	44484	266	1133	7324			
CSA120_K1_2	230	4281	26125	266	621	4188			
CSA_017_K1_1	173	385	2336	182	351	2064	191	444	2690
CSA_017_K1_2	173	433	2823	182	397	2389	191	512	3126
CSA110_K1_1	159	1616	9340	168	474	2819	186	957	5500
CSA110_K1_2	159	1426	8277	168	423	2554	186	808	4684
Positive control_K1_1	212	5410	29298						
Positive control_K1_2	212	5363	28986						
Negative control_K1_1	-	-	-						
Negative control_K1_2	-	-	-						

The variation of peak height and area was calculated in order to determine which of the two parameters was most reproducible. Table 5 shows the average proportion of K1, MAD20 and FC27 alleles in percentage and the variation when estimated by peak height and peak area. For the three alleles (K1, MAD20 and FC27), their average abundance (33%-47%) was not different both by peak height or area and also between SMA cases and UM malaria ($P = 0.05$).

Table 5 Relative abundance of *msp-1* and *msp-2* allele families as calculated by peak height and peak area in SMA cases and UM controls.

Allelic family	SMA Cases		UM Controls	
	Percentage relative abundance		Percentage relative abundance	
	Peak height (SD)	Peak Area (SD)	Peak height(SD)	Peak Area(SD)
K1	33.82 (21.97)	33.75 (21.97)	35.56 (28.02)	34.09 (26.66)
MAD20	42.86 (28.96)	42.86 (28.53)	33.33 (24.38)	33.33 (24.10)
FC27	44.44 (25.06)	44.44 (25.00)	46.67 (29.98)	46.67 (30.38)

RO33 and IC3D7 alleles were not included in estimating the relative clonal abundance because only single alleles (monoclonal infections) were amplified for these loci; therefore calculating relative abundance (which is basically a proportion of all the alleles of a particular loci in a sample) would give a 100% abundance which can be misinterpreted to mean high clonal density of these alleles.

Peak height and area data was then compared between the SMA cases and the UM controls (paired data). RO33 allele had the highest peak height and peak area values (Table 6) for both the SMA (peak height= 20179 rfu, peak area = 196581) cases and UM (Peak height = 25163 536 rfu, peak area= 243775) control. High peak height and peak area values recorded for RO33 are not a direct reflection of clone abundance. This may be due to the ability of capillary-based instruments to detect shortest fragments more readily and the high efficiency of PCR for shorter sequences, resulting in high peak height and peak area for alleles with fewest repeats (Selkoe and Toonen, 2006), hence the high peak height and area values for RO33 (fragment size = 160 bps) as compared to IC3D7 (fragment size = 470-700 bps).

Table 6 Allele height and Allele area values obtained for the SMA cases and UM controls

Allele family	Average Allele Height (rfu)		Average Allele area	
	SMA	UM	SMA	UM
K1	4330 (605)	4703 (596)	29911 (4435)	28556 (4362)
MAD20	4344 (487)	1455 (169)	33828 (4178)	10997 (1349)
RO33*	20179 (2144)	25163 (1720)	196581 (22325)	243775 (18309)
FC27	7986 (1175)	10577 (1233)	67034 (9052)	88044 (9376)
IC3D7	610 (51)	936 (128)	10950 (918)	17519 (2278)

*Significantly high allele height and allele area values ($P<0.0001$) (Standard Error of Mean)

When the average allele height and area was compared between the cases and controls no significant difference was observed ($P>0.05$) for all allelic families.

CHAPTER FIVE

5.0 Discussion

In endemic areas malaria has a complex presentation: severe malaria anemia mainly occurs in children <2years, cerebral malaria that tends to occur in older children (3-5 years), placental malaria in pregnant women and in some cases asymptomatic malaria in children and adults (Greenwood *et al.,* 1991). It's unclear as to whether these syndromes are caused by genetically distinct parasites.

Advances in genotyping techniques have revealed that in a malaria infection, hosts are infected with more than one genotype (clone) of the same pathogen. (Kyabayinze *et al.,* 2008). Multiclone infections are thought to arise from infection with genetically diverse innoculum or reinfection before an existing infection is cleared (Read and Taylor, 2001). Such multiple clone infections are believed to play an important role in the development of clone specific immunity. For malaria, two kinds of datasets are important when scoring the clonal diversity within an infection: the number of genetically distinct clones (often referred to as MOI) and their relative abundance (Vardo-Zalik *et al.,* 2009). The first class of data is used in studies on transmission intensity (Anderson, et al., 2000; Branch et al., 2010) while relative abundance of clones is relevant for studies on the dynamics of infection (Read and Taylor, 2001). While MOI is relatively easy to determine, the clonal abundance requires techniques that are quantitative. The recent introduction of capillary electrophoresis of fluorescently labeled PCR amplicons has made this possible. In this study, the population structure of malaria parasites obtained from children with SMA and those with UM were studied by comparing MOI, clonal diversity and abundance.

MOI defined as the number of distinguishable *P. falciparum* clones between SMA and UM ranged from one to four clones per patient (Figure 6). In the SMA, 43% of the infections occurred as single

parasite clone compared to 34% in the control group. Overall, 68% of the infections in controls and 77% in cases were attributable to either one or two clones. Although this difference is not significant (P>0.05), it shows a slight reduction in MOI in SMA cases. Similarly Robert (1996) reported a lower MOI in severe compared to uncomplicated malaria and high parasite diversity in both groups (Robert *et al.,* 1996), and Waitumbi (2009) in a vaccine trial reported a reduction in MOI in vaccinated groups as compared to controls. On the contrary, a study by Mockenhaupt (2003) showed significant association of low hemoglobin levels and severe anaemia with high MOI (Mockenhaupt *et al.,* 2003). Although this results show that more SMA cases were infected with single parasite clones (Figure 6) than the controls, the overall difference in MOI (UM = 2.17, N = 35 and SMA = 2.0, N = 35) was not significant ($P = 0.54$).

In regard to the *msp-1* and *msp-2* allele prevalence, K1 allelic family was the most dominant (37.2%) compared to FC27 (21.9%), MAD20 (15.6%), RO33 (15.6%), and IC3D7 (9.7%), ($P<0.0001$, Tukey HSD, Figure 12). The high prevalence of the K1 allele in this study is consistent with previous studies in Michenga village in Tanzania and Asar village in Sudan (two regions with different transmission intensities) (Babiker *et al.,* 1997). When allelic prevalence was compared between the SMA cases and the UM controls, the K1 allele was still the most prevalent but no significant difference between the two groups was observed ($P>0.05$). Other studies have however shown dominance of different alleles. For example, a study by Ranjit (2005) showed that MAD20 (200 bp) and 3D7 (550bp) clones were overrepresented in severe cases of malaria. The RO33 allele has always been reported to be the least polymorphic with some studies showing an allele of 150bp (Ranjit *et al.,* 2005) or 160 bp (Sahu, et al., 2008). In this study four RO33 alleles were identified with fragment sizes of; 155bp, 158bp, 160bp, and 161bp. Of these four alleles, the 160 bp allele was more prevalent (>80%, Figure 9) in both groups.

With the low resolution of agarose gels, it would have been impossible to differentiate these alleles on agarose gels, clearly indicating the added value of CE.

Allelic frequency varied for both *msp-1* and *msp-2* alleles, some alleles were overrepresented in SMA cases whereas others were overrepresented in UM controls. For instance, the 420 bp of FC27 and the 492 bp of IC3D7 allele were overrepresented (19% and 15% respectively) in UM controls and the 337 bp of FC27 and the 516 bp of IC3D7 were overrepresented (23% and 22% respectively) in SMA cases (Figure 10 and Figure 11). Allele frequency has been shown to vary depending on transmission intensity. Ranjitt (2005) showed a 550 bp IC3D7 allele to be overrepresented in severe cases (Ranjit *et al.*, 2005). These findings do not support contention that SMA is caused by particular genotypes of *P. falciparum,* and therefore more studies on the contribution of other confounding factors would have to be investigated.

To quantify the relative abundance of parasite clones, two parameters afforded by CE, namely peak height and peak area were used. All samples were run in duplicates to ascertain the reproducibility of these two parameters and also determine which of the two has least variability. 100% reproducibility was achieved in the allele size and numbers for duplicate samples. However, peak height and area data in replicate samples were not reproducible (Table 4). To determine preferable index of relative clonal abundance in an infection, peak area and peak height was used to estimate relative allele abundance. Table 5 shows that for any single allele both peak height and peak area signals precisely predicted relative allele abundance. The finding that either peak height or peak area can be used to determine relative clonal abundance contradicts results from studies by Roberto (2007) that showed peak height to be less variable and therefore preferable for estimating relative clonal abundance. Table 6 shows the RO33 allele family had the highest allele height and allele area in cases and controls ($P<0.0001$) but no

difference was observed between SMA and UM ($P>0.05$). Capillary based instruments detect shorter fragments more readily than larger fragments. Because of the relatively small size of PCR amplicons from this locus (142 bps), we speculate this resulted to high peak height and peak area values exhibited in the RO33 allele.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Findings from this study have shown that the frequencies of the different *msp-1* and *msp-2* genotypes did not have a particular pattern, in that while some alleles were dominant in SMA cases others were dominant in UM controls. Although more cases (43%) were found to have monoclonal infections, the overall MOI between SMA cases and UM did not reach statistical significance ($P = 0.54$), therefore implying that severity of *P. falciparum* malaria is not restricted to single clones that outgrow other genotypes or multiple clones in an infection.

K1 allele was reported to be the most prevalent allele in the study population and this was consistent with a study in East Africa that showed a high prevalence of the K1 allele (Babiker *et al.*, 1997).

Peak height and peak area values between SMA cases and UM controls did not differ significantly. RO33 allele had significantly high ($P<0.0001$) peak height and area values as compared to the other alleles. Contrary to a study by Roberto (2007) that Peak height is less variable than peak area, this study has shown that both peak height and peak area are suitable measures of relative clonal abundance in an infection.

6.2 Recommendations for future research

This study was unable to as associate malaria severity with any particular genotypes in the study population. Progression to severe disease is therefore not attributable to parasite genotype only but is dependent on other factors such as premmunition (can provide some tolerance to certain genotypes due to prior exposure), age and host genetics. However, this study did not investigate the role of age and host immunity in progression to severe disease due to the nature of the study, whereby recruitment of subjects into the study did not examine if the patients had a prior malaria infection and if treatment was administered (this might provide some tolerance). We therefore recommend more comprehensive studies to determine the collective contribution of these factors to development of severe disease.

We were able to show that peak height and peak area profiles can be used to estimate relative clonal abundance. However, to minimize the variation observed in peak height and are profiles, we recommend well controlled studies to be set by simulating infections of known clones to generate a standard curve against which subsequent profiles are compared. Accurate estimation of relative clonal density can be useful in determining temporal changes in parasite clone densities in antimalarial drug trials.

In addition estimation of clonal abundance was done following PCR amplification. This means that products were collected at the plateau phase of the reaction when all fragments have been amplified to equal quantities. For accurate quantification, fragments should be harvested at the exponential phase, when clonal abundance is proportional to the quantities of the starting material.

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