THE SWAIN-LANGLEY AND McCOY BLOOD GROUP POLYMORPHISMS OF COMPLEMENT RECEPTOR 1: ROLE IN SEVERE *PLASMODIUM FALCIPARUM* MALARIA

BY

BERNARD OCHIENG' GUYAH (Msc.)

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Declaration

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

Signature 

Bernard Ochieng' Guyah

We confirm that the work reported in this thesis was carried out by the candidate under our supervision:

Signature 

Prof. Alloys S. S. Orago, Ph.D., MPH, D.Sc Epid.
Department of Pathology,
School of Health Sciences,
Kenyatta University, Nairobi, Kenya

Signature 

Dr. Michael F. Otieno, M.Ap.Sc., PhD.
Department of Pathology
School of Health Sciences,
Kenyatta University, Nairobi, Kenya.

Signature 

Dr. Vandana Thathy, PhD
Department of Immunology
KEMRI/Wellcome Trust Research Laboratories
Kilifi, Kenya

Signature 

Dr. Jose A. Stoute, MD
Associate Professor of Medicine, and Microbiology and Immunology
Dept. of Medicine
Penn State University College of Medicine, Hersley, PA.

Dedication

This thesis is dedicated to my younger brother who was shot dead in the post election violence and my family, chidren (Agnes and Joachim) and friends who helped me through the numerous difficult moments and celebrated with me during the joyful times. To my wife Magdalene: without your support and numerous sacrificial moments, I would not be able to have put this thesis together. Your love and patience I will not be able to repay. To my mother Conselata: without your motherly encouragement and fervent prayers I would not have had the determination to stay on through this work. The perseverance I saw in you and the trust you had in me will always reverberate in my mind.

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List of Abbreviations

aa	amino acid
ADCI	Antibody-dependent cell-mediated inhibition
AMA1	Apical membrane antigen 1
amp	ampicilin
BLAST	Basic local alignment search tool
bp	base pair
BSA	Bovine serum albumin
CCP	Control protein repeats
cDNA	complementary deoxynucleic acid
CIDR	Cystein-rich interdomain region
CI	Confidence interval
CM	Cerebral malaria
CMI	Cell-mediated immunity
CR1	Complement receptor 1
CSA	Chondroitin sulfate A
CSP	Circumsporozoite surface protein
C-terminus	Carboxy terminus
CYT	Cytoplasmic
DAPI	4', 6-diaminodino-2-phenylindone
DBL	Duffy binding ligand
DEPC	diethylpyrocarbonate
DMEM	Dulbeco's modified eagle medium

DMSO	Dimethyl sulfoxide
dNTPs	deoxynucleotides
DTT	Dithiothreitol
EBA-175	Erythrocyte binding antigen 175
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ELAM-1	Endothelial leukocyte adhesion molecule 1
EST	Expressed sequence tags
FACS	Fluoresence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
gDNA	genomic deoxylnucleic acid
GFP	Green fluorescent protein
GLURP	Glutamate rich protein
GPI	Glycosylphosphatidylinositol
HA	Hyaluronic acid
Hb	Haemoglobin
hr	hours
HEPES	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid
HIS	Hyperimmune sera
HRP	Horseradish peroxidase
HS	heparin sulfate
HSV gD1	Herpes simplex virus glycoprotein D1

ICAM1	Intracellular adhesion molecule 1
IC	Immune complex
IFA	Immuofluorescence assay
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRBC	Infected red blood cell
KAHRP	Knob associated histidine-rich protein
kan	kanamycin
kB	kilobases
kDA	kilodaltons
KN	Knops
LB	Luria Bertani
LHR	Long homologous region
LSA3	Liver stage antigen 3
LTR	Long terminal repeat
mAb	monoclonal antibody
McC	McCoy
min	minutes
MIRL	Membraneinhibitor of reactive lysis
mL	mililitre
MSP1,2,3	Merozoite surface protein 1, 2, 3

NCBI	National Centre for Biotechnology Information
NK cell	Natural killer cell
NIS	Nonimmune serum
NO	Nitric oxide
NOS	Nitric oxide synthase
N-terminus	Amino-terminus
OD	Optical density
OR	Odds ratio
ORF	Open reading frame
PCR	Polymerse chain reaction
PE	Phycoerythryn
PECAM-1	Platelet endothelial adhesion molecule 1
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
RCA	Regulators of complement activation
RESA	Ring-infected erythrocyte surface antigen
RFLP	Restriction fragment length polymorphism
rpm	revolution per minute
RPMI	Roswell Park Memorial Institute
RSV	Rour sarcoma virus
RT	Room temperature
RT-PCR	Reverrse transcriptase polymerase chain reaction
sec	seconds
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sl	Swain Langley
SMA	Severe malarial anaemia
SNPs	Single nucleotide polymorphism
TAE	tris-acetate buffer
TBE	tris-borate buffer
TM	Trasmembrane
TNFα	Tumour necrosis factor alpha
tRNA	total deoxylribonucleic acid
TSP	Thrombospondin
VCAM-1	Vascular cell adhesion molecule 1
x-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Abstract

Binding of *Plasmodium falciparum*-infected red blood cells (RBCs) to uninfected RBCs to form rosettes has been associated with severe malaria in Africa. The major parasite ligand that mediates rosetting is a high molecular weight product of the var gene family, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 interacts with several receptors on uninfected RBCs, including complement receptor 1 (CR1). The *CR1* gene encodes the McCoy and Swain-Langley Knops blood group antigens with respective alleles McC^a/McC^b and *Sl1/Sl2*. McC^b and *Sl2* allele frequencies are significantly elevated in Africans compared to other ethnic groups. This has led to the hypothesis that the McC^b and *Sl2* alleles may have been selected for in malaria-endemic regions by conferring protection against severe malaria. This case-control study sought to establish any associations between McC^b and *Sl2* and severe forms of malaria (cerebral malaria and severe malarial anaemia). The results demonstrated that individuals with *Sl2/2* genotype are more resistant to severe malaria in general, (OR 0.3, 95% CI 0.1 to 0.8, *P-value 0.01*) and cerebral malaria in particular (OR= 0.2, 95% CI= 0.1 to 0.9, *P-value 0.03*) than individuals with the *Sl1/1* genotype. However, individuals with $McC^{b/b}$ did not show any protective advantage against severe malaria. The data also observed that children with the *Sl2/2* $McC^{a/b}$ genotype combination were more resistant to severe malaria in general (OR = 0.2, 95% CI 0.1 to 0.7, *P-value* 0.01), and in particular cerebral malaria (OR = 0.2, 95% CI 0.0 to 0.9, *P-value* 0.03), than those with the *Sl1/1* $McC^{a/b}$ genotype. In order to study how age-dependent prevalence of African alleles, a cross-sectional survey was conducted and crosstabulation showed that the frequency of Sl2/2 was highest among age group $\leq$ 48 to $\geq$ 96 months. Logistic regression analysis demonstrated that the same age group had higher relative risk of Sl2/2 than any other group (OR = 3.36, 95% CI 1.36 to 8.30, *P value* = 0.01. The most predominant cloned sequence of DBL1α from the parasite isolate was the group 2 sequence. The lack of DBL1α constructs to bind erythrocytes was likely due to their inability to be trafficked to cell surface. Taken together, these observations support the conclusion that *Sl2* and McC^b alleles have evolved in the context of malaria transmission and in certain combination may confer some protective effects against severe forms of the disease. Although the data have demonstrated the protective effects of these CR1 polymorphic alleles, the mechanisms of this protection are not clear but rosetting is thaught to be involved. Understanding the molecular mechanisms of the protection may provide more insights into the pathogenesis of severe *P.falciaprum* malaria and inform the development of therapeutic agents against the disease.

CHAPTER 1: INTRODUCTION

1.1 Background information

Malaria is a disease transmitted by the bite of anopheline mosquitoes and caused by repeated cycles of invasion and intracellular growth of the protozoan parasite *Plasmodium* within erythrocytes. Most of the deaths due to malaria are caused by *P. falciparum* and occur in children under the age of five years. They account for nearly 25% of total child mortality in Africa (WHO, 1998). These childhood deaths occur as a result of complications such as severe malarial anaemia and cerebral malaria (coma). In the lowlands of the Lake Victoria basin region of Western Kenya, where malaria is holoendemic and the transmission rate is one of the highest in the world, severe malarial anaemia in children under the age of two years is the most common complication (Waitumbi *et al.*, 2000). However, in the highlands of Western Kenya and other regions of the world where transmission rate is lower, cerebral malaria is the major complication. It occurs in older children and the risk of developing the complication increases with age (Snow and Marsh, 1998).

Despite the huge amounts of genetic and biochemical data currently available for *P. falciparum* and its human host, the pathogenesis of severe anaemia and cerebral malaria remains poorly understood. Susceptibility to severe malaria is governed by a complex interplay of parasite, host, environmental and social factors. The pattern of clinical disease varies with age, acquired immunity and transmission rates (Snow and Marsh, 1998).

Human genetic variations undoubtedly play a pivotal role in determining susceptibility to severe malaria. Numerous susceptibility and resistance genetic factors

have been defined via population-based association studies, including primarily structural and regulatory polymorphisms of genes encoding erythrocyte proteins and genes of the immune system (Kwiatkowski, 2000).

The particular virulence of *P. falciparum* compared with other human malaria species has been ascribed to its unique ability to induce a number of novel modifications on the surface of the erythrocytes it infects such that infected cells become adherent to the vascular endothelium (cytoadherance) or to uninfected erythrocytes (rosette formation) (Newbold *et al.*, 1999; Chen *et al.*, 2000; Miller *et al.*, 2002). Cytoadherance and rosetting are mediated primarily by interactions between different host cell receptors and the major parasite adhesin expressed at the surface of infected erythrocytes, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is encoded by the var gene family (Miller *et al.*, 2002).

Rosetting is thought to contribute to the pathogenesis of cerebral malaria by participating in the blockade of post-capillary venules in the brain thereby decreasing cerebral blood flow (Kaul *et al*, 1991).

Several receptors have been identified on uninfected RBCs that mediate rosetting through interactions with PfEMP1 (*reviewed in* Chen *et al.*, 2000), that include complement receptor 1 (also called CR1, CD35 or C3b/C4b receptor) (Rowe *et al.*, 1997, 2000). CR1 is an immune regulatory integral membrane protein found on erythrocytes and most leukocytes (Fearon, 1980). It functions as a complement regulatory protein and as an immunological sponge that mops up immune complexes from the circulation and transports them to the liver and spleen for removal by fixed macrophages (Krych-Goldberg and Atkinson, 2001; Birmingham and Hebert, 2001).

CR1 contains the Knops blood group antigens, including the Swain-Langley (Sl 1,-2 and Sl -1,2) and McCoy (McC(a+) and McC(b+)) with respective alleles *Sl1/Sl2* and McC^a/McC^b (Moulds *et al.*, 1991; Rao *et al.*, 1991; Daniels *et al.*, 2003). The *Sl2* and McC^b alleles result from single amino acid variations in CR1 and are very rare in ethnic groups that are not of African descent (Moulds *et al.*, 2000, 2001). This observation has led to the hypothesis that the *Sl2* and McC^b alleles may have been selected for in malaria-endemic regions by providing protection against severe malaria. Part of this study that sought to investigate whether there is an association between resistance or susceptibility to severe malaria and the Swain-Langley and McCoy polymorphisms in malaria-endemic western Kenya has already been published (Thathy *et al.*, 2005). The results showed that individuals with the *Sl2/2* genotype were less likely to have severe malaria, in particular cerebral malaria, than individuals with the *Sl1/1* genotype. This association was even stronger in individuals with the *Sl2/2* $McC^{a/b}$ genotype compared to those with the *Sl1/1* $McC^{a/a}$ combination. These data demonstrated for the first time that *Sl2* and McC^b alleles are common in Africans because in certain combinations they may confer protection against *P. falciparum* malaria.

In order to understand the mechanisms of this protection, the study further hypothesized that the effects of this protection could be due to reduced CR1-mediated rosetting and those erythrocytes with these polymorphic variants (*Sl2* and McC^b) rosette less well compared with wild types (*Sl1* and McC^b). Therefore, an experimental design was formulated to investigate how the *Sl2* and McC^b polymorphisms could affect the structure-function relationships between CR1 and its ligand (PfEMP1) and how such

changes impact on either protection (less rosetting) or susceptibility (more rosetting) to severe malaria.

1.2. Statement of problem

Severe malarial anaemia and cerebral malaria are the major complications that contribute to the vast majority of deaths. The pathogenesis of these complications is poorly understood. The high mortality caused by *P. falciparum* malaria in endemic regions has resulted in the selection of polymorphisms in human genes that affect correlates of disease severity such as cytoadherance of infected RBCs to the microvasculature and rosetting. CR1 on RBCs has been implicated in the pathogenesis of severe malaria because of its function as a regulator of complement activation on RBCs and its ability to interact with *P. falciparum*-infected RBCs leading to rosette formation. Studies have reported that some CR1 polymorphic alleles (Sl2 and McCb) are found in significantly high frequencies in Africa and have been designated African alleles (Zimmerman *et al.* 2003). Although several studies converge to support the finding that Knops blood group antigen erythrocyte variants are associated with reduced rosetting, those that have sought to associate these blood group alleles on CR1 gene have reported mixed results (Zimmerman *et al.*, 2003, Thathy *et al*,. 2005). Since it has been shown that DBL1α domain of PfEMP1 binds to the active site of CR1 (Rowe *et al.*, 1997), it might therefore inhibit CR1 known major functions such as ligand binding, cofactor activity, decay-accelerating factor. This could lead into more deleterious effects like excessive complement activation resulting to inflammation. In the background of these possibilities, it is absolutely essential to obtain a much thorough understanding of the interaction of CR1 and PfEMP1 as well as the role of CR1 polymorphisms in malaria pathogenesis.

This understanding is required to inform any preventive strategy that involves blocking CR1-PfEMP1 interaction to prevent development of severe disease.

1.3 Research Questions

1. Do the mutations that give rise to the McC^b and/or *Sl2* CR1 alleles confer protection against severe forms of malaria?
2. Do *Sl2* CR1 allele frequencies change with age?
3. Do SA075 parasite isolate express rosetting DBL1α sequences?
4. Do the McC^b and/or *Sl2* variant erythrocytes rosette less well compared with wild types (McC^a and/or *Sl1*)?

1.4 Hypotheses

a) Individuals who harbour the African alleles (*Sl2*and McC^b) on CR1 gene are more protected against severe forms of malaria.

b) Mutations that result in McC^b and *Sl2* genotypes affect the interaction of CR1 with PfEMP1 thus leading to less rosette formation.

1.5 Objectives

1.5.1 General Objective

To understand how Knops antigen blood group polymorphisms on CR1 gene relate to protection and/or susceptibility to severe forms of malaria in order to elucidate the molecular basis of their effect on CR1 function and development of severe malaria.

1.5.2 Specific Objectives

1. To genotype children for CR1 Knops blood group polymorphisms using restriction fragment length polymorphism (RFLP).
2. To establish an association between different CR1 blood group variant types and severe forms of *P. falciparum* malaria (severe anaemia and cerebral malaria).
3. To clone and express the CR1-interacting domain of *P. falciparum* (DBLα) in COS7 cells.
4. To test the capacity of constructs expressing DBLα sequence tags to form rosettes with red blood cells harbouring CR1 alleles (*Sl2* and $McC^{a/b}$ and *Sl1* and $McC^{a/a}$).

1.6 Significance Of The Study

Evidence from several laboratories demonstrates the involvement of CR1 in the pathogenesis of severe malaria (Cockburn *et al.*, 2003; Rowe *et al.*, 2000). However, the exact role of CR1in the pathogenesis of severe malaria is not known. A study done in the Gambia failed to demonstrate an association between CR1 Knops blood group alleles with severe *P. falciparum* malaria. However, data from this study demonstrates the evidence that Sl2/*2* or *Sl2/2* $McC^{a/b}$ genotypes of CR1 may protect individuals against cerebral malaria and possibly also severe malarial anemia (Thathy *et al.*, 2005). The mechanism for the protection remains unclear. Perhaps the underlying mechanism of resistance to cerebral malaria is a decreased ability of *Sl2/2* $McC^{a/b}$-expressing RBCs to form rosettes and obstruct the cerebral microvasculature due to impaired binding of these CR1 variants to PfEMP1. At the same time, *Sl2/2* $McC^{a/b}$ RBCs may be less susceptible

to complement-mediated lysis or have an increased capacity to bind ICs and remove them more efficiently from the circulation, thereby reducing the likelihood of developing severe malarial anaemia.

In the background of these observations, the study sought to identify the molecular mechanism by which the *Sl2* and McC^b alleles of CR1 may confer protection from severe malaria. These findings could provide an insight into the pathogenesis of severe anaemia and cerebral malaria. The data could be useful for the development of new treatment strategies as well as in the formulation of effective antimalarial vaccines that would reduce the disease burden in sub Sahara Africa where it causes most deaths.

CHAPTER 2: LITERATURE REVIEW

2.1 Economic Importance Of Malaria

Malaria still remains as one of the most severe public health problems worldwide. The greatest burden due to the disease is borne by countries in the poorest continent (Africa) where it is the leading cause of morbidity and mortality. The most vulnerable individuals are those with little or no protective immunity and include young children, pregnant women and migrants coming from areas with little or no malaria transmission.

2.1.1 The Human *Plasmodiumsp Secies*

Most of the disease burden and virtually all deaths caused by human malaria are attributable to *Plasmodim falciparum (P. falciparum)*, the most lethal of all the other three *plasmodium* species (*P. vivax, P. malariae and P. ovale*) that infect the human host. The differences that exist between the four major human *Plasmodium* species are based on blood stage morphology, disease and clinical manifestation. *P. falciparum* is reported to lack the preference for erythrocyte subclasses and erythrocytes infected with mature pigmented stage of *P. falciparum* sequester in the microvasculature and are not found in the peripheral circulation. These survival properties of *P.falciparum* are thought to be the underlying factors that make it most lethal compared with the other three species. However, molecular methods have revealed the possible existence of other species or morphological variants. For example, sequencing of the gene for the circumsporozoite surface protein (CSP) revealed that some individuals diagnosed with *P. vivax* infections were actually infected with a distinct species more closely related to *P. simiovale*. *P. simiovale* is a simian malaria parasite which is morphologically identical to *P. vivax* (Qari

et al., 1993). More molecular analyses have shown the existence of morphological variants of *P. malariae*. Kawamoto et al (2002) reported that some morphological variants of *P. malariae* are distinct parasites related to *P. malariae* and *P. brasilianum*. *P. brasilianum* is a simian parasite of the South and Central America. It has recently been revealed in Malaysia that it is no longer an unusual occurrence that humans are naturally infected with the simian parasite, *P. knowlesi* (Cox-Singh *et al.*, 2007). The study demonstrated that nearly all of the cases identified by microscopy as *P. malariae* were proved to be *P. knowlesi* by PCR.

2.1.2 Epidemiology Of Malaria

Malaria is primarily a disease of the tropics and subtropics and is widespread in hot humid regions of Africa, Asia and South and Central America. About 40 percent of the world's population lives in areas with malaria risk (Aultman *et al.*, 2002). There are an estimated 300-500 million cases of malaria each year (Kirkpatrick, 2003), resulting in up to 2.7 million deaths. Most of these deaths are due to infection with *P. falciparum* and occur among children and pregnant women in the developing world (WHO 2003, 2005; Snow *et al.*, 1997, 2005). Depending on the intensity of transmission, malaria can be either stable (endemic) or unstable (epidemic). Stable malaria refers to a situation in which there is a measurable incidence of natural transmission over several years. Under stable endemic conditions, variation of malaria transmission from year to year is minimal, although seasonal fluctuations may take place. Different areas can experience different levels of incidence rates and this is often denoted by: hypoendemic (very intermittent transmission), mesoendemic (regular seasonal transmission), hyperendemic (intense, but with periods of no transmission during dry season) and holoendemic (transmission occurs

all year long). The intensity of malaria transmission is a crucial factor that influences the epidemiology and rate of development of immunity. For example, persons living in highly endemic areas usually exhibit a high level of immunity and tolerate the infection well (Snow *et al.*, 1998, Gupta *et al.*, 1999). Unstable, or epidemic, malaria refers to an increase in malaria in areas of low endemicity or to outbreaks in areas previously without malaria or among non-immune persons. These outbreaks can usually be attributed to changes in human host factors and behaviour or effects on the environment (Theander 1998, Luxemburger *et al.*, 1996). Unstable malaria is very specific for *P.vivax*, although sharp outbreaks may also occur with *P.falciparum.* Humans are the only significant reservoir for the parasite and sustained transmission depends upon contact between infected humans and anopheline mosquitoes. The vector capacity of a mosquito depends not only on its ability to support sporogony, but also on factors such as feeding habits, longevity and density. Climatic and ecological factors can also affect mosquito behavior and population. Transmission also depends on a pool of infected humans serving as reservoirs. This necessitates that the parasite be relatively long-lived within the human host, especially in the case of seasonal transmission and in areas of low endemicity. *P. vivax* and *P. ovale* are capable of relapses and *P. malariae* is well-known for long-term chronic infections. Roper *et al.*, (1996) reported that *P. falciparum* can also produce long-term chronic infections. Malaria transmission has been eliminated in many countries of the world, including the United States and countries of Western Europe. However, cases of malaria still occur in these countries, mostly in returning travelers or immigrants. This type of malaria has been designated 'imported malaria.'

The current malaria management strategies include the development of vaccines and chemotherapeutic agents, vector control, insecticides, education, and insecticide-treated bed nets. However, resistance to drugs by both the mosquito and the parasite is a growing obstacle in the battle against malaria. This has not been helped by the fact that there is no vaccine in clinical practice.

2.2 The pathology Of *P. falciparum* Malaria

The pathology and clinical manifestation of malaria vary remarkably depending on the parasite species, age, health and nutritional status of the host, the level of previous exposure and several other factors. For example, children under 5 years living in endemic areas are at higher risk of mortality and morbidity. The number of clinical episodes decline with an increase in age and this has been attributed to the gradual acquisition of immunity over many years of exposure (Bull and Marsh 2002). Therefore, very young children in highly endemic regions and travelers with no previous exposure to malaria parasite suffer the most morbidity and mortality. The symptoms of malaria include fever, chills, headache, muscle aches, tiredness, nausea and vomiting, diarrhoea, anaemia, and jaundice (yellow colouring of the skin and eyes). Convulsions, coma, severe anaemia and kidney failure can also occur. Two major complications are cerebral malaria and severe malarial anaemia.

Cerebral malaria is associated with sequestration of parasite-infected erythrocytes deep within the vascular beds of vital organs such as the brain and the major clinical signs are unrousable coma and convulsions. Although, the pathophysiology of cerebral malaria is not completely understood, it is thought to involve multiple factors and

complex interactions between the host and parasite. For example, parasite exo-antigens, which are released at erythrocyte rupture, are known to stimulate macrophages to secrete tumour necrosis factor-α (TNF-α) which upregulates the expression of adhesion molecules such as intercellular adhesion molecule 1 (CAM-1) on the surface of brain endothelial cells. This would lead to increased binding of infected erythrocytes and this would subsequently influence the amplification of various effects such as vascular blockage, soluble mediators, and metabolic effects.

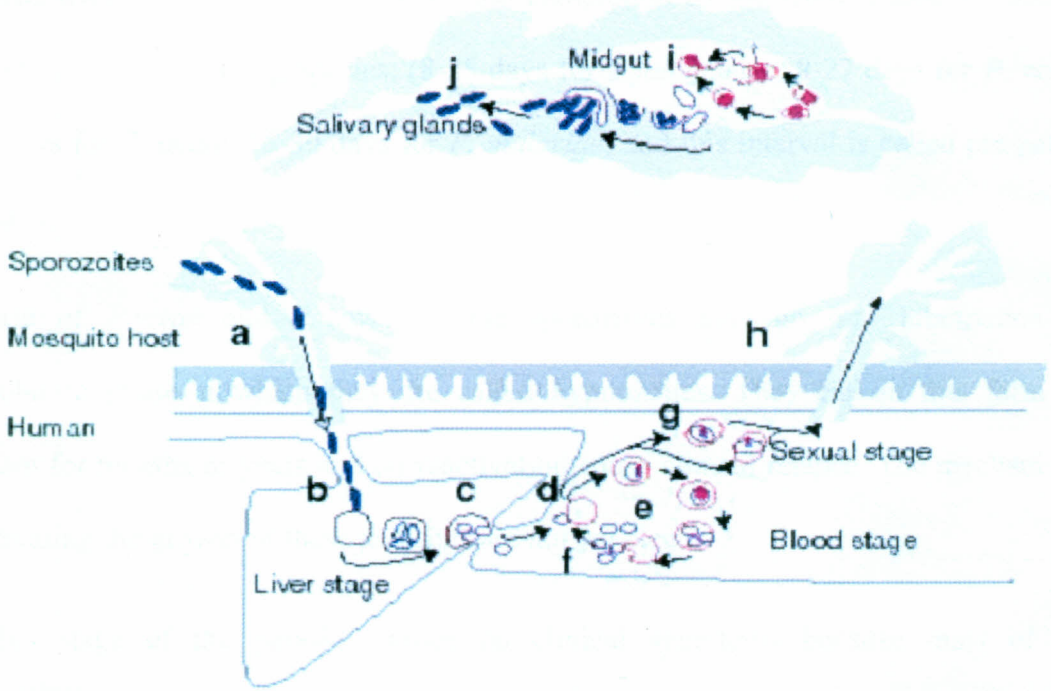
Severe anaemia is caused by lysis of erythrocytes and suppression of erythropoiesis in the bone marrow (Menendez *et al.*, 2000). In areas where transmission of malaria is very high, the common complication is severe malarial anaemia. On the other hand, cerebral malaria is the common complication in areas where transmission is low (Marsh, 1992).

It has been difficult to provide a gold standard definition of severe malarial anaemia. However, the definitions currently used comprise a set of clinical and laboratory parameters associated with an increased risk of death (Marsh *et al.*, 1995), combined with the presence of *Plasmodium falciparum* parasitemia (Hien *et al.*, 1996). In young children, these criteria are predominantly altered consciousness, severe anemia, and respiratory distress (WHO, 2000), a much broader range of criteria is applicable to adolescents and adults (Hien *et al.*, 1996). Nevertheless, severe anemia (hemoglobin concentration less than 5.0 g per deciliter) is a major cause of sickness and death among children living in high endemic regions in sub-Saharan Africa (English *et al.*, 2002). In various settings, 12 to 29% of hospitalized children are severely anaemic (Newton, *et al.*, 1997) and the in-hospital case fatality rate in these children is 8 to 17%.

Malaria is therefore a potentially deadly disease which can assume pathologically different forms. However, huge loses of lives among children could be averted if an early treatment was sought and the disease correctly diagnosed.

2.3 The life cycle of the parasite

Figure 1: The life cycle of *Plasmodium falciparum* parasite



(Adapted from Carucci *et al.*, 1998).

2. 3.1 Pre-erythrocytic schizogony

This is the stage in the life-cycle which involves the development of the schizoid forms of the parasite before reaching the red blood cells. The phase begins with the

injection of sporozoites into the subcutaneous tissue or directly into the human blood by the bite of a female anopholese mosquito. The sporozoites travel to the liver where they invade the hepatocytes. This takes about half an hour. The mechanism of targeting and invading the hepatocytes with this rapidity is fully known. However the circumsporozoite protein (CSP) is believed to play vital role in mediating the invasion of hepatocytes. Within the liver cells, the trophozoites that manage to successfully invade the cells begin their intracellular asexual division and each sporozoite divides into several merozoites. At the end of this phase, thousands of extra erythrocytic merozoites are released from each ruptured liver cell. The time taken for the completion of the tissue phase is variable depending on the infecting species; (8-25 days for *P.falciparum*, 8-27 days for *P. vivax,* 9-17 days for *P. ovale*, 15-30 days for *P. malariae*) and this interval is called pre-patent period.

In case of *P.vivax* and *P. ovale*, some sporozoites may go into hibernation-the cryptobiotic phase-in which they are called hypnozoites. They can in this form lie dormant for months or years and on reactivation cause clinical relapse. The mechanisms of activating the growth of these parasites are not yet clear.

This stage of the parasite causes no clinical symptoms because most of the sporozoites are destroyed by the phagocytes and only a few hepaotcytes are infected. However, in rare instances, some vague aches and pains, headache, nausea may be present. The sporozoite manipulates the host immune system by wrapping itself in the cell membrane of the infected host liver cells (Sturm *et al.*, 2006).

2.3.2 Erythrocytic Schizogony

This is the stage in which the parasite undergoes asexual multiplication in the erythrocytes. The phase starts when the merozoites released form the hepatocytes attaché to the red blood cells membrane and by a process of invagination, enter the erythrocyte. Within the red blood cell, the asexual division begins and the parasites develop through the stages of rings, trophozoites, early and mature schizonts. Each schizont contains several of erythrocytic merozoites. When these schizonts rupture, the merozoites are released and immediately invade uninfected erythrocytes. This repetitive cycle of invasion-multiplication-release-invasion continues and thus depletes the body of oxygen as a result of massive destruction of erythrocytes due to parasite infection. The intra erythrocytic cycle takes about 48 hr in *P. vivax*, *P. ovale* and *P. falciparum* infections and 72 hr in case of *P. malariae* infection. It is highly synchronous and the merozoites are released at approximately the same time of the day.The bursting of the infected red blood cells and resulting release of their contents stimulate Tumour Necrosis Factor alpha (TNFα) and other cytokines which culminates into the characteristic clinical manifestations of the disease.

Some merozoites in the red blood cells undergo transformation into gametocytes- male and female. The mechanisms of this phenomenon are not clearly understood. Mature gametocytes remain in the peripheral blood for a variable period and are picked up by the mosquito when it feeds on an infected person. Fertilization and sexual recombination of the parasite occurs in the mosquito's gut, thereby defining the mosquito as the primary host of the disease.

It is the asexual blood stage that is responsible for the clinical symptoms of the disease and considerable efforts to develop a vaccine against this stage in parasite development has been intense. Asexual blood stage vaccine would limit parasite growth and consequently minimize or prevent clinical disease.

2.3.3 Sporogony

The gametocytes continue their development in the mosquito. The fusion of the male and female gametes results into the formation of a zygote. The zygote transforms into an ookinete (invasive form) which penetrates the gut wall and becomes an oocyst. The oocyst undergoes asexual division to form numerous sporozoites which reach the salivary gland of the mosquito. The sporozoites are deposited into the human host when the mosquito bites during a blood meal to begin another cycle. The sporogony in the mosquito takes about 10-20 days and thereafter the mosquito remains infective for 1-2 months.

It is this complex life-cycle of the parasite which is one of the challenges that slows down the development of a malaria vaccine. It is therefore appreciated that a successful malaria vaccine development critically depends upon our thorough understanding of immunity to various stages of the parasite life-cycle.

2.4 Immunity To Malaria

The exact nature of the protective immune response to malaria is not clearly marked out. However, it is apparent that both innate and acquired immunity against malaria parasite occur and each of these immunological arms operates together for optimal and effective immune responses against malaria and other infections. Innate immunity

involves various genetic diseases and polymorphisms, which have been associated with decreased malaria infections. Naturally acquired immunity is species-, strain-, stage- and variant-specific and is never sterile (Andrysiak *et. al.*, 1986, Fandeur and Chalvet 1998, Rotman *et. al.*, 1999). Generally, acquisition of immunity to malaria is slow and usually requires repeated exposure to be maintained (Baird 1998). This instability is has been thought to be caused by numerous number of factors such as genetic variability of both the host and the parasite, parasite-induced immunosuppression among other factors.

2.4.1 Humoral Immune Responses

The fact that many people in endemic areas are infected with the parasite but do not have the disease gives evidence to the existence of acquired immunity. In such individuals, malaria infection induces strong humoral immune responses involving production of predominantly IgM and IgG but also of other immunoglobulin isotypes. In protected individuals cytophylic antibodies of IgG1 and IgG3 isotypes have been shown to be the most protective against *P. falciparum* malaria in humans (Bouharoun-Tayoun and Druilhe 1992, Aucan *et al.*, 2001). These antibodies confer protection through their action by interaction with effector cells such as monocytes and macrophages to mediate opsonization and antibody-dependent cellular inhibition (ADCI) (Aucan *et al.*, 2001). Opsonization enhances the activity of phagocytic cells (Perraut *et al.*, 1995) or initiates complement-mediated damage (Ramasamy and Rajakaruna 1997, Giribaldi *et al.*, 2001). Passive transfer of IgG has been shown to protect against malaria by reducing parasitaemia and clinical disease (Jakobsen *et al.*, 1996). Perlmann *et al.*, (1994) reported an elevation of total IgE and IgE anti-malarial antibodies in humans as well as experimental animals.

There are a number of important antigens that would induce antibody responses. For example, antibodies against some merozoite antigens such as merozoite surface antigen type 1 (MSA1) and merozoite surface protein type 1 (MSP1) have been shown to inhibit erythrocyte invasion *in vitro* (Egan *et al.*, 1999) The mechanisms of this protection has been suggested to be by binding to merozoites and stopping their attachment to or eventual invasion of erythrocytes or by causing agglutination of infected erythrocytes, causing large clusters that are easier to clear from circulation. High levels of IgG2 to ring-infected erythrocyte surface antigen (RESA) and to MSP2 are associated with resistance to *P.falciparum* at the end of the transmission season and levels tend to be higher in older individuals who are better protected against infection and disease (Aucan *et al.*, 2000). Some antibodies have been shown to harmful to individuals. For example, levels of IgG4 to parasite extract, RESA, MSP1 and MSP2 are lower in individuals who do not develop malaria than in susceptible individuals and are positively correlated with risk of infection (Aucan et al., 2000).

Antibodies targeted against the variant surface antigen (VSA) have been reported. It has been documented that acquisition of antibodies to parasite-encoded variant surface antigens (VSA) on the infected erythrocyte membrane is important in the development of immunity and only those parasites that are not controlled by pre-existing antibodies against VSA go ahead to cause severe disease. The best-characterized VSA is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) and parasites can switch between different PfEMP1 forms. This switching is associated with changes in antigenicity and adhesion phenotype (Smith *et al.*, 1995). Antibodies to PfEMP1 (Smith *et al.*, 2000) and the Duffy binding protein (Michon *et al.*, 2000) have been reported to prevent

cytoadherance and thus reduce the risk of developing cerebral malaria. Carlson *et al.*, (1990) showed that antibodies can disrupt spontaneous binding of uninfected red blood cells to *Plasmodium*-infected red blood cells (rosetting). It is speculated that the slow acquisition of protective immunity to malaria reflects the necessity to build up a broad repertoire of PfEMP1-specific antibodies (Bull *et al.*, 1998).

Since each immunological arm influences one another, the humoral response is bolstered by a variety of non-specific effector mechanisms which are mediated by neutrophils, macrophages and natural killer (NK) cells. For instance, antibodies can link parasitized cells through Fc receptors to mononuclear cells or polymorphonuclear cells (Bouharoun-Tayoun *et al.*, 1990, Kharazni and Jespen 1984), thus mediating antibody-dependent cell-mediated inhibition (ADCI). *In vitro* experiments have also shown that antibodies can opsonise merozoites and then through their Fc receptors bind them to monocytes or macrophages. This binding will activate the monocytes or macrophages to produce toxic cytokines such as tumor necrosis factor alpha (TNFα) (Bouharoun-Tayoun *et al.*, 1995). Antibodies therefore play a significant role in protection against malaria infections.

2.4.2 Cell-mediated Immunity

Although the importance of antibodies in protection against malaria has been established, this humoral response is only achieved before merozoites re-invade new erythrocyte since during the asexual-stage; there is little opportunity for the antibodies to interact directly with the parasite. It is even less possible because the merozoites are only exposed to the antibodies for such a short time after which the parasite remains

intracellular for the rest of the erythrocytic life cycle. During the intra-erythrocytic stage, the antibodies can only interact with parasite antigens expressed on the surface of the infected cells. However, it has been reported that cell-mediated immune responses become crucial for protection against the parasite at this intra-erythrocytic stage. T cells play a pivotal role in the elimination of blood stage malaria parasite through the release of cytokines which activate other effector cells. Cytokines implicated in immunity to malaria include IL-12, IFNγ and TNFα.

Studies have shown that children with mild malaria have higher levels of plasma IL-12 compared with children who suffer severe infection and that the levels of IL-12 inversely correlated with parasitaemia (Seder et al., 1993, Paul and Seder 1994). This protective immunity is due to the ability of IL-12 to induce INFγ production by NK cells and CD4 T cells (Paul and Seder 1994).

IFNγ is an important effector cell which plays a significant role in resistance to blood stage malaria infection. Peripheral blood mononuclear cells from children with mild *P.falciparum* infection produces high levels of IFNγ when stimulated in vitro with merozoite antigen, and have lower risk of re-infection (Ockenhouse *et al.*, 1984). IFN-γ is thought to play a role both in the development of protective immunity (Luty *et al.*, 1999) and in the inflammatory response, which may be involved in the pathology of malaria (Day *et al.*, 1999).

TNFα production level is significantly elevated in plasma of patients with malaria (Luty *et al.*, 2000, Perkins *et al.*, 2000) suggesting that this inflammatory cytokine may be involved in the development of immunity to malaria infection. Studies have

demonstrated that treatment of *P.falciparum*-infected children with anti-TNFα antibodies reduces fever (Van Hensbroek *et al.*, 1996). Pathological effects of TNFα have also been reported (Clark *et al.*, 1997). For example, high plasma TNFα levels in children with *P.falciparum* have been associated with poor prognosis in cases of cerebral malaria (Grau *et al.*, 1989, Kwiatkowski *et al.*, 1990).

$CD8^+$ T cells have been implicated as critical effector cells in protection against the pre-erythrocytic stage of malaria. The target of these malaria specific $CD8^+$ T cells has been suggested to be the infected hepatocyte, which presents, on its surface, parasite-derived epitopes in association with class I major histocompatibility complex (MHC) molecules. The $CD8^+$ T cells subsequently secrete IFN-γ, which in turn induces the infected hepatocytes to produce nitric oxide (NO) that eliminates the infected hepatocytes or inactivates the intracellular parasite (Schofield *et al.* 1987, Nussler *et al.* 1993, Seguin *et al.* 1994, Klotz *et al.* 1995).

The other subset of T cells that have been involved in protection against malaria is the γδT cells. An increase in γδT cells has been reported in adult individuals who have had limited or no previous exposure to *P. falciparum* malaria. It could therefore be summarized that although humoral and cellular effector mechanisms are involved in the defense against malaria parasite, cell-mediated immunity seems to be the major defensive arm against the disease.

2.5 Malaria Control Strategies

The aim of the WHO global malaria control strategy is to halve the number of annual deaths from malaria by 2010 and vector control is an important part of this control

strategy. This is so because in areas where malaria has successfully been eliminated, the control strategy has targeted the vector (*Anophelese* mosquito). A multidimensional approach should be used in the control plan, such as good management of clinical malaria (use of drugs), indoor spraying of insecticides such as DDT, personal protection measures including the use of insecticide-impregnated materials (bed nets and curtains), larval control, environmental control and vaccine research. However, each of these malaria control strategies has its sizable share of challenges and constraints. For instance, insecticide resistant anopheles mosquitoes (Himeidan *et al.*, 2007) have been reported and a coverage limitation of insecticide-treated bed nets (ITNs) has been a major setback in their full implementation.

The current malarial control strategies consist of chemotherapy (the use of drug to target disease) and prevention of mosquito vector/human contact using insecticide-impregnated bed nets and, to some degree, indoor residual insecticide spraying and environmental control for reducing mosquito breeding sites. Insecticide-treated bed nets have been shown to reduce severe disease and mortality due to malaria in endemic regions. In community-wide trials in several African settings, ITNs have been shown to substantially reduce all-cause mortality (Eisele *et al.*, 2005). There is current testing of new kinds of bed nets dabbed; long-lasting insecticide-treated nets (LLINs) that retain lethal concentrations of insecticide for at least 3 years (WHO, 2007). This is expected to improve the use of insecticide-treated bed nets since they will not have to be re-treated quite often.

Despite extensive work on malaria vaccine development, there is still no malaria vaccine in clinical practice. However, the completion in sequencing of the genomes of

humans, *P. falciparum*, and *An. gambiae* has ushered in a new era of hope that genomics research will result in the development of new and better tools for malaria control.

2.6 Current Status Of Malaria Vaccine Development

It is now several decades since it was shown that a vaccine that would protect man against malaria was a possibility. Despite tremendous efforts and improved investment in malaria vaccine development, no effective vaccine has been put to clinical use and it is still some years away before one reaches the market. However, all is not lost as many promising advancements have been made in malaria vaccine development.

Several antigens from various stages of development of malaria parasite have been tested for their immunogenecity in both animals' experiments and human trials. Since the asexual blood stage is responsible for malaria disease, several antigens thought to be targets of protective immune responses have been identified (Crewther *et al.*, 1990; Cooper 1993; Good *et al.*, 1998) with merozoite surface protein 1 (MSP1) and the apical membrane antigen 1 (AMA-1) topping the list (Good, 1998). *In vitro* studies have shown that MSP1-19 specific antibodies inhibited merozoite invasion (Egan *et al.*, 1999). MSP1-19 is the portion of MPS1 that has been shown to be the target of human protective antibodies (O'Donnell *et al.*, 2001). MSP3-based vaccines have also been suggested and IgG3 antibodies that recognize MSP3 are reported to have strong association with the development of long-lasting natural protection against malaria (Roussilhon *et al.*, 2007). AMA1 is another blood-stage antigen that is involved in the merozoite invasion of human erythrocytes (Kato *et al.*, 2005).

Efforts to develop a transmission blocking (TB) vaccine have also yielded promising results. For example, antibodies that exclusively target conformational epitopes of Pfs48/45 protein are reported to prevent fertilization (Vermeulen *et al.*, 1985; Roeffen *et al.*, 1995). Pfs48/45 is a TB target protein expressed by gametocytes (Carter *et al.*, 2000) and present on the surface of the sporogonic (macrogametes) stages of the malaria parasites. Furthermore, anti-Pfs48/45 antibodies are present in human sera from endemic areas (Roeffen *et al.*, 1995) and correlate with TB activity (Bousema *et al.*, 2007).

Although both asexual and sexual vaccine candidates have been tested, the most successful malaria vaccine candidate has been derived from pre-erythrocytic stage, the circumsporozoite surface protein (CSP). RTS,S is a vaccine candidate based on *P.falciparum* circumsporozoite surface antigen which has shown great promise for development of safe, tolerable and immunogenic malaria vaccine. RTS,S/AS02A is designed to prevent infection and is currently the most advanced vaccine candidate in development for clinical trials (Bojang *et al.*, 2001, Alonso *et al.*, 2004; Apante *et al.*, 2007). Clinical trials with RTS,S have been developed through to Phase IIb and have shown that the vaccine is safe, tolerable and immunogenic. RTS,S/AS02D was shown to induce high titres of anti-circumsporozoite antibodies (Aponte *et al.*, 2007). These findings have set the foundation for an expanded Phase III studies to confirm the efficacy of the vaccine against clinical malaria disease.

As malaria vaccine development focuses more on the CSP-based vaccine candidate RTS,S, trials with vaccine candidates based on other stages of parasite development need to be pushed forward as well. Even the search for new antigens need to be intensified.

This is necessary because it is generally accepted that only a multicomponent vaccine could be effective against malaria.

2.7 Human Genetics And Innate Resistance

Certain genetic diseases and polymorphisms have been associated with decrease in infection by *P.falciparum* as well as *P.vivax.* For example, individuals who lack the Duffy blood-group antigen are refractory to *P. vivax* (Miller *et al.*, 1976; Horuk *et al.*, 1993). A large proportion of the populations in western Africa are Duffy negative, thus accounting for the low levels of *P. vivax* in West Africa. The Duffy antigens are expressed on red blood cells and other cells in the body and whose expression on blood cells is encoded by Fy genes (Fya, Fyb, Fyc). *Plasmodium vivax* malaria uses the Duffy antigen to enter blood cells and Fy-/Fy- genotype confers complete resistance to *P.vivax* infection. The genotype is very rare in European, Asian and American populations, but is found in almost all of the indigenous population of West and Central Africa (Carter and Mendis, 2002). This innate resistance led to the identification of the Duffy antigen as the erythrocyte receptor for merozoite invasion.

Most of these polymorphisms arise from several erythrocyte disorders found predominantly in malaria endemic areas and at frequencies much higher than expected. This has lead to speculation that these disorders confer some protection against malaria. For example, Southeast Asian ovalcytosis is due to a mutation in an erythrocyte membrane protein called band 3 (Mueller and Murrison, 1977). This mutation causes the erythrocyte membrane to become more rigid and more refractory to merozoite invasion (Zimmerman *et al.*, 2003). The other inherited blood disorder, α^{+}-thalassaemia, which is common in Africa and Southeast Asia, provides protection against malaria. Most studies

show that α^+-thalassaemia homozygotes have considerable protection against SMA compared to heterozygotes (Williams *et al.*, 2004; May *et al.*, 2007). Alpha thalassaemia results in the formation of smaller red blood cells, which contain less of the oxygen-carrying, iron-containing pigment, haemoglobin.

Other common disorders of Hb that appear to provide relative protection include carriers of β-thalassemia and homozygous Hb E (Fairbanks *et al.*, 1979). The mechanism(s) of these protective effects are not clearly understood. However, Fowkes *et al.* (2008) reported an increased erythrocyte count and microcytosis in children homozygous for α^+-thalassaemia, and suggested that this observation may contribute to substantial protection of such children against SMA. It is speculated that the combination of the defect and infection leads to premature lysis or clearance of the infected erythrocyte. For example, glucose-6-phosphate dehydrogenase (G6PD) deficient erythrocytes would have an impaired ability to handle oxidative stress. The additional oxidants produced as a result of parasite metabolism and the digestion of hemoglobin may overwhelm the infected erythrocyte and lead to its destruction before the parasite is able to complete schizogony.

Another inherited red blood cell disorder, sickle cell disease, protects against malaria. Sickle cell trait (genotype HbAS) confers a high degree of resistance to severe and complicated malaria (Aidoo *et al.*, 2002). The mechanism by which HbAS protects against malaria is not well established. However, while some studies indicate that it could be partly influenced by the physical characteristics of HbAS erythrocytes, a number of studies suggest that HbAS may also enhance the acquisition of natural immunity (Marsh *et al.*, 1989).

It is therefore crucial to understand the mechanisms by which these human host genetic factors contribute to protection against severe forms of malaria in order to inform the development of effective and long lasting antidisease measures. Several studies have pointed out that severity of *P. falciparum* malaria is due to the ability of the parasite to adhere to the vascular endothelium and rosette formation (Rowe *et al.*, 1997). Understanding the molecular mechanisms of rosette formation is a crucial knowledge in designing approaches that will alleviate disease severity and reduce the enormous burden of malaria.

2.8 Pathogenesis Of Complicated Malaria

Severe malaria is a complex condition that does not have a simple definition. The development of severe malaria probably results from a combination of parasite-specific factors, such as adhesion and sequestration in the vasculature and the release of bioactive molecules, together with host inflammatory responses. These include cytokine and chemokine production and cellular infiltrates. Most of the deaths are due to complications associated with *P. falciparum* with cerebral malaria (CM) being the most notable and frequent cause of death. The pathogenesis of CM is complex and still poorly understood. However, several studies implicate various factors in the pathology of this life-threatening complication due to malaria. Inflammatory cytokines and chemokines and sequestration of infected erythrocytes have been suggested to play a major role in the pathogenesis of cerebral malaria (Taylor *et al.*, 2004, Clark *et al.*, 1991, Montegomery *et al.*, 2006, Armah *et al.*, 2005).

CM is characterized by an impaired consciousness. The presenting symptoms are severe headache followed by drowsiness, confusion, and ultimately coma. Convulsions

are also frequently associated with cerebral malaria. These neurological manifestations are believed to be due to the sequestration of the infected erythrocytes in the cerebral microvasculature. Sequestration refers to the cytoadherance of trophozoite- and schizont-infected erythrocytes,to endothelial cells of deep vascular beds in vital organs, especially brain, lung, gut, heart and placenta. This phenomenon is believed to provide several advantages to the parasite. The major advantage is the avoidance of the spleen and the subsequent elimination of infected erythrocytes. In addition, the low oxygen tensions in the deep tissues may provide a better metabolic environment for the parasite to thrive in.

The other common life-threatening complication due to *P. falciparum* malaria is severe malarial anaemia (SMA).This complication is caused by a variety of pathophysiological mechanisms. Although both the molecular and cellular basis of SMA is not clear, it is thought to arise from both decreased red blood cell (RBC) production and increased RBC destruction. Destruction of RBCs can occur as a result of parasite invasion and replication. However, in malaria-endemic areas SMA is consistently observed at relatively low parasite burdens suggesting that the destruction of uninfected RBC is the major cause of hemoglobin (Hb) loss (Ekvall, 2003). Increased destruction of uninfected RBCs has been proposed to result from mechanisms such as bystander intravascular hemolysis or accelerated senescence, arising from lipid peroxidation (Das and Nanda, 1999), reduced red cell deformability (Dandorp *et al.*, 1999), modification by surface-bound IgG or complement (Goka *et al.*, 2001), up-regulation of host phagocytic function (Looareesuwan *et al.*, 1987), loss of red blood cell complement proteins (Stoute *et al* 2004) and adsorption of parasite-derived antigens (Layez *et al.*, 2005). Many more

mechanisms that lead to the huge destruction of RBC in malarial anaemic patients could exist.

More recently, respiratory distress has been included in the list of indicators of severe malaria besides CM and SMA.

2.8.1 Sequestration Of Erythrocytes And Severe Malaria

Sequestration is the phenomenon where erythrocytes infected with mature trophozoites and schizonts are removed from the peripheral circulation by binding to endothelial cells commonly in postcapillary venules of deep tissues (Chen *et al.*, 2000). This adhesion to endothelium has a vital role in the pathogenecity of malaria causing occlusion of small vessels and contributing to the failure of many organs (Miller *et al.*, 1994). It is widely believed that massive sequestration in the brain is the main underlying cause of coma in cerebral malaria.

It is not clear why parasites sequester, although, it is thought that sequestration could lead to better parasite maturation in an oxygen-depleted environment and that it would allow the parasite to escape spleen-dependent destruction.

2.8.2 Parasite Ligands For Cytoadherance

There are several known parasite proteins expressed on the surface of *P. falciparum* infected erythrocytes that participate in cytoadherance (see Table 1). Subtelomeric variant open reading frame (STEVOR) proteins are expressed in sequestered gametocytes where no PfEMP1 is detected, hence it is suggested that these proteins could be mediating cytoadherance (Blythe *et al.*, 2004). Of the other identified parasite ligands

implicated in cytoadherance listed in Table 1, only PfEMP1 is discussed here in detail due to its proven role in mediating adhesion (Howard *et al.*, 1983, Leech *et al.*, 1984).

PfEMP1 is a parasite host-derived highly polymorphic polypeptide of high molecular mass (200-350 KDa). The molecule is structured into several intracellular Duffy binding-like domains (DBL1-5), cysteine-rich interdomain regions (CIDR1-2), a transmembrane (TM) region and intracellular acidic segment (ATS). PfEMP1 is encoded by the multiple var gene family and in 3D7 (*P. falciparum* clone), the protein is encoded by about 60 var genes (Baruch *et al.*, 1995, Gardner *et al.*, 2002, Su *et al.*, 1995, Smith *et al.*, 1995).

PfEMP1 is expressed on the surface of infected erythrocytes and has been the main focus for protective immunity (Su *et al.*, 1995, Smith *et al.*, 1995, Bull *et al.*, 1998, Dodoo *et al.*, 2001, Gardner *et al.*, 2002). The most conserved regions of PfEMP1 are located in both the N-and-C

Table 1: Host cell receptors and ligands for adhesion for *P.falciparum*-infected RBCs

Host receptor (s)	Receptor location	Parasite ligand
CD35	RBC	PfEMP1 (DDL-1α)
HS-like GAGs	RBC	PfEMP1 (DDL-1α)
ABO blood group antigen	RBC	PfEMP1?
TSP	Serum, endothelium	PfEMP1?
CD36	Endothelium, RBC	PfEMP1 (CIDR-1α)
ICAM-1	Endothelium	PfEMP1 (DBL-2β)
CD31	Endothelium	PfEMP1?
VCAM-1	Endothelium	?
E-selectin	Endothelium	?
CSA	Endothelium	PfEMP1 (DBL-3γ)
IgM and IgG	Serum	PfEMP1?

terminal domains. The N-terminal region contains cysteine-rich domains reported to be involved in the sequestration of parasitized erythrocytes in the microvasculature (Baruch et al., 1995, Smith *et al.*, 1995, Su *et al.*, 1995). Fernandez *et al.* (1998) reported a striking association between the adhesion of parasitized erythrocytes to several receptors and presence of PfEMP1. The involvement of PfEMP1 as an adhesive parasite ligand to various receptors has led to its implication in the pathogenecity in severe malaria. This pathological significance of PfEMP1 has made it a potential candidate for antimalarial

vaccine. Substances that block interaction of PfEMP1 and host receptors would prevent cytoadherance and sequestration in the microvasculature. Furthermore, chemicals that would block the intracellular transport of PfEMP1 to its final surface destination could form potential therapeutic and preventive management for malaria.

2.8.2.1 The PfEMP1 Expression And Malaria

PfEMP1 is the most prominent member of the variant surface antigens (VSA). It is the major parasite antigen expressed on the surface of erythrocytes infected with *P. falciparum* and is subject to continuous assault by the host immune system. The protein is expressed on the surface of infected erythrocytes where it mediates binding to endothelial receptors. PfEMP1 is encoded by the highly diverse *var* gene family and switching expression of different var genes conveys different antigenic and adhesive properties to infected erythrocytes (Noviyanti *et al.*, 2001).

2.8.2.1.1 Functions Of PfEMP1

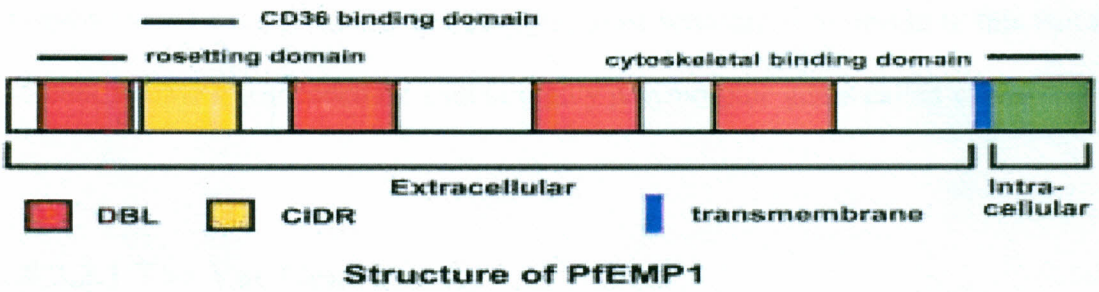
PfEMP1 is a family of transmembrane proteins that play a central role in malaria pathogenesis by mediating sequestration and rosetting (Miller *et al.,* 2002). PfEMP1 undergoes clonal antigenic variation on the red cell surface and is responsible for the adhesive properties of infected cells. The *P. falciparum* genome is estimated to contain approximately 60 *var* genes (Gardner *et al.,* 2002), but only one major transcript is expressed by each parasite in an infected RBC at any given time. Transcriptional switching from one *var* to another results in antigenic variation of the PfEMP1 proteins, allowing the parasite to evade the host immune system as well as vary the cytoadherent properties of infected RBCs (*reviewed in* Miller *et al.*, 2002). Thus, severe malaria may

be caused by parasites expressing PfEMP1 variants that afford parasites optimal sequestration in immunologically naïve individuals.

2.8.2.1.2 Structure Of PfEMP1

The extracelullar portion of the protein is organized into a variable number of adhesive Duffy-binding-like (DBL) domains interspersed with cysteine-rich interdomain regions (CIDR) that can simultaneously recognize different host cell receptors (Fig. 2; *reviewed in* Smith *et al.*, 2001).

Figure 2: Schematic diagram of PfEMP



Adapted from Smith et al., 2001

2.8.2.2 The PfEMP1 And Antigenic Variation

Antigenic variation is the ability of a clonal population to switch its antigenic phenotype. In the context of *P. falciparum*, antigenic variation is the mechanism of immune evasion. The process is mediated by PfEMP1. This molecule has the ability to express itself in different variants on the surface of infected erythrocytes. The PfEMP1 derives this ability from the differential expression of var genes leading to the exposure of phenotypically different PfEMP1 variants on the surface of *P. falciparum*-infected

erythrocytes. The antigenic variation has been suggested to have many implications on malaria immunity.

Sterile immunity that prevents infection may never develop. However, significant antidisease immunity is acquired relatively rapidly (Marsh, 1992). While the protective targets of antidisease immunity are largely unknown, the parasite variant antigens exposed at the erythrocyte surface are considered strong candidates with PfEMP1 reported to be the most immunologically assaulted variant protein. This extensive antigen variation has been a major challenge for malaria vaccine development and partly a crucial causative factor in pathogenesis of severe disease. Although some other variant surface antigens (VSA) exist, PfEMP1 has been the most scrutinized molecule in this family of proteins. PfEMP1 is encoded by a set of highly polymorphic genes called var genes.

2.8.2.2.1 The Var Gene Family

The var gene family is a group of approximately 60 highly diverse genes that have been found to encode *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Su *et al.*, 1995). This group of genes has been shown to have large open reading frames (ORFs) which encode polypeptides with varied molecular masses of 200-350 kDa. The name *var* was coined for these genes because their sequences show great variability. The primary structure of PfEMP1 consists of a putative transmembrane region (TM) followed by a conserved acidic terminal segment (ATS). The extracelullar portion of the protein is organized into a variable number of adhesive Duffy-binding-like (DBL) domains interspersed with cysteine-rich interdomain regions (CIDR) that can simultaneously recognize different host cell receptors (Figure 2; Smith *et al.*, 2001). These regions of the

gene can however, be assembled in three major blocks: N-terminal segment (NTS), Duffy binding-like (DBL) domain and Cysteine-rich interdomain region (CIDR).

The structural organization of the var gene is complex .They are composed of two exons which are separated by an intron of approximately 1 kb. The first exon encode the extracelullar domain which is composed of variable number of semi-conserved Duffy-binding-like (DBL) domains of different sequence classes (DBLα,- β, -γ, -δ, -ε and several other DBLx). These DBL domains are interspersed with cysteine-rich interdomain regions which are also of different classes (CIDRα, -β, and -γ). The Duffy binding-like domain alpha (DBL1α) located in the N-terminal head structure is found in all PfEMP1 and has been shown to mediate rosetting and endothelial binding of infected erythrocytes (Rowe *et al.*, 1997, Chen *et al.*, 1998, Vogt *et al.*, 2003). The second exon encodes the intracellular region, transmembrane and more conserved highly acidic terminal end. The var genes appear to be scattered on all chromosomes and are not necessarily distributed evenly among chromosomes since chromosomes 4, 7 and 12 have been reported to harbour more var genes than other chromosomes (Rubio *et al.*, 1996).

Var genes have been found to exist in all *P. falciparum* parasites (Smith *et al.*, 1995; Su *et al.*, 1995; Thompson *et al.*, 1997). Most of the var genes are located at the telomeric region on the chromosomes and have been shown to be highly polymorphic whereas those harboured at the centromere are quite conserved (Thompson *et al.*, 1997). It has been suggested that possession of this relatively large reservoir of var genes enables the parasite to vary its antigenic profile in order to evade elimination by the host immune responses. This ability of the parasite to change the PfEMP1 expressed on the infected red blood cell surface is the basis of antigenic variation in *P. falciparum* and most

parasites perhaps would not survive for more than a few generations. The huge var diversity makes immunoprevention based on var PfEMP1 sequences quite challenging even though some conserved regions and amino acid residues are indeed found in PfEMP1.

2.8.2.2.2 Classification Of Var Genes

Grouping var genes is as diverse as the genes themselves. Elucidation of the genome sequence of *P. falciparum* has revealed that *var* genes can be categorized into different classes, each with distinct 5′ flanking sequences, chromosomal locations (telomeric or internal position), gene orientations (transcription is either towards the telomere or cetromere) and whether they belong to a small set of long genes that encode PfEMP1 molecules with 6-9 domains or to a much larger set of short genes that encode short PfEMP1 molecules with only 4 domains. The genes have been grouped into five distinct groups (UpsA, UpsB, UpsC, UpsD and UpsE) depending on their association with the 5' upstream regulatory sequences (Voss *et al.*, 2003; Lavstsen *et al.*, 2003; Gardner *et al.*, 2002). Recently, Kraemer *et al.*, (2007) grouped the former UpsD with UpsA reducing the number of groups to four. It has been shown that genes do not fall randomly into these categories. For example, most of the long var genes have DBL2β and are associated with UpsA, whereas most short var genes have DBL2δ. Moreover, of the telomeric var genes, those that are transcribed towards the telomere are associates with UpsA compared with those that are transcribed towards the cetromere which are associated with UpsB (Kraemer *et al.*, 2003; Lavstsen *et al.*, 2003). This apparent genetic structuring is thought to have a functional specialization and distinct immunological properties for UpsA var gene transcription is more in rosetting parasites whereas both UpsA and UpsB transcripts

are more in children with severe malaria (Bull *et al.*, 2005; Kaestli *et al.*, 2006; Rottmann *et al.*, 2006). Jensen *et al.*, (2004) reported that UpsA sequences were more recognized by antibodies in semi-immune children thereby suggesting their role in malaria.

It has been difficult to amplify the whole var sequence due to the huge variability in its sequence and many studies have reliably amplified and sequenced short sequence tags of about 350 nucleotides (Bull *et al.*, 2005; Albretch *et al.*, 2006; Barry *et al.*, 2007). These sequence tags have been amplified from priming sites within DBLα domain which is one of the few domains present in most var genes (Taylor *et al.*, 2000a).

DBLα sequences have been further classified on the basis of number of cysteine residues found in them. In this classification, DBLα sequences have been assigned to six groups. Sequence group 1-3 are those that contain 2 cysteines (Cys2), and sequence group 4 and 5 are those that contain 4 cysteines (Cys4) whereas sequence group 6 includes those DBLα sequences that contain either 1,3 or 5 cysteine(s) (CysX) (Bull *et al.*, 2005). Studies have revealed an association between certain DBLα sequence groups and severe malaria. For example, Bull *et al.*, (2005) reported a striking association between the expression of cys2 group and rosette formation. This study support another one conducted in Brazil which showed an increased expression of DBLα sequences of reduced number of cysteines in children with severe malaria (Kirchgatter and del Portillo, 2002). From the existing data, it is apparent that var gene sequences are organized in certain groups that play significant functions and influence the outcome of malaria disease.

2.8.2.2.3 PfEMP1 As A Vaccine Candidate

Surface proteins of *Plasmodium* are potential malaria vaccine candidates because they are targets of the humoral immune response by the host. Antibodies induced by certain of these proteins can confer immune protection by interfering with functions critical for the survival of the parasite. Naturally acquired antibodies to PfEMP1 expressed by parasites causing severe malaria are suggested to be protective and of major interest for the development of a vaccine against severe disease. The involvement of PfEMP1 as a vaccine candidate has been demonstrated mostly in pregnancy-associated malaria. The first *var* gene implicated in placental adhesion, *FCR3varCSA* (Buffet *et al.*, 1999) has been the most intensively studied. The DBL3γ domain of *FCR3varCSA*, which binds to chondroitin sulfate A (CSA), is considered to be a potential vaccine candidate although different studies have produced mixed results. For example, Douki *et al.*, (2002) and Costa *et al.*, (2003) reported that antibodies raised to the DBL3γ domain of *var1CSA* from the IT/FCR3 parasite line recognized the surface of infected erythrocytes of a wide range of different CSA-binding parasite lines. The observation suggested that if PfEMP1 variant encoded by *var1CSA* was used as a vaccine candidate, it would elicit antibodies that could recognize a diverse number of CSA-binding *P. falciparum* isolates. However, *var1CSA* gene is rarely transcribed in parasites derived from placental infection even though it occurs in virtually all parasite isolates (Rowe *et al.*, 2002) and that transcription of the gene does not correlate with CSA-binding phenotype (Kyes *et al.*, 2003, Salanti *et al.*, 2003). The other sub family of var which has been poised to be a leading vaccine candidate for pregnancy-associated malaria is *var*2CSA. Var2CSA is suggested to be the major ligand for placental adhesion and that a disruption of var2CSA gene leads to an

impairment of pregnancy-associated adhesion phenotype (Viebig *et al.*, 2007). Other domains of PfEMP1 that mediate binding to specific receptors besides CSA that could be used as malaria vaccine candidates have also been reported. Chen *et al.*, (2004) have shown that immunization with PfEMP1-DBLα generates antibodies that disrupt rosettes and protects against sequestration of *P.falciparum*-infected erythrocytes. Moreover, immunization of *Aotus* Monkeys with Recombinant cysteine-rich interdomain region 1α has been reported to be protective against severe disease during *P.falciparum* reinfection (Makobongo *et al.*, 2006). Although there is encouraging evidence that PfEMP1 could be a potential vaccine candidate against malaria, more research work is still needed to be done in order to clearly understand the expression of var genes and their contribution to malaria immunity.

2.8.2.3 Host Receptors For Cytoadherance

Several molecules have been identified as endothelial receptors based on their ability to support adhesion of *P. falciparum* infected erythrocytes during *in vitro* assays. CD36 and thrombospondin (TSP) were the first molecules to be described as cytoadherance receptors that bound parasitized erythrocytes (Roberts *et al.*, 1995; Barnwell *et al.*, 1985, 1989). TSP has been demonstrated to bind to erythrocyte membrane protein, designated PfEMP1 (Baruch *et al.*, 1996), a parasite derived variant protein that has been implicated in several aspects of severe malaria and its pathogenesis.

CD36, another host receptor involved in cytoadherance is found distributed on monocytes, endothelial cells, platelets and erythroblasts (Barnwell *et al.*, 1989). CD36 binds PfEMP1 via its CIDRα domain (Baruch *et al.*, 1997) and that monoclonal antibodies (mAbs) specific to CD36 and soluble CD36 (sCD36) block the binding of

parasitized erythrocytes to melanoma cells and CD36-expressing COS cells (Barnwell *et al.*, 1985; 1989; Howard and Gilladoga, 1989). More receptors identified include vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1, endothelial leukocyte adhesion molecule 1 (ELAM-1) or E-selectin, Platelet endothelial adhesion molecule 1 (PECAM-1) or CD31, heparan sulfate (HS), hyaluronic acid (HA), chondroitin sulfate A (CSA), complement receptor 1 (CR1) or CD35 or C3b/C4b receptor. VCAM-1 and ELAM-1 or E-selectin has been identified as potential receptors for sequestration of infected red blood cells (Ockenhouse *et al.*, 1992).

ICAM-1 has been shown to play a significant role in cerebral malaria (Fernandez-Reyes *et al.*, 1997). Attempts to map its binding site (Berendt *et al.*, 1989) revealed DBL2β and C2 regions of PfEMP1 as the active domains (Smith *et al.*, 2000a). PECAM-1 or CD31 is implicated in cytoadherance of field isolates and has been shown to use PfEMP1 as a ligand (Treutiger *et al.*, 1997).

CSA is one of the receptors crucial for binding of infected erythrocytes to the placenta and uses PfEMP1 domains as ligands such as DBL3 (Buffet *et al.*, 1999) and CIDR (Reeder *et al.*, 1999). The other receptor involved in sequestration in the placenta is HA (Beeson *et al.*, 2000). Moreover, HS, a negatively charged glycosaminoglycan (GAG) has been confirmed as a host receptor, mediating cytoadherance to endothelial cells via DBLα domain of PfEMP1 (Barragan *et al.*, 2000b; Vogt *et al.*, 2003).

The affinity of *P. falciparum* for binding to endothelial receptors is diverse and different receptors can act synergically to influence the final pattern of adhesion (McCormick *et al.*, 1997). It is believed that parasites take advantage to binding with broad specificity to various receptors to enhance their growth and survival. The multiple receptor binding is

therefore an adaptive property that the parasite has developed to anchour itself to the endothelia where it would mature and multiply without being taken to the reticuloendothelial system for elimination.

2.8.2.3.1 Complement Receptor 1 (CR1) And Its Expression

In human peripheral blood, complement receptor type 1 (CR1,CD35, C3b/C4b receptor, immununе adherence receptor) is found principally expressed on erythrocytes, monocytes, neutrophils, B cells and some T lymphocytes (Fearon, 1980). In tissues, CR1 is found on mast cells, glomerular podocytes and follicular dendritic cells and on astrocytes (Krych-Goldberg and Atkinson, 2001). The number of CR1 molecules on erythrocytes in normal individuals varies remarkably. Different healthy individuals may show up to 10-fold variation in the number of CR1 molecules per cell (Wilson *et al.*, 1986). This expression is partly determined by the two codominant alleles, one controlling high CR1 copy numbers while the other controls low expression of CR1 molecules per cell.

2.8.2.3.2. Function Of Complement Receptor 1 (CR1)

CR1 is an immune regulatory molecule responsible for the phenomenon of "immune adherence", which refers to the attachment of red cells to antibody-antigen complexes in the presence of complement, an event essential for the destruction of microbes and initiation of an immune response (Nelson, 1953). CR1 on RBCs serves as the immune adherence receptor for the complement fragments C3b and C4b (Krych-Goldberg and Atkinson, 2001). CR1 acts as an immune adherence receptor that binds C3b or C4b-opsonized immune complexes and transports them to the liver and spleen where they are

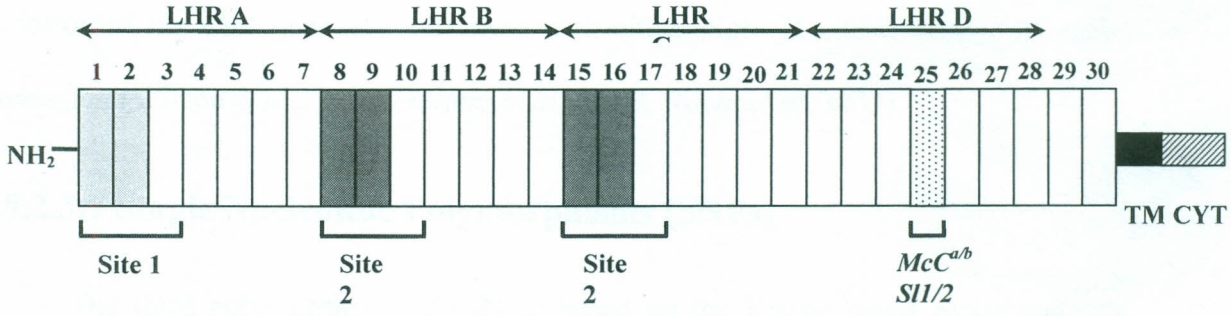
transferred to fixed macrophages and processed. In addition CR1 binds the complement component C1q and mannan-binding lectin, and these ligands may also play a role in immune adherence. CR1 is a member of the regulators of complement activation (RCA) protein family. Together with other members such as CD55 (decay accelerating factor) and CD59 (MIRL, membrane inhibitor of reactive lysis), CR1 protects the surface of RBCs and other host cells from attack by complement. CR1 accelerates the decay of enzymes that activate the complement cascade, the C3 and C5 convertases, and exhibits cofactor activity for the inactivation of C3b and C4b by Factor I (Krych-Goldberg and Atkinson, 2001).

2.8.2.3.3 Structure Of Complement Receptor 1 (CR1)

In humans, CR1 is encoded by a single copy gene on chromosome 1q32 that spans 105-160 Kb and contains 38-46 exons, depending on the structural allotype (Cohen *et al.*, 1999). CR1 is a single chain, multi-modular type I transmembrane glycoprotein of approximately 200 kDa. Like all members of the RCA protein family, the extracelullar portion of CR1 is composed of a variable number of independently folding globular domains called complement control protein repeats (CCPs) or short consensus repeats. Each CCP is composed of approximately 60 amino acids and contains a hydrophobic core formed by conserved amino acids including 4 invariant cysteines that are held together by 2 disulfide bonds. Based on a high degree of internal sequence homology, all except the 2 carboxyl terminal CCPs are further arranged into long homologous regions (LHRs) arranged in tandem and each composed of 7 CCPs (*reviewed in* Krych-Goldberg and Atkinson, 2001). The most common of the four structural variants of CR1 (CR1*1, F allele) has its amino terminal (NH_2) extracellular portion composed of 30 CCPs

organized into 4 LHRs (Figure 3). LHR-A binds mainly C4b and has convertase decay accelerating activity via active site 1 (CCPs 1-3). LHR-B and -C bind both C4b and with greater affinity, C3b via active site 2 (CCPs 8-10 and CCPs 15-17). Site 2 also possesses high cofactor activity for C3b and C4b as well as acting as the binding site for PfEMP1. The functional differences in sites 1 and 2 are determined by amino acid sequence differences. LHR-D binds mannan-binding lectin (Ghiran *et al,* 2000) and C1q (Klickstein *et al,* 1997). Swain Langley (Sl) and McCoy (McC) Knops blood group antigens have been localized at CCP 25 (Moulds *et al.*, 2001). The CCP repeats are followed by a transmembrane domain (TM) and a cytoplasmic tail (CYT).

Figure 3: Schematic diagram of the extracelullar domain of complement receptor type 1 (CR1)



Adapted from Krych-Goldberg and Atkinson, 2001

2.8.2.3.4. CR1 Polymorphisms And Role In Blood Groups

There are three types of polymorphisms identified in human CR1, namely molecular weight, erythrocyte copy number and Knops (KN) blood group antigens. Polymorphisms in CR1 have been associated with different aspects of severe malaria outcome.

2.8.2.3.5 Allotype Polymorphisms

There are four structural allotypes of CR1 with CR1*1 (220 kD/A or F allele), and CR1*2 (250 kD/B or S allele) being the two most common in all populations studied to date (Cohen *et al.*, 1999). The other two CR1 size polymorphisms are type C (190 kD) and type D (280 kD). These size polymorphisms are derived from four codominant inherited alleles. It has been suggested that these size variants evolved through duplication or deletion of a highly homologous unit rather than an alternative mRNA splicing (Hourcade *et al.*, 1989).

2.8.2.3.6 CR1 Density Polymorphisms

Erythrocyte CR1 copy number constitutes the second polymorphism. Quantitative expression of CR1 is controlled by an unknown genetic element that in Caucasians but not Africans, is linked to a *Hind*III restriction fragment length polymorphism (RFLP) in an intron of the *CR1* gene and correlates with either high (H allele) or low (L allele) expression (Wilson *et al.*, 1986; Herrera *et al.*, 1998; Rowe *et al*, 2002).

2.8.2.3.7 Single Nucleotide Polymorphisms (SNPs)

The third polymorphism of CR1 is based on the Knops blood group antigens, including Swain-Langley and McCoy and their alleles, *Sl1*/*Sl2* and McC^a/McC^b, respectively (Moulds *et al.*, 1991; Rao *et al.*, 1991). The Knops antigen, which is the 25th blood group system recognized, is known to lie within the CR1 protein repeats. Racial differences exist in the frequency of these antigens: 98.5% and 96.7% of American Caucasians and Africans, respectively, are positive for McC(a). 36% of a Mali population were Kn(a) and 14% of them exhibited the null (or Helgeson) phenotype compared with

only 1% in the American population. The serological phenotypes for Swain-Langley are designated Sl 1,-2 and Sl -1, 2, and for McCoy, McC(a+) and McC(b+) (Daniels *et al*, 2003). The frequencies of McC (b) and Sl (2) are higher in Africans compared with Europeans and while the frequency of McC (b) was similar between Africans from the USA or Mali, the Sl (b) phenotype is significantly more common in Mali - 39% and 65% respectively. In Gambia the Sl (2)/McC(b) phenotype appears to have been positively selected - presumably due to malaria. 80% of Papua New Guineans have the Hegelson phenotype and case control studies suggest this phenotype has a protective effect against severe malaria.

These phenotypes are occasionally the result of low erythrocyte CR1 copy number but most often due to single nucleotide polymorphisms (SNPs) that cause specific amino acid changes in exon 29 encoding CCP 25 in LHR-D of CR1 (Moulds *et al*., 2001). The substitution of glycine, a neutral amino acid, for the basic amino acid arginine at position 1601 (R1601G) results in the *Sl2* allele and expression of the Sl -1, 2 phenotype. The substitution of the acidic amino acid glutamic acid for the basic amino acid lysine at position 1590 (K1590E) results in the McC^{b} allele and expression of the McC(b+) phenotype. A simple PCR-RFLP method has been developed to genotype individuals for these polymorphisms (Moulds *et al*, 2004). The fact that these polymorphisms are common in malaria endemic regions has led to suggestions that they could play some significant role in severe malaria pathogenesis and as such formed an interesting theme among malaria researchers.

2.8.2.4 CR1 And Its Role In Cerebral Malaria

Rosetting is the spontaneous binding of uninfected red blood cells to *P. falciparum* infected red blood cells (Handunetti *et al.*, 1989, David *et al.*, 1988). Rosetting has been demonstrated to be closely associated with severe *falciparum* malaria, particularly cerebral malaria (Carson *et al.*, 1990; Newbold *et al.*, 1997; Roberts *et al.*, 2000; Rowe *et al.*, 2000; Fairhurst *et al.*, 2005). Studies from most malaria endemic regions have shown that parasite isolates from children with cerebral malaria have higher rosette frequency compared with those from children with mild malaria (Carson *et al.*, 1990; Rowe *et al.*, 1995). Moreover, children with cerebral malaria have been reported to be less likely to have anti-rosetting antibodies, suggesting the contribution of rosetting to pathogenesis of cerebral malaria (Treutiger *et al.*, 1992; Carson *et al.*, 1990). Data by Wahlgren *et al.*, (1991) also revealed that nearly 50% of parasites form rosettes when cultured *in vitro*. It is therefore most likely that rosette formation plays some pivotal role in the pathogenesis of severe malaria and cerebral malaria in particular.

Numerous binding combinations exist between different host receptors and parasite ligands that can induce rosette formation. The first host receptor to be identified in rosetting process is the ABO blood group particularly blood group A antigens (Carson and Wahlgren, 1992; Barragan *et al.*, 2000a). Previous studies have shown that rosetting is reduced in blood group O erythrocytes in *P. falciparum* strains (Carlson *et al.*, 1992) and field isolates (Rowe *et al.*, 1995). Most recently, Rowe *et al.*, (2007) compared rosette frequency of parasite isolates from blood group O children with isolates from non-O groups. This study concluded that the protective effect of blood group O operates through the mechanism of reduced *P. falciparum* rosetting. Other related studies have

documented that the protective effects of thalassemia, sickle-cell trait and heamoglobin C (HbC) may be partly caused by reduced rosetting (Carlson *et al.*, 1994; Fairhurst *et al.*, 2005).

The other receptors that are involved in rosette formation by interacting with PfEMP1 are CD36 (Handunetti *et al.*, 1992) although the molecule is expressed in low levels in mature red blood cells and even then, rarely participates in rosetting process (Wahlgren, 1992). Serum proteins mainly IgM and/or IgG have been shown to play a role in rosetting of some strains of parasites via PfEMP1 (Scholander *et al.*, 1996; Clough *et al.*, 1998a). Apart from IgM and IgG, some other immunoglobulins in normal serum have been shown to play a role in rosetting. For instance, Treutiger *et al.*, (1999) found that fibrinogen and albumin promote rosetting and that pro-rosetting effect of fibrinogen could be reversed by anti-fibrinogen antibodies. Some other more receptor involved in rosetting is heparin-like sulfate sugars (Rowe *et al.*, 2005). This observation is supported by the finding that parasite strains cultured in type O blood treated with heparinase was unable to form rosette (Chen *et al.*, 1998; Barragan *et al.*, 1998).

Rosetting is a heterogeneous phenomenon involving numerous red blood cell receptors suggesting multiple pathways of rosette formation. However, complement receptor 1 (CR1, CD35, C3b/C4b) has been reported to play a more common role in rosette process by binding to PfEMP1 (Rowe *et al.*, 1997). The functional domain of PfEMP1 that mediates adhesion of uninfected erythrocytes by CR1 has been established to be DBLα (Rowe *et al.*, 1997, 2000).

The finding that SI(a-) and McC(b+) CR1 blood group polymorphic alleles occur in higher frequency in population of African descent suggests the protective role these

alleles could play against severe malaria (Rowe *et al.*, 1997). CR1 copy numbers has also been linked to rosette formation. For example, some parasites have been shown to lose their capacity to form rosettes when cultured in CR1-deficient red blood cells and that soluble CR1 disrupts rosettes and block rosette reformation in some parasite strains (Rowe *et al.*, 1997). Thathy *et al.*, (2005) conducted a case-control study which demonstrated that *SI2* and *SI2/2/McC*$^{a/b}$ were more strongly associated with resistance to cerebral malaria than severe anaemia. However, a previous case-control study in the Gambia reported negative association of the same CR1 knops blood group alleles and severe malaria (Zimmerman *et al.*, 2003).

Overall, these results suggest that selection pressure induced by *P. falciparum* has resulted in the accumulation of numerous red blood cell variants that support rosetting less well to confer protection from severe malaria. CR1 has definitely been demonstrated to have a bigger role to play in rosette formation by interacting with its parasite ligand PfEMP1 (Rowe *et al.*, 1997).

2.8.2.5 The Possible Mechanisms Of Rosetting

It is not clearly understood why parasites form rosettes. However, numerous hypotheses have been formulated. It has been suggested that rosetting could enhance parasite growth and survival by increasing microvascular obstruction of blood flow (Kaul *et al.*, 1991), since parasites have been shown to do better in low oxygen levels. Rosetting could also enhance invasion of uninfected red blood cells much faster by allowing merozoites to move directly from ruptured schizonts into uninfected red blood cells forming rosettes (Wahlgren *et al.*, 1992), but this has no experimental evidence (Clough *et al.*, 1998).

The other hypothesis is that rosetting could shield the infected erythrocytes from the host phagocytic cells or antibodies that would lead to clearance of the infected erythrocytes by the immune system, one of the main mechanisms of anti-parasitic immunity (Bouharoun-Tayoun *et al.*, 1995). Although many experimental and clinical studies have shown positive correlation between rosetting and severe malaria, the mechanism for the pathogenesis is not clearly known. In fact, whether rosetting itself plays a direct role in the pathogenesis of severe malaria or it is just a marker for some other causal factors remains to be established. However, having reported that *SI2/2* and $McC^{a/b}$ confer protection to severe malaria, particularly cerebral malaria (Thathy *et al.*,2005), it would be prudent to confirm these findings by giving them molecular definitions in order to understand the mechanisms of their protective effects. This knowledge could form the basis of novel treatment strategies.

2.8.2.5 CR1 And Its Role In Severe Malarial Anaemia

Besides cerebral malaria, the other severe complication of *P. falciparum* malaria is severe anaemia. Although the mechanisms of severe malarial anaemia are unclear, Waitumbi *et al.*, (2000) reported the alterations in the expression of the complement regulatory proteins CR1, CD55 and CD59 on the RBC surface of patients with severe malarial anemia. The biological function of these proteins on the surface of host cells is to protect such cells from attack by complement (Weiss *et al.*, 1989; Holguin and Parker, 1992; Nicholson-Weller, 1992 and Kazatchkine *et al.*, 1987). The importance of CR1 in preventing complement-mediated damage is exemplified by diseases such as systemic lupus erythematosus, characterized by acquired CR1 deficiency, defective clearance of immune complexes (ICs) and hemolytic anemia (Walport and Lachmann, 1988).

Circulating ICs and complement activation are also prominent features of malaria infection (Adam *et al.,* 1981; Jhaveri *et al.*, 1997; Stoute *et al.*, 2003). Stoute and his colleagues studied the role of complement regulatory proteins, including CR1, in the pathogenesis of severe anaemia caused by *P. falciparum*. In the case-control study, they demonstrated that children with profound malarial anaemia in western Kenya have significantly higher levels of circulating ICs than those with symptomatic uncomplicated malaria (Stoute *et al.*, 2003). Moreover, erythrocytes of children with severe malarial anaemia have higher levels of surface IgG and C3b, acquired deficiencies of CR1 and are more susceptible to complement-mediated phagocytosis than those of uncomplicated malaria controls (Waitumbi *et al.*, 2000). These studies have led to the hypothesis that a decline in CR1 and other complement regulatory proteins, together with an increase in circulating IC levels places parasitemic children at risk for the development of severe anaemia. Decline of CR1 levels below a critical point would cause uncontrolled complement activation and IC deposition resulting in increased susceptibility of erythrocytes to phagocytosis or complement-mediated lysis. This mechanism could account for the accelerated destruction of uninfected erythrocytes that has been well documented during malaria infection (Price *et al.*, 2001; Woodruff *et al.*, 1979).

2.8.2.6 CR1 Polymorphisms And Role In Malaria

Data from several scientific experiments have pointed out that mutations in the CR1 gene are associated with either protection or susceptibility to severe malaria. Cockburn *et al.* (2004) have shown in a case-control study that RBC CR1 deficiency is extremely common in malaria-endemic areas of Papua New Guinea and that polymorphisms associated with CR1 deficiency (L allele) may confer protection against severe malaria.

Heterozygotes for the low-expression allele (HL genotype) but not homozygotes (LL) were found to be significantly protected from severe malaria as a whole. Surprisingly, no association was found between these polymorphisms and cerebral malaria per se.

A case-control study of children with severe malaria compared to non-malaria controls in the Gambia failed to show any association between either the *Sl2* or McC^b CR1 variant alleles and severe malaria (Zimmerman *et al.,* 2003). However, a recent case-control study was conducted by our group in western Kenya in which, unlike the Gambian study, children with severe malarial anemia or cerebral malaria were matched to controls with symptomatic uncomplicated malaria by age and gender (Thathy *et al.,* 2005), This study demonstrated that individuals with the *Sl2/2* genotype were significantly protected from cerebral malaria or severe malaria in general. Association with resistance to severe malaria appeared to be strongest in individuals who had the genotype *Sl2/2* $McC^{a/b}$.

The mechanisms by which the *Sl2* and McC^b alleles of CR1 may confer protection from severe malaria are unknown. These polymorphisms are located near the C-terminus of the CR1 molecule (CCP 25 in LHR D), distant from the binding sites for C3b, C4b and PfEMP-1 (active site 2 in CCPs 8-10 (LHR B) and CCPs 15-17 (LHR C); (Figure 3). The amino acid changes that result in the *Sl2* and McC^b alleles (R1601G and K1590E, respectively) could induce significant conformational changes on the CR1 molecule such that there is altered binding of ligands (C3b, C4b or PfEMP-1) to either copy of site 2 located in LHR -B or –C. Consequently this could affect immune complex binding capacity, cofactor activity for inactivation of C3b or rosetting ability of RBCs expressing these polymorphisms. In addition, aggregation of CR1 molecules on the RBC surface

enhances the binding affinity of CR1 for its ligands (Paccaud *et al.*, 1990). Aggregation of CR1 may also alter the efficiency of rosetting with infected RBCs. Perhaps the amino acid substitutions in CCP 25 also affect the ability of functional aggregates to form due to conformational incompatibility or charge interference between different CR1 molecules.

2.8.2.7 CR1-PfEMP1 Interaction And Pathogenesis Of Severe Malaria

Erythrocyte CR1 has been implicated in the pathogenesis of severe *P. falciparum* malaria because of its ability to bind to PfEMP1 and mediate rosette formation with infected RBCs *in vitro* (Rowe *et al.*, 1997). This was found to be true for several different laboratory-adapted *P. falciparum* strains as well as field isolates from Kenya and Malawi (Rowe *et al.*, 2000). Although rosetting has generally been known as a parasite virulent factor (Carlson *et al.*, 1990; Rowe *et al.*, 1995), its biological significance still remains unclear. The association between rosetting and the pathogenesis of severe malaria is highlighted by several studies in Africa that show a positive correlation between severe disease, particularly cerebral malaria, and *P. falciparum* isolates that tend to form rosettes *in vitro* (Carlson *et al.*, 1990; Treutiger *et al.*, 1992; Rowe *et al.*, 1995; Ringwald *et al.*, 1993; Kun *et al.*, 1998). Moreover, some studies have shown that anti-rosette immunity appears to be important in determining disease outcome (Chen *et al*, 2000). However, other studies conducted in Southeast Asia (Ho *et al.*, 1991) or Papua New Guinea (al-Yaman *et al.*, 1995), have shown no association between rosetting and severe disease.

Rowe *et al.* (1997) demonstrated that erythrocytes infected with some *P. falciparum* clones rosette efficiently with uninfected erythrocytes that have normal CR1 copy number than with erythrocytes which have deficiencies in CR1 copy number (<100

number than with erythrocytes which have deficiencies in CR1 copy number (<100 molecules per RBC). By expressing individual PfEMP1 domains on the surface of COS7 cells, they showed that uninfected RBCs bound to the DBLα domain of PfEMP1. Soluble CR1, anti-CR1 monoclonal antibodies or C3b dimers were able to inhibit rosette formation, showing the requirement for CR1 in rosetting (Rowe *et al.*, 1997, 2000). The DBLα-binding site on CR1 was mapped to the major C3b-binding site, site 2; Fig. 1), and the interaction occurred independent of C3b-binding (Rowe *et al.*, 2000). Thus, it appears that the DBLα domain of several *P. falciparum* clones or isolates mediates rosetting through direct interactions with the C3b-binding site of CR1 on uninfected RBCs. However, a direct interaction has not yet been proven using purified peptide domains.

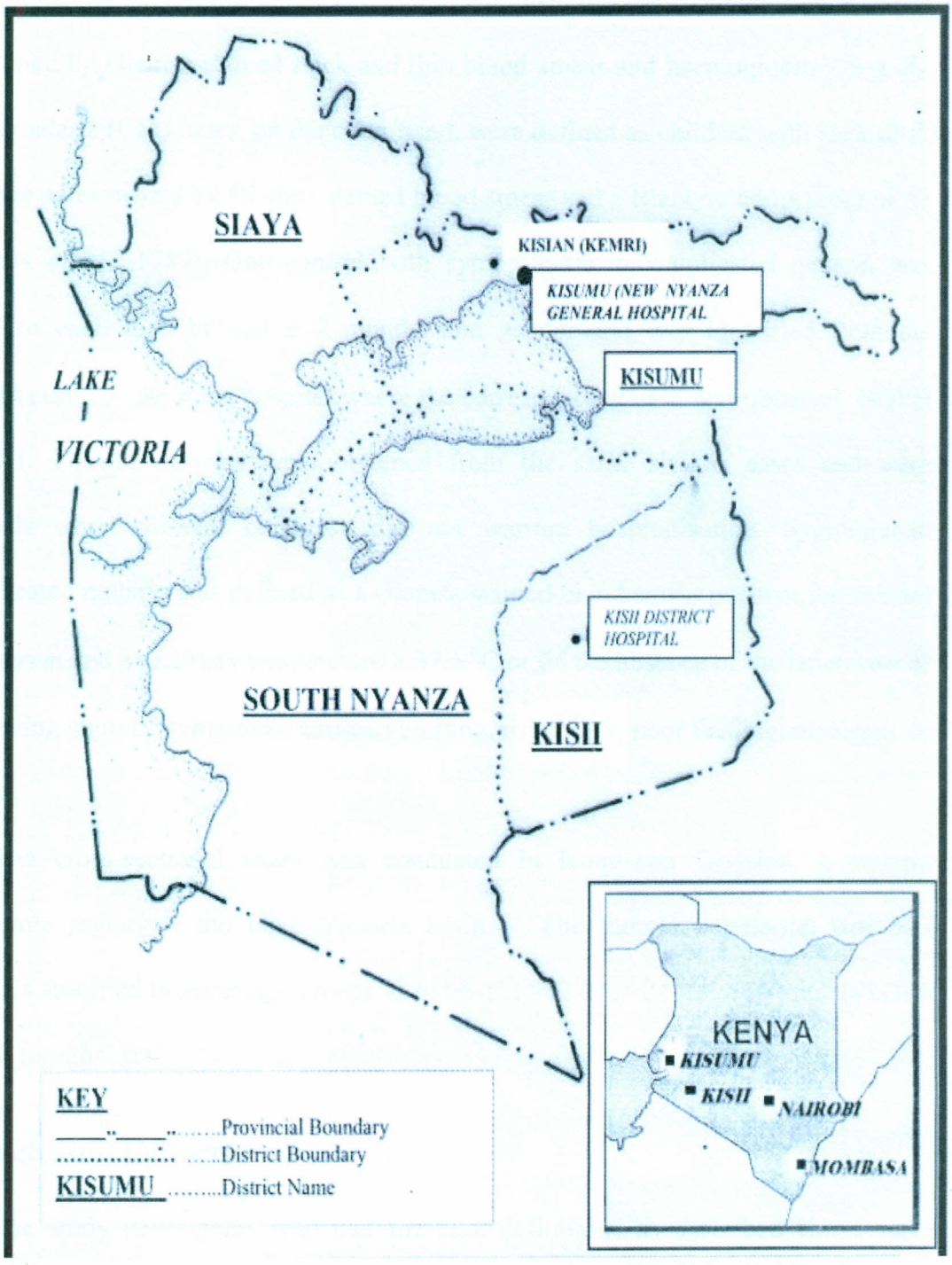
CHAPTER 3: MATERIALS AND METHODS

3.1 Study Site And Population

All assays were carried out at KEMRI-Walter Reed research laboratories in Kisumu (*Figure 4*).Children who met the study criteria were recruited from the paediatric ward of the Nyanza Provincial General Hospital (NPGH), Kisumu, Kenya. NPGH is a referral hospital and is the catchmet area of the malaria holoendemic region of the Lake Victoria basin, western Kenya. The area is inhabited predominantly by the Luo ethnic group. Because cerebral malaria (CM) is uncommon in the Lake Victoria basin, CM cases were recruited from the paediatric ward of the Kisii District Hospital (KDH) as well as a few cases from NGPH. KDH is situated in the highlands of western Kenya where malaria transmission is seasonal and thus receives many more CM cases than NGPH (Mash and Snow, 1999). The predominant ethnic group in Kisii is the Abagusi.

Sampes for the cross-sectional survey were collected from Kombewa.

Figure 4: Map showing location of the study sites, the Nyanza Provincial General Hospital and Kisii District Hospital



3.2 Study Design And Case Definitions

This was a case-control study of 460 children. Cases for severe malarial anaemia (SMA) were defined as those children who had asexual *P. falciparum* parasites as confirmed by Giemsa-stained thick and thin blood smear and haemoglobin ≤ 5 g/dL. Cerebral malaria (CM) cases, on the other hand, were defined as children with asexual *P. falciparum* parasitaemia by Giemsa-stained blood smear and a Blantyre coma score of ≤2 (Molyneux *et al.*, 1989). One control with symptomatic uncomplicated malaria was matched to each case by age ± 2 months and gender and was identified from the outpatient clinic of the same hospital where the corresponding case was obtained, NGPH and KDH. Control samples were obtained from the same site as cases and were individuals whose disease condition did not warrant hospitalization. Symptomatic uncomplicated malaria was defined as a Giemsa-stained blood smear positive for asexual *P. falciparum* and an axillary temperature ≥ 37.5 °C or, in the absence of the latter, two of the following signs or symptoms: nausea/vomiting, irritability, poor feeding, myalgias, or headache.

The cross-sectional study was conducted in Kombewa Division, a malaria holoendemic region of the Lake Victoria basin. The sample population was 345 individuals stratified in seven age groups: 0-6; >6-12; >12-24; >24-48; >48-96; >192-384 and >384 months.

3.2.1 Inclusion Criteria

All the study participants who met the case definitions as described above were included in case-control study.

3.2.2 Exclusion Criteria

Children were excluded from participation if there was evidence of other concomitant serious infections such as meningitis, pneumonia, sepsis or chronic illness. In addition, because some of the studies at KEMRI-Walter Reed Project laboratories also included the measurement of erythrocyte complement regulatory proteins, children were excluded if they had a history of blood transfusion in the three months preceding enrolment to avoid the influence of donor erythrocytes.

3.3 Blood Sample Collection

This study was part of a bigger one in which blood samples were collected aseptically into EDTA tubes by trained phlebotomists and transported to the laboratory. An aliquot of 200 µl of whole blood was transferred into a sterile 1.5 mL eppendorf tube and used for laboratory procedures. In cases where the samples got to the laboratory late in the evening, they were store at 4°C until the following day.

3.4 Experimental Procedures

3.4.1 ABO Blood Group Typing

After blood samples were received in the laboratory, the samples were typed for ABO blood group by standard haemagglutination techniques. This method is heavily hinged on the antigens naturally present on the red blood cells. The analysis is done in terms of the presence or absence of agglutination. Based on this serological procedure, there are four main blood group types: A, B, AB and O. Blood group type A has antigen A and anti-B antibodies, type B has antigen B and anti-A antibodies whereas type AB has both A and

B but has none of the antibodies. Type O which has none of the antigens has been referred to as null type. However, it has both anti-A and anti-B antibodies. ABO typing procedure involved mixing an individual blood sample with monoclonal antibodies, anti-A, anti-B and anti-AB and the sample observed to see if there was agglutination. If agglutination occurs, it means the blood reacted with its corresponding antibody. Therefore, two sets of 10 µl of the blood sample from same individual was applied on to a glass slide and mixed with a drop of either anti-A or anti-B monoclonal antibodies. The samples were observed if the red blood cells stuck together. The results were recorded accordingly. If there was agglutination for both anti-A and anti-B, that individual was typed AB. In instances where there was no agglutination with all the antibody types, the sample was typed as O. Blood typing was also done to tell whether or not an individual had a substance called Rh factor on the surface of their red blood cells. Those who have this substance are considered Rh^+ (positive). Those without it are considered Rh^- (negative). Rh typing uses a method similar to ABO typing. For example, if an individual blood sample reacts with anti-A monoclonal antibody and is positive for Rh factor, that individual is typed as A^+. The ABO data was important for subsequent analysis. Studies have shown that ABO blood group influences the frequency, size and robustness of rosettes (Rowe *et al.*, 1995; Barragan *et al.*, 2000; Carlson *et al.*, 1992).

3.4.2 Genotyping Of CR1 Knops Blood Group Polymorphisms

3.4.2.1. Genomic DNA Extraction

200 µl of whole blood sample aseptically collected in EDTA-tube was aliquoted into a clean 1.5 mL eppendorf tube and stored at 4°C if the sample was not used immediately.

In some cases the sample was blotted onto a filter paper and dried for future DNA extraction. DNA was extracted from the whole blood using the QIAamp DNA blood extraction kit (Qiagen, Valencia, CA) as described by the manufacturer. The protocol involves lysing the red blood cell membrane, precipitating out the lysed membrane components and other debris, binding the genomic DNA on a spin column, washing and elution as its final step. 20 µl of QIAGEN protease was added into the bottom of the sterile 1.5 ml eppendorf tube and 200 µl of the blood sample added into the tube. 200 µl of lysis buffer was added to the microcentrifuge and mixed by vortexing for 15 sec. Vortexing must be sufficient in order to release genomic DNA completely. The tube was incubated at 56°C for 10 min. and briefly spun to remove drops from inside of the lid. 200 µl of absolute ethanol was added to the sample and mixed by vortexing before spinning briefly to collect drops from inside of the lid. The mixture was then carefully applied to the QIAamp spin column and spun at 8000 rpm for a minute. The spin column was transferred to a clean 2 ml collection tube and carefully opened. 500 µl of AW1 was added without wetting the rim of the column and centrifuged at 8000 rpm for one minute. The QIAamp spin column was again transferred to a clean 2 ml collection tube and 500 µl of wash buffer added again being careful not to wet the rim of the spin column. The coulumn was centrifuged at maximum speed for 3 min. To remove residual wash buffer, the spin column was transferred to a new 2 ml collection tube and spun at maximum speed for an additional 1 min. The QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube. The spin column was carefully opened, 200 µl of buffer elution buffer added. The spin column was incubated at room temperature for 5 min. and centrifuged for a minute at 8000 rpm. The incubation period increased genomic DNA

yield. The extracted genomic DNA was used in PCR or stored frozen at -20°C for future use.

3.4.3 Polymerase Chain Reaction

Before the actual procedures were begun, PCR reagents and buffers save for DNA polymerase was thawed out on ice. The amplification reactions were carried out on ~500 ng of genomic DNA in a 100 µl final volume containing 1X GeneAmp PCR Buffer II, 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.2 µM each primer and 5 U AmpliTaq Gold® DNA Polymerase. PCR master mix containing the components as shown in Table 2 was prepared. The master mix was made in a sterile tube using barrier pipet tips and the volumes adjusted according to the number of samples to be run. The tube containing the master mix was placed on ice. The master mix was always prepared in excess by one extra tube. The forward primer (24KnNde) had the following sequence, 5'-ACC AGT GCC ACA CTG GAC CAG ATG GAG AAC AGC TGT TTG AGC AT-3' whereas (25Rb) 5'-GGA GGA GTG TGG CAG CTT G-3' was used as the reverse primer. After all the PCR components except the template were added and thoroughly but gently mixed, 98 µl of the master mix was aliquoted into a thin-walled PCR tube clearly labeled with the specific sample identity. 2 µl of the genomic DNA was added into the respective tubes and mixed gently. The samples were then loaded onto a programmable DNA analyzer (Applied Biosystems, CA USA). The heated lid was enabled to promote uniform distribution of heat within the reaction tubes. Thermal cycling conditions were a 9-min hot-start step for the activation of the enzyme, then a 1-min denaturation step at 94°C followed by 44 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C and terminated by

an elongation step of 10 min at 72°C. After the final extension, the PCR products were ready for gel analysis or stored at 4°C for later analysis.

Table 2 : **PCR components required in the preparation of the master mix**

Component (Final Concentration)	*Volume (µl) per 100 µl reaction*
10X GeneAmp PCR buffer II (1X)	10.0
25 mM $MgCl_2$ (2.5 mM)	10.0
10 mM dNTPs (200 µM)	2.0
100 µM 24KnNde (0.2 µM)	0.2
100 µM 25Rb (0.2 µM)	0.2
5 U/µl AmpliTaq-Gold (5 U)	1.0
Sterile water	74.6
Template : Genomic DNA (~500 ng)	2.0

3.4.4 Gel Analysis Of PCR Products

The PCR products were resolved on a 2% agarose gel. To prepare the gel, 2 grams of agarose (Invitrogen) was added to 100 mL of 1X TAE buffer (electrophoresis buffer) in pyrex bottle and swirled to mix. The agarose was then cooked in a microwave for 3 min. The cooked gel was allowed to cool warm enough to add 5 µl of 10 mg/ml ethidium bromide to have a final concentration of 0.5 µg/ml. Ethidium bromide is a fluorescent dye that intercalates between bases of DNA. It was incorporated into the gel so that staining would occur during electrophoresis, but the gel could also be stained after electrophoresis

by soaking in a dilute solution of ethidium bromide. Since the dye is a known mutagen and should be handled as a hazardous chemical, exposure to the dye was minimized as much as possible and protective gloves were strictly worn at all times when handling it.

The bottle was gently swirled to mix and the molten gel poured into a gel casting tray ensuring that there was no air bubble introduced in the process. The gel casting tray was then fitted with sample combs of appropriate sizes. The open ends of the trays were closed with tape while the gel was being cast and later removed prior to electrophoresis. It generally took 20 min to set the gel. When the gel was ready, it was placed into a mini gel electrophoresis tank and submerged in 1X TAE. The sample combs were removed to create the wells where the samples would be loaded. This was done taking care not to rip the bottom of the wells or breaking the walls of the wells. The samples were prepared for loading by aliquoting 10 µl of each PCR product and mixed with 1 µl of 10X DNA loading dye. The loading dye contained glycerol which allowed the samples to "sit" into the sample wells and bromophenol blue as a tracking dye, which migrated in the gel and allowed visual monitoring of how far the electrophoresis had proceeded. To size the PCR products, a lane was loaded with 5 µl of 1 Kb Plus DNA ladder as a standard molecular size maker. After all the samples were loaded into the wells, the electrophoresis chamber was covered with its lid and current applied. DNA would migrate towards the anode (positive electrode) which is usually coloured red in the gel electrophoresis tank. The gel was run at a constant voltage of 100V for 30 min. The distance DNA had migrated in the gel was judged by visually monitoring migration of the tracking dye. To visualize DNA, the ethidium bromide-stained gel was placed on an ultraviolet transilluminator immediately after the end of electrophoresis. This was done because DNA has been

shown to diffuse within the gel over time, and it is recommended that examination or photography should take place shortly after the end of electrophoresis. The expected PCR product was a 305 bp fragment. It was this product that was subjected to subsequent analysis by endonuclease digestion.

3.4.5 Restriction Fragment Length Polymorphisms (RFLP)

RFLP is a technique that involves the fragmentation of genomic DNA by a restriction enzyme. A restriction enzyme is a molecular scissors that can recognize and cut DNA wherever a specific short sequence occurs. The process is termed restriction digestion. The restriction enzyme will "walk" through the whole sequence and only digests at positions where it recognizes its cleavage site. Two restriction enzymes were used to genotype the amplified PCR products. The endonucleases used are recorded in *Table 3*.

Table 3: Recognition sites for restriction enzymes used in RFLP

Enzyme	*Source*	*Recognition sequence*	*Cut*
BsmI	***Bacilus***	**5'...GAATGCN$^{\vee}$...3'**	**5'...GAATGCN...3'**
	stearothermophilus	**3'...CTTAC$^{\wedge}$GN...5'**	**3'...CTTAC GN...5'**
MfeI	***Mycoplasma***	**5'...C$^{\vee}$AATTG...3'**	**5'....C AATTG...3'**
	fermentas	**3'...GTTAA$^{\wedge}$C...5'**	**3'....GTTAA C...5'**

$^{\vee\wedge}$ denotes recognition site whereas N denotes C or G or T or A.

The right PCR products were digested with either Bsm1 for McC^{a}/McC^{b} genotyping or Mfe1 for *Sl1/Sl2* genotyping (*Table 4*). The enzyme digest reaction was set in a 30 µl

reaction volume in a 0.6 ml eppendorf tube. 28 µl of each PCR product was aliquoted into two separate 0.6 mL eppendorf tubes which were correctly labeled with the sample identity and the type of enzyme used for digestion. For the samples that were to be digested with BsmI, for McC^a/McC^b detection, 2 µl of BsmI was added into the tubes containing 28 µl of PCR product. The enzymes were brought from the freezer in a cooler box. The tubes were briefly vortexed to mix and quickly spun to collect the sample at the bottom of the tube. To detect *SI1/SI2,* MfeI was used and the same procedure for BsmI applied. The samples were then incubated at 37°C overnight for MfeI digestion. For BsmI, the samples were digested at 65°C in thermocycler for 4 hr. However, an incubation of 4 hr would be sufficient for even for MfeI digestion. These conditions were optimal for complete digestion of genomic DNA into expetcted number of resolvable restriction fragments.

Table 4: Expected band sizes (base pair) for McCoy and Swain-Langley Knops genotyping

Genotype	***Base pair change***	***Amino acid change***	***Restriction enzyme for RFLP analysis***	***Homozygous wild type* (McC$^{a/a}$ or SI1/1)**	***Heterozygous* (McC$^{a/b}$ or SI1/2)**	***Homozygous variant* (McC$^{b/b}$ or SI2/2)**
McC^a/McC^b	4795A >G	K1590E	BsmI	305	305, 166, 139	161, 144
SI1/S2	4828A> G	R1601G	MfeI	305	305, 161, 144	166, 139

After the completion of incubation period, the PCR digests were resolved on a 2.5% high resolution agarose gel. In situations where DNA was to be analyzed later, the products were stored at 4°C. The percentage of the gel was increased because smaller

fragments were expected to be resolved. To prepare the gel, 2.5g of Wide range/standard 3:1 agarose (Invitrogen, CA USA) was dissolved in 100 ml of 1X TAE buffer as previously described in the method for preparing 2% agarose gel. 5 µl of 10 mg/ml ethidium bromide was added when the gel was cool enough and poured into the gel casting tray fixed onto a mini gel electrophoresis tank preset with samples combs of appropriate sizes. Any air bubble that might have been introduced during the process was removed. Molecular oxygen interferes with current flow since it offers resistance to current flow during gel electrophoresis. The gel was allowed to solidify.

When the gel was setting, samples were prepared for loading by mixing each samples with 3 µl of 10X DNA loading dye. The samples were then loaded into the wells and electrophorosed at a constant voltage of 100V for 1 hr 30 min taking an early picture of the gel at 40 min and a later one at the end of electrophoresis. The expected band sizes for respective alleles are shown in Table 4. The sample could either be homozygous wild type or variant or heterozygous for both alleles. The McC^b and *Sl2* variant allelic PCR products were digested by the restriction enzymes BsmI and MfeI, respectively. In situations where for some reasons it was not clear to genotype the samples with a particular enzyme, the RFLP procedure was repeated until it was without any doubts clear for genotyping. However, such cases were very rare.

3.5 Parasite Culture

3.5.1 *P. falciparum* Clone SA075

P. falciparum clone used in this study originated from SA075, a field isolate from western Kenya, East Africa. *P. falciparum* strain SA075, consistently exhibits high

rosetting rates *in vitro* (~50 to ~80%). Rosetting appears to be largely CR1- mediated in this parasite line since anti-CR1 $F(ab')_2$ fragments are able to inhibit rosetting by 70-80% (Stoute, unpublished results). The parasite was cloned by limiting dilution from the original stock and subsequently maintained in continuous culture with periodic enrichment for the rosetting phenotype. Cloning by limiting-dilution involves a procedure that separates cells based on the assumption that if a suspension of cells is diluted with adequate culture medium, a concentration of cells will be generated such that an accurately measured volume of the diluted suspension dispensed into a well will contain one cell. If this single cell remains viable and proliferates, then an isolated clone of cells will have been established in the well. Limiting-dilution cloning of parasitized red blood cells (pRBC) was performed as previously described by Udomsangpetch *et al.*, (1989). Parasitized cells to be cloned were counted by determining parasitaemia in Giemsa-stained thin smear. The parasitized cells were diluted by resuspending in plain RPMI-1640 such that there were $1x10^4$ parasitized cell/ml (10 parasitized cells/µl). For each 96-well plate, 100 parasites were mixed with 19.2 ml of RPMI-1640 medium and 0.8 ml of 50% haematocrit. 200 µl of this mixture were added to each well in a 96-well plate to obtain a final dilution of 1 parasite per well at 2% haematocrit. The culture medium was changed and 0.4% haematocrit added on day 7 and day 14. On day 18, the presence of parasites was detected microscopically. In the well where parasites were observed, a second round of cloning by limiting dilution was performed to ensure the production of what was considered a true clone. After the second round of cloning, the isolated clone was expanded by seeding into 24-well culture plate and supplemented with 300 µl of fresh erythrocyte suspension at 2% haematocrit per well. Finally the parasites were

transferred into a T25 culture flask for routine cultivation. It was vital to count the parasitized cells correctly for the dilution procedures depended heavily on these calculations. It was also necessary that the dilution process be done carefully to ensure that several wells contained predominantly single parasitized cells for this was the general assumption of the procedure. Although many wells did not contain any parasite at all, parasites were observed in a few wells.

3.5.2. Routine Maintenance Of *P. falciparum* Culture

Heparinized O^+ blood was washed with RPMI1640 (Sigma, IL USA) and stored at 50% heamatocrit at 4°C prior to use. This wash was meant to free the erythrocytes of plasma and leucocytes. *P. falciparum* SA075R+ clone was thawed out and cultivated at 5% haematocrit in complete medium (RPMI-1640 supplemented with 25 mg/ml HEPES, 2 mg/ml sodium bicarbonate, 50 µg/mL gentamicin and 10% heat-inactivated human O^+ serum). The final pH of the culture medium solution was 7.4. All cultures were maintained at 37°C in an atmosphere of 5% CO2, 5% O2, and 90% N2 with daily media replacements depending on levels of parasitaemia. Parasite development and parasitaemic levels were monitored by standard method of preparing Giemsa-stained thin blood smears. The smears were stained for 30 min in 10% Giemsa, allowed to dry and then read under a light microscope using X100 objective with immersion oil.

3.5.3. Enrichment Of Rosetting And Non-rosetting Parasites Strains

Rosetting ring and young trophozoite-infected erythrocytes were selected by gelatin sedimentation method. The basis of this method of selecting rosetting parasite line is that parasites which are in rosette will sediment at the bottom of the tube since they are

heavier than those that do not rosette. The culture to be enriched was spun down at 2500 rpm for 5 minutes at room temperature and the resulting pellet resuspended in 0.75% gelatin. The suspension was incubated in a water bath at 37°C for 30 minutes. To enrich for nonrosetting parasites, the layer of cells floating at the interface was collected into a sterile 15ml eppendorf tube, washed twice in incomplete culture medium (plain RPMI). Each wash involved spinning at 2500 rpm for 5 minutes at room temperature with a final wash in complete culture medium (RPMI supplemented with 10% serum and gentamycin). The pellet was resuspended in complete culture medium and haematocrit adjusted at 5% with fresh O^+ uninfected human erythrocytes. The parasites were then returned to culture and maintained at 37°C in an atmosphere of 90% N_2, 5% CO_2 and 5% O2. To generate and maintain the non-rosetting parasite line, the selection procedure was repeated three times. The parasite line thus generated was designated SA075R-.

However, to enrich for rosetting parasites, the cells sedimenting through the gelatin was collected in a pellet, treated with 20 mg/ml heparin to disrupt the rosettes. Heparin has been shown to disrupt rosettes *in vitro* (Carlson *et al.*, 1992). The pellet was resuspended in 10 ml of incomplete medium containing 20 mg/ml heparin and incubated at 37°C in a water bath for 30 minutes. The resulting supernatant (basically containing the rosetting parasites) was transferred into a sterile 15 ml eppendorf tube and spun for 5 minutes at 2500 rpm at room temperature. The pellet was washed three times in incomplete medium to remove any traces of heparin. The rest of the procedures were similar to those for R- selection. The parasites thus generated were labeled SA075R+.

The cultures were synchronized once a week by sorbitol lysis to maintain a synchronous culture. Each time the cultures were synchronized, the first generation of

schizonts- were subjected to same round of rosette selection. There were three rounds of rosette selection before the parasites were used in RNA isolation. This continued rounds of selection enriched the parasite culture for rosetting parasites. Separate culture flasks were maintained for both R+ and R- parasite lines. Rosetting rate of the SA075R+ parasite line was confirmed by mounting a wet preparation on a glass slide and observing under a light microscope. For SA075R+, the rosetting rate was more than 65% whereas for SA075R-, the rate was less than 5%. As the parasites propagated, the cultures were expanded into larger culture flasks (T75) before harvesting. When the parasitaemia was high enough (~10%), the *P. falciparum*-infected erythrocytes were purified using Percoll gradient step purification method.

3.5.4. Sorbitol Synchronization Of *P. Falciparum*-Infected Erythrocytes

Sorbitol treatment of *P. falciparum* cultures is a procedure meant to synchronize the cultures and is based on the ability of the parasite to modify the erythrocyte it infects. Since the parasite continues, as it matures, to impart both chemical and morphological changes on the infected erythrocytes, these changes render the cells permeable and allow sorbitol to enter the erythrocytes causing their lysis. However, uninfected erythrocytes and those that are infected with ring stage of the parasite are impermeable to sorbitol. This treatment therefore results in cell suspension containing only ring stage parasites estimated to be up to 18 hr old (Jensen, 1988).

To achieve uniform ring stage, parasitized erythrocytes with ring forms were treated with 5% aqueous D-sorbitol. Culture contents were pelleted down by centrifuging at 2400 rpm for 5 minutes at room temperature. The supernatant was aspirated and packed cells suspended in 5 times volume of 5% (wt/vol) D-sorbitol solution. The suspension

was incubated at room temperature for 15 minutes, gently mixing by inverting the tubes up and down several times after every 3 minutes. After the completion of the incubation period, the cells were spun down at 2400 rpm for 5 minutes at room temperature and the supernatant discarded. This was followed by two washes in RPMI 1640 medium without serum (incomplete medium) to remove sorbitol. The cells were then resuspended in complete culture medium and haematocrit adjusted with unparasitized O^+ erythrocytes before returning to culture to allow continued development and multiplication of the parasites. Immediately after sorbitol treatment, cultures consisted mainly of single or multiple ring-form infections and uninfected erythrocytes. Synchronization was confirmed and the level of parasitaemia established by standard microscopy. Highly synchronous *P. falciparum* ring stage parasites were obtained using this method.

3.5.5. Percoll Enrichment Of Early Stages Of *P. falciparum*

Percoll gradient step centrifugation method has been successfully used for obtaining highly purified preparations of viable different stages of *P .falciparum* parasites. Percoll (1.13 g/ml) was made isosmotic by the addition of one volume of 10X incomplete culture medium to nine volumes of stock Percoll solution (90%). This solution was then diluted with 1X incomplete culture medium to make 80%, 70% and 40% percoll solutions. 10 ml of 90% Percoll was added into a sterile oakridge tube. 5 ml of the subsequent Percoll dilutions were run slowly down the tilted oakridge tube starting with the heaviest (80%, 70%, and 40%), to form the layered Percoll gradient. The tube with layered Percoll gradients was placed in a place where it would not be disturbed. The cultures with predominantly ring stage of the parasite were harvested by spinning down at 2500 rpm

predominantly ring stage of the parasite were harvested by spinning down at 2500 rpm for 5 minutes at room temperature. The pellet was resuspended in 5 ml of incomplete culture medium. The cell suspension was layered by allowing it to slowly trickle down the side of the oackridge tube. The tube was then centrifuged at room temperature (20°C to 24°C) for 20 minutes at 10,000 rpm. Upon completion of centrifugation, the different stages of the asexual cell cycle of the parasite were separated according to their density in a continuous gradient of Percoll. A few contaminating schizonts would be found at the top layer and just below this portion, mature trophozoites (if in some cases they were there) would be separated. The ring stage and very early trophozoite stage would be positioned at the layer just below the trophozoite portion. Uninfected erythrocytes would be found settled at the bottom of the tube.

Using a sterile Pasteur pipette, the layer containing ring and early trophozoite stages were transferred into a sterile 50 ml conical tube. The parasitized erythrocytes were washed three times in incomplete culture medium. The efficiency of this method to enrich for rings and early trophozoites was confirmed by standard microscopy. There was over 90% purity of the early forms of *P. falciparum* cell cycle. It is important to note that the viability of the parasite is not impaired by this procedure for parasites separated by this method can still grow and develop normally. The parasites were then ready for RNA isolation. In cases where the parasites were meant for culture, the last wash would be done in complete culture medium.

3.6 Total RNA Isolation, cDNA Synthesis And RT-PCR

3.6.1. Isolation Of Total RNA From *P. falciparum* Purified Infected Erythrocytes

Total RNA was isolated using TRIzol reagent (invitrogen). This is a modification of guanidine thiocyanate method already described by Chomczynski and Sacchi (1987). The harvested cells were lysed for 5 minutes in 0.1% saponin prepared in 1X PBS. High quality RNA was essential for successful full-length complementary DNA (cDNA) synthesis. This therefore required that the RNA should be devoid of any RNase contamination and aseptic conditions must be maintained at all time.

Isolation of RNA by this method has four major steps: homogenization, phase separation, precipitation and washing. After the saponin lysis of the parasitized erythrocytes in a 15 ml conical tube, the lysate was pelleted and resuspended in 1 ml of pre-warmed TRIzol Reagent™. The mixture was thoroughly mixed for uniform homogenization. For phase separation, 0.2 mL of chloroform per 1 ml of TRIzol Reagent used was added and shaken vigorously for 20 sec. The tube was incubated at room temperature for 3 min and sample spun at 5000 x g for 30 min at 4°C. After centrifugation, the mixture separated into a lower red, phenol chloroform phase, an intermediate phase, and a colourless upper aqueous phase. RNA is found exclusively in the aqueous phase. Using sterile Pasteur pipette, the aqueous phase was transferred to a fresh tube and precipitated by mixing with 0.5 ml isopropyl per 1 ml of TRIzol Reagent. The sample was mixed by repeated inversion before incubating at room temperature for 10 min. The sample was then spun at 12,000 x g for 10 min at 4°C. The supernatant was then discarded and the RNA washed in 1 ml of 75% ethanol per 1 ml of TRIzol Reagent and mixed to dislodge the RNA and other wash salts. The 75% ethanol was prepared in

by inverting the tube for 5 min taking care not to over dry the RNA. The RNA was dissolved in 100 µl of DEPC-treated water and incubated at 65°C for 10 min. This incubation was helpful in dissolving the RNA. Aliquots in smaller volumes were prepared for storage at -80°C. This was done to avoid shearing of RNA due to repeated freeze-thawing cycles.

An aliquot was used for the quantification of RNA. This was done by taking the optic densities (ODs) at 260 nm and 280 nm using a spectrophotometer. The concentration of RNA was calculated as follows: OD^{260} x dilution factor x 40 µg/µl. $OD^{260/280}$ ratio was calculated to assess the purity of the isolated RNA. A ratio above 1.8 was considered pure and of good quality whereas any ratio below 1.8 was taken to contain contaminants and was not good enough for the subsequent assays. The RNA used in these experiments had an $OD^{260/280}$ ratio of at least 1.8.

3.6.2 cDNA Synthesis

A complementary deoxyribonucleic acid (cDNA) is a single-stranded DNA synthesized from an RNA (usually messenger RNA) template using reverse transcriptase. The synthesis of cDNA was done using the Superscript (Invitrogen) kit and followed the steps as they were described by the manufacturer. To get rid of any genomic DNA contamination, 10 ng of ring or early trophozoite-stage total RNA was treated with RNase-free DNaseI (Invitrogen). 1 µg of RNA, 1 µl of 10X DNaseI reaction buffer, 1 µl DNaseI, amp grade, 1U/µl and 10 µl of DEPC-treated water were mixed in an DNase/RNase-free tube and the mixture incubated at room temperature for 15 min. The volumes were adjusted accordingly depending on the amount of total RNA needed for an

DNase/RNase-free tube and the mixture incubated at room temperature for 15 min. The volumes were adjusted accordingly depending on the amount of total RNA needed for an RT-PCR reaction. After the end of the incubation the activity of DNaseI was terminated by adding 1 µl of 25 mM EDTA. EDTA is an exonuclease inhibitor and DNaseI is a 5' exonuclease. The reaction was then incubated at 65°C for 10 min to heat inactivate the enzyme before setting on ice for 1 min. The total RNA was ready for cDNA synthesis at this point. It was always stored at -20°C if it was not used.

For first-strand cDNA synthesis, SuperScriptTM II (Invitrogen) was used. A 20-µl reaction volume was used and the following components were added in a nuclease-free microcentrifuge tube: 2 µl of 50 ng/µl random hexamers, 1 µl of 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH). The volume of the total RNA (R+ or R-) added to the mix varied depending on the concentration of the isolated RNA. However, 5 µl of total RNA was used for the cDNA synthesis. All these components were diluted in DEPC-treated water in a final volume of 13 µl. The mixture was heated at 65°C for 5 min and chilled on ice for 4 min. The contents of the tube were collected by brief centrifugation before the addition of the following components. 4 µl of 5X First-Strand Buffer, 1 µl of 0.1 M DTT, 1 µl of 40 units/µl RNaseOUT and 1 µl of 200 units/µl SuperScript™ III RT. The random hexamers were preferred as primers to either oligo-dT or gene-specific primers due to their ability to hybridize anywhere on the RNA. The components were mixed by gently pipetting up and down and incubated at room temperature (25°C) for 5 min. The tube was then loaded onto a thermocycler and incubated for 45 minutes at 50°C followed by a termination step at 70°C for 15 minutes to inactivate the reaction. To remove the RNA strand complementary to the cDNA, 1 µl of

3.6.3. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

3.6.3.1 DBLα Sequences Amplification

The RT-PCR was done with primers that would amplify DBLα sequences. The oligonucleotides DBLαAF' (GCACG(A/C)AGTTT(C*/T)GC and DBLαBR (GCCCATTC(G/C)TCGAACCA (Bull *et al.*, 2005) were used to amplify var DBLα domain sequence tags. αAF and αBR are universal primers validated for unbiased amplification of the duffy binding-like 1α (DBL1α) domain of PfEMP1 from var genes (Taylor *et al.*, 2000).

This set of primer should yield a PCR product of ~400 bp. The PCR components were added in a reaction tube in a final volume of 50 µl as shown in Table 5.

Table 5: An outline of PCR components for amplification of DBLα domain

PCR component	*Required volume*	*Final concentration*
10X HiFi Buffer	5 µL	1X
50 mM $MgSO_4$	2 µL	2.8 mM
10 mM dNTP mix	1 µL	0.2 Mm
10 µM DBLαAF'	2 µL	0.4 µM
10 µM DBLαBR	2 µL	0.4 µM
5 U/µL Taq DNA polymerase (HiFi)	0.5 µL	0.05U
cDNA (from first-strand reaction)	4 µL	
RNase/DNase-free distilled water	33.5 µL	

To set the PCR reaction, a premix of the above reagents, excluding the template was made in a sterile 1.5 mL microcentrifuge tube on ice. 45 µl of the mastermix was then added into a labeled PCR tube and 5 µl of the respective cDNA added. The tube was mixed gently before loading for amplification. Thermocycling conditions were a 2 minutes hot-start step and a 30 second denaturation step at 94°C followed by 35 cycles of 30 sec at 94°C, 1 min at 42°C, 30 sec at 65°C and a final elongation step of 7 min at 65°C. The PCR products were stored at 4°C until gel analysis.

3.6.3.2 DBL1α-CIDR1α Sequences Amplification

To amplify sequences between DBLα and CIDRα, the same forward primer (DBLαAF') was used with a reverse primer, BetaR. The PCR layout was similar with

that of amplifying DBLα sequences. The cycling conditions were also similar and the major difference was in the reverse primer. Like other PCR products, the product obtained with this primer set was purified to obtain pure inserts for cloning purposes.

3.6.3.3 Amplification Of cDNA Upstream Of DBL1α

To amplify sequences upstream of DBLα, the oligonucleotides shown in *Table 6* were used.

Table 6: Oligonucleotide sequences for DBLα upstream region

Oligo Name	***Oligo Sequence***
UpsA750	AACATKGTTCTATTTTCTC
UpsB	TTGCCTCTDTTGTTATCTC
LYLD3'	TTCATGATCAAGGTATAAATC
PTYF3'	ACGTAGTCAAAATATGTGG
PTNL3'	ACGTTAATCTAAATTGGTAG
MFLP3'	TTATTAGGTAAAAACATATC

The DBLα domain of var-genes/PfEMP1 was amplified from the cDNA template synthesized from R+ and R- *P. falciparum* parasite strains using four pairs of primer sets. Eight PCR tubes were set up and labeled with the right primer set (UpsA with all the reverse primers or UpsB with all the reverse primers as outlined in *Table 6*). The type of template cDNA used (either R+ or R-) is also shown. A premix was prepared ensuring that the right primer set was added into the right tube and an aliquot transferred into

labeled PCR tubes. The final PCR reaction volume was 50 µl. This has already been described elsewhere in the methods. The set up was the same as the one outlined in Table 6 with adjusted volumes depending on the number of reaction tubes needed to be run. The PCR conditions ,were the same as the DBLαAF'/DBLαBR primers. The cycling conditions were a 2-min denaturation step followed by 35 cycles of 20 sec at 94°C, 30 sec at 47°C, 1 min at 65°C and a final elongation step of 7 min at 65°C. The PCR products obtained by these primer sets were analyzed on an ethidium bromide-stained agarose gel as been described in the previous methods.

Table 7: A layout of PCR for amplification of sequences upstream of DBLα domain

	Reverse Primer			
Forward Primer	LYLD3'	PTYF3'	PTNL3'	MFLP3'
UpsA750	(R+)	(R+)	(R+)	(R+)
	(R-)	(R-)	(R-)	(R-)
UpsB	(R+)	(R+)	(R+)	(R+)
	(R-)	(R-)	(R-)	(R-)

3.6.3.4 Amplification Of Cdna Downstream Of DBL1α

The following oligonucleotide sequences were used to amplify sequence fragments downstream of DBL1α as outlined in Table 8.

Table 8: Oligonucleotide sequences for amplification of DBLα upstream domain

Oligonucleotide Name	Oligonucleotide sequence
BetaR	GA/CCCAC/TTCIGC/TCATCCA
LYLD5'	GATTTATACCTTGATCATGAA
PTYF5'	CCACATATTTTGACTACG
PTNL5'	CTACCAATTTAGATTACGT
MFLP5'	GATATGTTTTTACCTAATAA

The PCR requirements and cycling conditions were pretty similar with those of the upstream domain amplification parameters. The only changes were the primer sets used as indicated in Table 9.

Table 9: A layout of PCR for amplification of sequences downstream of DBL1α domain

	Forward Primer			
Reverse Primer	LYLD5'	PTYF5'	PTNL5'	MFLP5'
BetaR	(R+)	(R+)	(R+)	(R+)
	(R-)	(R-)	(R-)	(R-)

This kind of a table was used to record the products generated by PCR reactions using various primer sets. BetaR was used as the reverse primer with different four forward primers.

3.6.3.5. Amplification Of Whole Semi-Conserved Head Structure Of DBL1α Domain

In order to amplify the whole head structure of DBL1α domain from cDNA generated from a rosetting parasite line SA075R+, primers spanning the region were designed from sequence alignment of both upstream and downstream of DBL1α domain. The primers were appended with restriction sites for cloning purposes. For confirmation of cell surface expression of the constructs, a primer was designed that contained a FLAG peptide tag.

3.6.4 Cloning And Sequencing

3.6.4.1 Purification Of DNA Fragments And Plasmids

Before DNA products were cloned into either a cloning or expression vector, the products were purified to get rid of substances which would inhibit or negatively influence the downstream experiments. To achieve this, two kits were used depending on the band patterns from gel analysis of the DNA products. In cases where there was only a single band, QIAquick PCR purification kit was used. In other cases where there were multiple band, QIAquick Gel extraction kit was applied. The principle of the QIAquick system is that, it uses the spin column technology combined with selective binding properties of a uniquely-designed silica-gel membrane. Each kit is provided with special buffers already optimized for efficient removal of contaminants and recovery of DNA. The DNA is adsorbed to the silica-membrane in the presence of high salt whereas the contaminants flow through the column. This ensures the complete removal of the impurities resulting into pure DNA products. Elution of DNA is done at low salt

concentration and is heavily dependent on pH. Maximum elution efficiency is achieved between pH 7.0 and 8.5. Although water (pH within this range) could be used for elution, DNA eluted in water would not last for long in storage since DNA degrades faster in the absence of a buffering agent. The impurities that are washed away by this system include: salts, enzymes, unincorporated nucleotides, agarose, dyes, mineral oil, detergents such as DMSO and many more.

3.6.4.2 QIAquick PCR Purification Kit Protocol

This was a kit obtained from QIAGEN (CA, USA) and is designed to purify DNA fragments from PCR and other enzymatic reactions. The procedure involved adding 5 volumes of DNA binding buffer to 1 volume of PCR sample and mix. The sample was applied to the QIAquick column placed on a 2 mL collection tube and spun for 1 min at 13,000 rpm on a table-top microcerntifuge. The flow through was discarded and the QIAquick column put back to the same tube. 750 µl of wash buffer was added to the column and spun for 1 min at 13,000 rpm discarding the resulting flow through. The column was put back to the same collection tube and spun for an additional 1 min to remove residual wash buffer that contains alcohol, which may inhibit some subsequent experimental procedures. After the last spin, the collection tube was discarded and the QIAquick column placed in a sterile 1.5 microcentrifuge tube and appropriate volume (at least 15 µl) of elution buffer (10mM Tris-Cl, pH 8.5) added directly to the centre of the QIAquick membrane and let the column stand for at least 1 min, and then centrifuged. This incubation enhanced DNA recovery and sometimes the more you incubated, the more you recovered DNA from the QIAquick membrane. The purified DNA was good

enough for many applications. It was ready for use immediately or stored at -20°C for later use.

3.6.4.3 QIAquick Gel Extraction Kit Protocol

In this procedure, the DNA fragment already separated on a 0.8% agarose gel was excised using a sterile, sharp scalpel. It was vital to minimize the size of the gel slice by just cutting closely around the band. The excised band slice was placed into a clean 1.5 mL microcentrifuge tube and weight recorded. 3 volumes of solubilization and binding buffer were added to 1 volume of gel (100 mg ~ 100 µl). This was followed with a 10 min incubation at 50°C mixing the tube by vortexing every 3 min during incubation to enhance dissolution of the gel. When the gel was completely dissolved, 1 volume of room temperature isopropanol was added to the sample and mixed. This step was done to increase the yield of DNA fragments less than 0.4 kb and more than 4 kb. The solution was transferred to a spin column in a 2 ml collection tube and DNA bound by spinning at 13,000 rpm for 1 min. For sample volumes of more than 800 µl, the spin column was reloaded and spun again. The flow through was discarded and the spin column put back to the collection tube. 500 µl of solubilization and DNA binding buffer was added and spun. This step was necessary to remove any traces of agarose from the membrane. The flow through was poured off and the spin column washed by adding 750 µl of wash buffer and spinning at 13,000 rpm for 1 min. For products that would be used for direct sequencing or blunt-end ligation procedures, the spin column was left to stand at room temperature for 5 min. before centrifugation. After this wash step, the flow through was discarded and the spin column dried by spinning for an addition minute at maximum speed. This was done to dry the membrane by removing the residual wash buffer that

could affect the downstream experiments. The flow-through was discarded together with the collection tube and spin column placed into a sterile 1.5 ml polypropylene microcentrifuge tube. DNA was eluted by adding appropriate volume (at least 15 µl) of elution buffer (10mM Tris-Cl, pH 8.5) directly to the centre of QIAquick membrane, incubated for at least 1 min. and spun for 1 min at maximum speed. The DNA was at this time ready for use or stored at -20°C for later use. To visually analyze the purified DNA, an aliquot (usually 1 µl) was run on an agarose gel. For quantitative analysis using spectrophotometer, 1 µl of the purified DNA was used. This analysis also showed a measure of DNA purity. DNA is usually pure enough for most applications when it has a 260/280 nm OD ratio of at least 1.8.

3.6.4.3 TOPO XL PCR Cloning Kit Procedure

The vector is linearized and has a single 3'-thymidine (T) overhangs for TA cloning and an enzyme (Topoisomerase) covalently bound to the plasmid. The plasmid is thus referred as "activated" vector. Cloning PCR products to this plasmid does not require ligase. The vector utilizes the ability of Taq polymerase to add a single deoxyadenosine (A) to the 3' ends of PCR products. The vector works by donating its single overhanging 3' deoxythymidine (T) residues which allows the PCR inserts generated with Taq polymerase (Platinum HiFi Taq polymerase) to ligate efficiently with the vector. Topoisomerase binds duplex DNA at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT in one strand (Shuman, 1991). The energy released is conserved by the formation of a covalent bond between the 3' phosphate of the cleaved strands and a tyrosyl residue (Tyr-274) of topoisomerase I. The phosph-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the

original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to successfully and efficiently clone PCR products.

The purified DNA containing DBL1α sequences was cloned into pCR®-XL-TOPO® vector (Invitrogen) as described in the kit. 5 µl TOPO^R cloning reaction was set in a sterile 0.6 ml microcentrifuge tube. The reaction components are shown in Table 10. The components were mixed gently and incubated at room temperature for strictly 5 min. The kit does not recommend longer incubation period since doing so leads to a decrease in the transformation efficiency. The reaction was stopped by adding 1 µl of the 6X TOPO® Cloning Stop Solution and mixed for several seconds before centrifuging to collect the contents at the bottom of the tube. The addition of the Stop Solution was meant to also increase the yield of transformants by an average of 2-fold for chemically competent cells that were to be used for transformation reaction. The TOPO® Cloning reaction was set on ice and was now ready for transforming competent cells. In instances where the reaction was not used immediately, it was kept at -20°C for at most 24 hr as recommended by the manufacturer. A cloning reaction was also set using TE buffer instead of insert to act as a negative control. Nearly all transformation procedures were performed with freshly prepared TOPO® cloning reactions. In examining the transformation efficiency, it was generally expected to find no colony on negative control plate whereas a successful transformation reaction would yield several colonies.

In some instances where the concentration of the purified products (insert) was very low, as much as 4 µl was added to the cloning reaction. In such cases, no water was added in

the cloning reaction tube in order to maintain the recommended final volume for TOPO vector cloning reaction of 5 µl.

Table 10: Requirements for TOPO vector Cloning reaction

Cloning Reaction Component	*Volume*
Gel-purified PCR product (insert)	0.5 to 3 µl
pCRR-XL-TOPOR vector	1µl
Sterile ddH_2O	Add to final volume of 4 µl
Final volume	5 µl

3.6.4.4 Transformation of TOP10 Cells By Chemical Method

A vial of One Shot^R TOP10 chemically competent cells (Invitrogen) was thawed out on ice. 2 µl of the TOPO^R Cloning reaction was added into the vial and mixed by gently tapping the vial. Mixing by pipetting up and down was avoided for this would destroy the cells. Chemically competent TOP10 cells have fragile cell membrane that can easily be broken by application of force. The reaction was incubated on ice for 30 min. A control vial was also included. For the control, pUC19 plasmid was used to transform the competent cells. 1 µl of the control DNA was added to the 50 µl vial of competent cells and incubated as the experimental DNA. During this incubation period, a water bath was set at 42°C and after the 30 min incubation period was over, the cells were heat-shocked strictly for 30 sec at 42°C without shaking. Sometimes the heat-shock step was done at 37°C for 5 min. This was done when transforming larger products. The tubes were then immediately transferred to ice and incubated for 2 min. 250 µl of room temperature

S.O.C was added, the tubes tightly capped and shaken horizontally for 1 hr at 37°C in a shaker incubator. When the transformed cells were being incubated to express the antibiotic resistance gene, kanamycin plate (for experimental DNA) and ampicilin plate (for control DNA) were placed in an incubator to warm. The plates contained 50 µg/ml kanamycin and 100 µg/ml ampicilin. For the experimental DNA, 50 µl of the transformants was spread on the pre-warmed kanamycin plate. The control transformants was diluted 1:10 and 50 µl of the diluted suspension spread on the prewarmed ampicilin plate. At the time of spreading the transformation reaction, 50 µl of S.O.C or LB was mixed with 50 µl of the bacterial cell suspension for easy spread. It was sometimes necessary to spread the transformation culture in different volumes (50 µl or 150 µl) to have at least a plate where the clones are spread wide a part to easily pick a single clone for inoculation. The plates were then inverted upside down and incubated at 37°C overnight. The following morning, the plates were visualized for any colony growth and removed from the incubator to be stored in the refrigerator until the evening of the same day when they were picked for growth in culture. In instances where there was need to inoculate a starter culture, a single colony was picked and incoculated into 5 ml LB broth containing an appropriate selective antibiotic and the starter culture grown for ~ 8 before making a dilution (usually 1:500) to have an overnight culture.

3.6.4.5 Analyzing Positive Clones

3.6.4.6 Culturing Transformants

The plates were removed from the refrigerator and ~15 colonies picked for an overnight culture in LB medium containing 50ug/ml kanamycin. The choice of the kind of antibiotic to be used for selection of transformants depended entirely on the type of

vector used for cloning the PCR products (insert). It was important to have a plate where the colonies were well distributed to ensure that only single colony was picked without contaminating it with another. A single colony was picked with a clean tooth pick or sterile pipette tip and dipped into a 14-mL conical tube containing 3 mL of LB broth containing an appropriate antibiotic (mostly 50μg/ml kanamycin or 100μg/ml ampicilin) depending on the selective marker of the used cloning vector. This preparation was done aseptically near a Bunsen burner to avoid contamination especially S.O.C and LB medium that did not contain antibiotics. The cultures were grown overnight with a constant shaking at a speed of 240 rpm in a 37°C shaker incubator.

3.6.4.7 Isolation Of Plasmid DNA

The isolation of plasmid DNA was done using QIAprep Spin miniprep kit (CA, USA). The kit has its principle based on alkaline lysis of bacterial cells followed by adsorption of DNA onto a unique silica membrane in the presence of high salt. The procedure consists of three basic steps: preparation and clearing of a bacterial lysate, adsorption of DNA onto the QIAprep membrane and washing. The last step is elution of plasmid DNA in low salt buffer. The unique silica membrane ensures that only DNA is bound and retained on the membrane while RNA, cellular proteins, and metabolites flow through into the collection tube.

1.5 ml of the overnight culture was aliquoted into 1.5 ml ependorff tube and pelleted down for 5 min at room temperature. The pellet was resuspended in 250 μl of cell resuspension buffer and mixed by pipetting up and down. For effective lysis of bacterial cells, it was ensured that there was no cell clumps remained at the resuspension step. 250 μl of the lysis buffer was then added and mixed for several seconds to lyse the cells. The

tube was mixed thoroughly but gently until the solution became viscous and slightly clear. However, lysis should not be let to proceed for more than 5 min because longer lysis reactions would negatively impact on product yield. This was an indication of complete lysis. 350 µl of neutralization buffer was added to the tube mixed immediately and thoroughly by inverting the tube about six times. Addition of neutralization buffer not only neutralizes the lysate, but also adjusts it to high-salt binding conditions in single step. The high salt concentration causes denatured proteins and other cellular debris plus detergents to precipitate, while the smaller plasmid DNA renatures correctly and remains in solution. The tube was then centrifuged for 10 min at 13,000 rpm at room temperature and the supernatant applied into the QIAprep spin column by pipetting. The spin column was spun down and the flow through discarded. The spin column was taken back to the collection tube and 500 µl buffer PB added and the spin column spun down. This step was done to remove endonuclease activity when using bacterial strains that have high levels of endonuclease which can reduce DNA quality. The flow through was discarded and the spin column taken back to the collection tube and washed by adding 750 µl of wash buffer and spun down. The flow through was discarded and the spin column dried by spinning down for 1 min. This additional spinning is essential to ensure removal of residual wash buffer. The spin column was then placed into a sterile 1.5 ml polypropylene microcentrifuge tube and 50 µl of elution buffer added directly to the centre of the membrane, the column incubated for at least 1 min at room temperature. To elute the DNA, the column was spun for 1 min and the plasmid DNA was ready for downstream applications or stored at -20^{o}C for later use. An aliquot was made for spectrophotometric analysis.

To analyze the DNA, 2 µl of the product was prepared for loading on 1% agarose gel as already been described. This was part of the analysis was done to confirm the presence of plasmid DNA in the bacterial culture before proceeding with the other procedures.

3.6.4.8 Restriction Analysis Of Plasmid DNA

To confirm the presence and correct orientation of the insert, plasmid DNA was restricted with KpnI, an endonuclease that linearized the vector by cutting once in it. The enzyme digestion was incubated at 37°C for 4 hr. 1 µl of the enzyme was used while the volume of DNA to be digested varied according to the stock concentration of isolated plasmid DNA. To release the insert and confirm the right orientation, the DNA was digested with EcoRI, an endonuclease that cuts twice in the vector. Again 1 µl of the enzyme was used in the reaction while the volume of the DNA digested varied accordingly. The incubation time was 4 hr at 37°C same as KpnI digestion. It was however, vital to have the nanodrop readings of the DNA products so that the enzymes were not overloaded with too much DNA to digest leading to incomplete digestion. The layout for this experiment is shown in Table 11a and the restriction sites for each endonuclease in Table 11b.

Table 11a: Endonuclease Digestion Requirements

Enzyme Type	***Water***	***DNA***	***Buffer***	***10X BSA***	***Enzyme***	***Total Vol.***
KpnI	x µl	x µl	NEB1 (1.5µl)	1.5 µl	1 µl	15 µl
EcoRI	x ul	x µl	EcoRI (1.5 µl)	-	1µl	15 µl

Table 11b: KpnI and EcoRI recognition sites

Enzyme	*Source*	*Recognition sequence*	*Cut*
KpnI	***Klebsiella***	**5'...GGTACᵛC...3'**	**5'...GGTAC C...3'**
	pneumoniae	**3'...CᐱCATGG...5'**	**3...C CATGG...5'**
EcoRI	***Escherichia coli***	**5'...Gᵛ AATTC...3'**	**5'...GGTAC C ...3'**
		3'...CTTAAᐱG...5'	**3'...C CATGG...5'**

3.6.4.9 Storage Of Bacterial Clone Stocks

Clones that after analysis were found to contain the correct insert were prepared for freezing. To do this, the starter culture (an overnight culture) was diluted 1:500 in 3 ml of LB supplemented with the appropriate antibiotic. The cultures were grown at 37°C with a constant shaking speed of 240 rpm for 3 hr. Bacterial cells are frozen when they are at their log phase of development. During this phase, cell division is at maximum rate and bacteria are most active and healthiest at this stage. 2 hr incubation period is enough for bacteria to reach the log phase. After this growth period in the incubator, an aliquot of 0.5 ml of bacterial culture was mixed with 0.5 ml of sterile 40% glycerol. The mixture was transferred into 1.2 cryotube and frozen at -80°C. This was a useful practice for it saved time and additional work when more of the DNA from a particular clone was needed for subsequent experimentations. Instead of doing another cloning reaction and going through the transformation protocol all over again, a bacterial stock was streaked on a selective plate and colonies picked for culture the following day. Sometimes, a bacterial

stock was inoculated directly into a selective culture medium and grown overnight. This would save a lot of time and resources.

3.6.5 Sequencing

After gel analyses of the plasmid products to size screen for the clones, the seemingly correct constructs were sequenced to confirm the correct orientation of the inserts. Sequencing was done with M13 Forward and M13 Reverse using Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems). The Big Dye was used at a concentration of 1:8. There were two tubes (each for M13Forward and M13Reverse sequencing primers) set for the preparation of a mastermix. Each tube contained 0.5 µl Big Dye (HiDi), 1.75 ul of 5X Big Dye Terminator Sequencing buffer, 1 µl of 10 µM of either M13 Forward or M13 Reverse primer. The volume of DNA depended on the amount of purified DNA estimated and the size of the fragment to be sequenced. The components were diluted in sterile water in a final volume of 10 µl. Once all the components were added they were mixed and aliquoted into PCR tubes or a 96-well plate and appropriate amounts of DNA added. The samples were run on a thermocycler with the following cycling conditions: a 24 cycles of 1°C per sec to 96°C, 30 sec at 96°C, 1°C per sec to 50°C, 15 sec at 50°C, 1°C per sec to 60°C and 4 min at 60°C.

The Big Dye PCR products were ethanol/sodium acetate precipitated in a 96-well plate. In cases where PCR tubes were used, the products were transferred to a 96-well plate for precipitation procedure. A mastermix containing 3 µl of 3 M sodium acetate, 62.5 ul of 95% ethanol, and 24.5 µl of distilled water was prepared depending on the number of samples to be run. 90 µl of the master mix was aliquoted into wells with 10 µl of the PCR products to be precipitated. The plate was sealed with a plate sealer and

incubated at room temperature for 30 min before spinning for 30 min at 3000 x g. The supernatant was removed without disturbing the pellet by placing a paper towel folded to the size of the plate on top and inverting the plate on it. The plate was spun at 50 x g for 1 min and 150 µl of cold (-20°C) of 70% ethanol added, the plate sealed and then spun at 3000 x g for 10 min. The supernatant was removed again without disturbing the pellet as already mentioned. The plate was then covered with paper towel and left to air dry for 30 min. The pellet was resuspended in 10 ul of HiDi and briefly spun before denaturing at 96°C for 3 min. The clones were run on an automated 3031xl Genetic analyzer (Applied Biosystems Sequencing machine). The sequences were identified and named by comparing how they aligned with previously described fragments of the DBLα region of *P. falciparum* 3D7 var genes. The alignments were done through searches of the non-redundant protein and DNA sequence databases at the National Centre for Biotechnology Information (NCBI) and edited using computer software called BioEdit.

3.6.6 Expression Of Constructs Containing DBLα Sequences

3.6.6.1 Construction Of Recombinant Plasmids For Surface Expression In COS-7 Cells

To clone both the sequences encoding DBLα and DBLα-CIDR regions, pRE4 plasmid was used. The plasmid was obtained from Tony Adam's laboratory (University of Notre Dame, Indiana, USA). This plasmid, already described by Cohen *et al.* (1988) has been used for cloning various *P. falciparum* gene domains for expression on mammalian cells (Chitnis and Miller, 1994; Ohas *et al.*, 2004; Semblat *et al.*, 2006). It contains a SV40 origin of replication (SV40 ori), a Rous Sarcoma virus long terminal

repeat (RSV LTR) as a promoter and the SV40 early polyadenylation signal (SV40 polyA). The plasmid contains a herpes simplex virus glycoprotein D1 (HSV gD1), which is cloned in the HindIII cloning site located downstream of the RSV LTR. The HSV gD1 has a signal sequence peptide at the NH2-terminal end and a transmembrane stretch with a cytoplasmic tail at the COOH-terminal end. HSV gD1 has unique ApaI and PvuII restriction sites. The signal sequence peptide and hydrophobic transmembrane region of HSV gD1 has been used to target various regions of malaria gene and/or proteins to the surface of mammalian cells, such as COS-7 cells.

To prepare the pRE4 plasmid for cloning the DBLα domains, the vector was restricted with ApaI and PvuII to cleave the central region of HSV gD1. This digestion cleaved the vector into two fragments. The restriction fragments were separated by gel electrophoresis. The vector was recovered using QIAGEN gel purification kit (QIAGEN, Valencia, CA) following manufacturer's protocol. DNA sequences encoding different regions of PfEMP1 protein were cloned in the vector using PvuII and ApaI sites to generate the following constructs: HSV gD1-DBL1α, HSV gD1-DBLα-CIDRα and HSV gD1-CIDR.

3.6.6.2 HSV gD1-DBLα Constructs

This construct contained sequences encoding DBLα domain of PfEMP1. PCR was used to amplify sequences encoding this region. To generate this construct, DBL1αSA075FPvuII 5'-TCTCGT**CAGCTG**GCAACTGCTAGTGGGGAAAGAGCTAGCACCACAGAT-3' was used as forward primer and DBL1αSA075RApaI 5'-

ACGAGT**GGGCCC**GGTTAAAAAACCTTGCACTGTTTCATAGTCATT-3' as reverse primer. The PvuII and ApaI sites are in bold. To confirm surface expression of this construct, another construct was generated that contained FLAG peptide tag. To prepare this construct, the oligonucleotide DBL1αSA075FPvuFL 5'-TCTCGT**CAGCTG**GACTACAAAGACGATGACGATAAGGCAACTGCT-3' was used as forward primer and DBL1αSA075RApa as the reverse primer. The cycling conditions were a 2-min denaturation step followed by 34 cycles of 20 sec at 94°C, 30 sec at 65°C, 1.20 min at 68°C and a final elongation step of 7 min at 68°C. The PCR products were restricted with PvuII and ApaI at 37°C overnight. The restriction fragments were purified using QIAGEN PCR purification kit (QIAGEN, Valencia, CA) following the procedures as outlined by the manufacturer. The PCR products were ligated with the pRE4 vector. The vector contains HSV gD1 whose ORF has unique PvuII and ApaI restriction sites where DBLα or DBLα-CIDR domain was cloned as an HSV gD1 chimera.

To improve expression levels and to use green fluorescent protein (GFP) as a marker of transfected cells, the plasmid pEGFP-NI (BD Biosciences Clontech, Palo Alto, CA) was used. Plasmid pEGFP-N1 (Clontech) encodes a red-shifted variant of the wild-type green fluorescent protein (GFP) transcribed by the immediate-early promoter of human CMV (Prasher *et al.*, 1992; Chalfie *et al.*, 1994). This plasmid allows expression of a recombinant fusion protein to the N-terminus of the EGFP used as a transfection marker.

The vector was obtained in the form of a chimeric protein, pEGFP-N1-EBA175RII/HSV gD1 from Tony Adam's laboratory. The vector had been used by the group to clone and express region II of EBA175 (Ohas *et al.*, 2004). The antigen was

inserted into this vector at the ApaI and PvuII unique sites. The EBA175RII was exchanged with DBL1α domains using HindIII and ApaI restriction sites. The HSV gD1-DBLα chimeric protein construct described above was restricted with HindIII and ApaI and cloned into HindIII and ApaI sites of the pEGFP-NI-EBA175rII-HSVg D1. The ligation was done at 16°C overnight in the presence of 1μl of T4 ligase.

The final construct were designated pEGFPN1-HSV gD1-DBL1α or pEGFPN1-HSV gD1-DBL1α-FLAG and were purified by QIAquick Miniprep Kit (QIAGEN, Valencia, CA) that produced endotoxin-free plasmid preparations. These are the constructs which were used in transient transfection experiments.

3.6.6.3 HSV gD1-DBLα-CIDRα Constructs

This construct contained sequences encoding DBLα-CIDR1α domains (the semi-conserved head structure) of PfEMP1. PCR was used to amplify sequences encoding this region. The construct was generated by PCR amplification from cDNA of SA075R+ using a forward primer containing with a PvuII site, DBL1αSA075FPvu: 5'-TCTCGT**CAGCTG**GCAACTGCTAGTGGGGAAAGAGCTAGCACCACAGAT-3'. The reverse primer was CIDR1SA075RApa with an ApaI site appended to it. 5'-ACGAGT**GGGCCC**TGATGAATCACGATGATCATTGGAATGACTGGTACT-3'. The PvuII and ApaI restriction sites in the forward and reverse primers, respectively, are in bold. The cycling conditions were a 2-min denaturation step followed by 34cycles of 20 sec at 94°C, 30 sec at 68°C, 2.20 min at 68°C and a final elongation step of 7 min at 68°C. The same procedure followed to prepare construct containing DBL1α domain sequences was used to prepare DBL1α-CIDR1α domain sequences. Again there was another construct prepared using a forward primer encoding a FLAG peptide sequence in

phase with the start codon as shown above. The PCR cycling conditions were similar with the non-FLAG construct. This construct was generated to verify surface expression of the final constructs on COS7 cells. The final constructs were as designated pEGFPN1-HSV gD1-DBL1α-CIDR or pEGFPN1-HSV gD1-DBL1α-CIDR-FLAG.

3.6.6.4 HSV gD1-CIDR Construct

This construct was generated to act as negative control in the RBC binding assays. Studies using constructs containing CIDR domain of PfEMP1 have shown that this region does not bind to RBCs *in vitro* (Rowe *et al.*, 1997). This construct was also generated by PCR amplification using cDNA of SA075R+ with forward primer: CIDRSA07FPvu: 5'- and reverse primer CIDRSA075RApa: 5'-ACGAGTGGGCCCTGATGAATCACGATGATCATTGGAATGACTGGTACT-3'. The cycling conditions were a 2-min denaturation step followed by 34cycles of 20 sec at 94°C, 30 sec at 68°C, 1.20 min at 68°C and a final elongation step of 7 min at 68°C. The rest of the procedures were similar with those for preparing other constructs described earlier in the methods. The final construct of a chimeric protein was named pEGFPN1-HSV gD1-CIDR.

3.6.7 Cell Line Culture, Transfection Experiments And Immunofluoresence Assays

3.6.7.1 Cell Line Culture

The cell line for transfection experiments was COS7 cells which are the African Green Monkey Kidney cells. This mammalian cell line has been used in both transient and stable transfection experiments in several studies. The cells grow as a single thickness cell layer (monolayer). The cells were cultured in Dulbeco's modified eagles'

medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (penstrep). To prepare complete culture medium, DMEM medium was brought into the biological safety cabinet and appropriate volume of thawed fetal calf serum added. The media was filtered, labeled and stored at 4°C. The culture medium was prepared under aseptic conditions to avoid contamination with bacteria or yeast that would compete with mammalian cells for nutrients and/or cause cell infection and cell death. One vial of COS-7 cells was thawed out in a 37°C water bath and transferred to a sterile 15 ml conical tube containing 10 ml of DMEM medium supplemented with penstrep. The sample was spun down at 2700 rpm for 5 min and supernatant discarded. The pellet was then resuspended in 10 ml DMEM and spun again at 2700 rpm for 5 min. The supernatant removed and the resulting cell pellet resuspended in 4 ml of complete cell line culture medium. The cell suspension was divided into two halves and transferred to two T25 culture flasks. The volumes were topped up to 5 ml with complete cell line culture medium. The caps were loosened and the flasks placed in a CO_2 incubator at 37°C, 5% CO_2 and 90% relative humidity. The high humidity was meant to cut down evaporation loses in unsealed culture flasks which would make the culture to become hypertonic thus stressing the cells to death. The cells were observed routinely to examine their status and health in an inverted microscope. This regular examination of cells ensured that the cells were keenly monitored for any form of contamination by micro-organisms and for the maintenance of healthy cells in culture. Generally, dead cells round up and become detached and are usually not refractile. During these regular monitoring of cultures, the culture medium was changed by aspirating the previous culture medium and adding fresh one. Since COS-7 cells are

adherent cells, the healthy cells would remain attached to the flask. The purpose of media changes was to replenish nutrients and avoid the build up of potentially harmful metabolic byproducts and dead cells. When the cultures were ~70% confluent, the cells were passaged. It is known that many cell cultures perform well if they are subcultured before they have reached confluency. Splitting was done to keep the cells in the active log phase of growth.

Passaging or splitting cells involved transferring a small number of cells into a new culture flask. Cells could be cultured for a longer time if they are split regularly, as this practice would avoid the senescence associated with prolonged high cell density. The cells were first detached by removing the culture medium and rinsing the cell sheet with DMEM medium. Appropriate amounts of dissociation medium (mixture of trypsin-EDTA) were added and the enzyme treatment incubated for 5 min at room temperature. The progress of enzyme treatment was monitored by observing the flasks under an inverted microscope. This treatment should not be elaborate for EDTA is toxic to the cells and trypsin can strip the cells of their necessary attaching proteins and may fail to re-attach. When the cells were all detached, the starter culture was diluted 1:2, 1:4 or 1:8 depending on when the cells were required for use. In some times the cells were diluted 1:1. This dilution meant that, a small amount of culture containing a few cells was diluted in a larger volume of fresh complete cell line culture media. The seeding would be done either in T25 or T75 culture flask. Again this would depend on the number of cells needed for the transfection experiment. If a lot of cells were required, then a small number of detached cells could then be used to seed a new T75 culture and vice versa. When cells were ready for harvesting, they were subjected to enzyme treatment to detach

them as already been described. A small portion (10 µl), of the detached cells was mixed gently with trypan blue and carefully loaded onto a clean haemocytometer for counting. This was done in cases where it was necessary to set up a culture at known concentrations.

3.6.8 Transfection Experiments

3.6.8.1 Preparation of DNA For Transfection

The DNA for transfection was prepared in relatively large quantities to have enough for all the experiments without having to prepare more quite often. This was essential for consistency purposes and the need to use DNA from same clone for transfection assays. Enough quantities were prepared from bacterial stock which contained the correct insert and occurring in the right orientation. This was established by enzyme digestions and confirmed by sequencing.

3.6.8.2 Transfection Assay

Transfection is a procedure by which experimental DNA (cloned DNA) is introduced into a cultured mammalian cell. It typically involves opening transient pores in the cell plasma membrane, to allow the uptake of a genetic material. Since the cloned DNA may have been extensively modified, transfection is often used to test whether a particular modification affects the function of a gene. A very efficient method is the inclusion of the DNA to be transfected in liposomes. Lipososmes are small, membrane-bounded bodies that are in some ways similar to the structure of a cell and can actually fuse with the cell membrane, releasing the DNA into the cell. Transfection can either be transient or stable.

Transient transfection is the procedure by which DNA is introduced into eukaryotic cell, retained there and expressed temporarily. The procedure provides an opportunity to identify genetic elements that control gene expression in malaria parasites. The procedure described here is that for transient transfection.

Before the actual day of transfection, COS7 cells were plated so they would be ~50 to ~70% confluent the next day. They were plated in medium without antibiotics for it has been reported that transfection efficiency is reduced in some cell lines when they are grown in antibiotics. The transfection was done using FuGENE® 6 Transfection Reagent (Roche) following the manufacturer's protocol. This is a lipid-cation based transfection method which involves mixing of a cationic lipid with the DNA to be transfected to produce liposomes, which then fuse with the cell plasma membrane and deposit the DNA into the COS7 cells.

It was first ensured that DNA to be used in transfection was of high quality with a 260/280 ratio of 1.8. The exact DNA concentration was also vital so that known amounts of DNA would be used to transfect COS-7 cells. FuGENE® 6 was removed from the refrigerator and allowed to warm to room temperature. During this time, the cells were removed from the incubator and observed microscopically to ensure that they looked healthy and were 50-70% confluent. Also brought to room temperature was serum-free DMEM (no additives). This was used to dilute FuGENE® 6 Transfection reagent. 94 µl to 100 µl of serum-free DMEM were aliquoted into 6 sterile eppendorf tubes. The number of tubes depended on the number of constructs to be transfected. The volumes depended on the amounts of FuGENE® 6 Transfection reagents added into the tube as shown in Table12. It was ensured that FuGENE® 6 Transfection reagent was added directly into

the serum-free medium avoiding any contact of the FuGENE reagent with the walls of the tube containing the serum-free medium. The tube was immediately flicked to mix and incubated at room temperature for 5 min. To prepare the complex (DNA-FuGENE complex), an appropriate amount of DNA depending on the starting concentration of DNA (final DNA mass of 1 µg) was added into each tube and mixed by tapping the tubes for 1 sec. The tubes were then incubated at room temperature for at least 15 min. Generally, the complex was incubated for 30 min. 100 µl of the complex was then added drop-wise to the cells into the appropriate well as outlined in *Table 12*. The plate was swirled to mix the complex in all the wells. The cells were incubated at CO_2 incubator at 37°C until the time of assay.

Table 12: Transfection layout of a 6-well plate

(A) Cell Control *No reagent* *No DNA*	*(B) Reagent Control* *6 µl reagent* *No DNA*	*(C) DNA Control* *No reagent* *1 µg DNA*
(D) 3:1 ratio **3 µl reagent** **1 µg DNA**	(E) 3:2 ratio 3 µl reagent 2 µg DNA	(F) 6:1 ratio 6 µl reagent 1 µg DNA

3.6.8.3 Preparation Of Expression Constructs

SA075R+DBLα sequence tag constructs were expressed on the surface of COS-7 cells by cloning it into plasmid pEGFP-N1 (Clontech) as a chimeric GFP fusion protein

with the herpes simplex virus (HSV) glycoprotein D1 signal sequence and transmembrane domain followed by the GFP open reading frame at the C-terminus (Rowe *et al.*, 1997; Ohas *et al.*, 2004). This construct has previously been used to express region II of erythrocyte-binding antigen 175 on the surface of COS-7 cells (Ohas *et al.*, 2004). COS-7 cells were transfected with 1 μg of pEGFP-SA075R+DBLα plasmid DNA or pEGFP alone (negative control) and transfection efficiency assessed by fluorescence microscopy 48-60 hr post transfection.

3.6.8.4 Immunological methods

3.6.8.4a Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) And Immunoblots

The immunological techniques were used to confirm and/or detect surface expression of DBL1α constructs on COS7 cells. The transfected cells were washed twice with plain PBS, each wash lasting 5 min. The cells were detached by incubating with 1 mL of PBS supplemented with 5mM EDTA per well of a 6-well plate. The cells were spun at 5000 rpm for 5 min at 4°C. The resulting pellet was lysed by resuspending on 50 μl lysis buffer (50mM Tris HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% TRITON® X-100, 1X protease inhibitor cocktail) and pipetting up and down 30 times before incubating on ice for 30 min. The lysate was spun at maximum speed (14,000 rpm) for 30 min at 4°C. The supernatant was carefully transferred into clean 1.5 mL tube and stored at -20°C for later use or immediately prepared for SDS-PAGE. 9 μl of the lysate was aliquoted into a clean 0.2 mL tube and mixed with 3 μl of 4X sample buffer before heating at 70°C for 10 min. The tubes were briefly spun to collect the drops into the tubes and samples loaded into 12% resolving polyacrylamide gel layered with a 4 % stacking gel. The proteins were

electrophoresed in 1X running buffer at 100V for 1 hr. The separated proteins were tranfered onto a nitrocellulose membrane or polyvinylidene difluoride (PVDF) at 30V for 1 hr in transfer buffer.

The transferred proteins on the nitrocellulose membranes were probed for the fused constructs using either anti-FLAG-hrp or anti-GFP-hrp antibodies. The blots were blocked with 1X casein overnight at 4°C. The blocking agent was removed by washing once with Tris Buffered Saline (TBS) pH 8.0, with 0.05% Tween-20. Each wash lasted 5 min. The blots were incubated with anti-GFP-hrp diluted in wash buffer at 1:1000 and anti-FLAG-hrp diluted 1:500 in the wash buffer for 1 hr at RT with constant agitation. Antibody solution was decanted off and the blots washed 5 times each wash lasting 5 min. The blots were developed using Chemiluminescent Peroxidase Substrate by incubating with the substrate for 5 min in the dark. The blots were drained briefly and wrapped in plastic film before exposing for a range of times from several seconds to 3 min. The films were developed, fixed in the dark and washed before drying. The films were read for analysis.

3.6.8.4b Immunofluorescence Assay

COS7 cells were grown and transfected on coverslips. 48 hr after transfection, the cells were washed to remove dead cells with plain PBS. The cells were fixed for 15 min at RT by incubating with PBS containing 4 % paraformaldehyde and 4% sucrose. Fixed cells were washed with PBS with each wash lasting 5 min at RT and blocked with 10% bovine serum albumin (BSA) in PBS for 30 min at 37°C. The blocking agent was decanted and the cells incubated with primary antibody, mAb anti-FLAG M2 antibody diluted 1:500 in 3% BSA/PBS for 2 hr at 37°C. The plates were washed three times with

PBS for 5 min (to wash off the primary antibody) and incubated with the secondary antibody, Texas Red diluted 1:50 in 3% BSA/PBS for 45 min at 37°C. The secondary antibody was washed off three times with PBS with each wash lasting 5 min. The coverslips were mounted with cells side down on glass slides using a small drop of mounting medium and sealed with molten agarose before examining by fluorescence microscopy.

3.6.8.4c Surface Immunofluorescence Assay And Flow Cytometry

Transfected COS7 cells on a 6-well culture plate were washed with plain PBS and detached with PBS/5mM EDTA. The detached cells were transferred into a 15 ml conical tube and washed twice by spinning at 2500 rpm at 4°C for 5 min. The cells were resuspended in 1 ml plain PBS and an aliquot diluted 1:20 for counting on a haemocytometer. A suspension of 200,000 cells was made and transferred to 1.5 ml eppendorf tube and pelleted down at 2500 rpm for 5 min at 4°C. The supernatant was aspirated and the cells resuspended in 200 µl of PBS/1% BSA and blocked for 30 min at RT. The tubes were spun down and incubated with hyperimmune serum or control (Nonimmune) serum diluted 1:12 in PBS/0.5% BSA for 30 min at RT. After this incubation, the cells were washed thrice before incubating with the secondary antibody, mouse anti-human IgG, Spectral Red® (SPRD) conjugate (PC5) diluted 1:500 at RT for 30 min with constant mixing. The secondary antibody was washed off by adding 200 µl of wash buffer (PBS/0.5% BSA) and spinning at 2500 rpm for 5 min at RT. This wash was repeated twice and the cells fixed in 100 µl of fixative (2% formaldehyde). The cells

CHAPTER 4 RESULTS

4.1 CR1 Polymorphisms Are Associated With Severe Malaria

4.1.1 Group Demographics

Table 13 describes the total number of cases and controls per group and hospital. There were no significant differences in the age, gender or ethnic group distribution between cases and controls. Cases and controls recruited from the NPGH were predominantly of the Luo ethnic group whereas most of the cases and controls recruited from the KDH were of the Abagusii ethnic group.

Table 13: Characteristics of the study participants

	Nyanza Provincial General Hospital N = 320				Kisii District Hospital N = 140	
	Severe anaemia		Cerebral malaria		Cerebral malaria	
	Cases N = 137	Controls N = 137	Cases N = 23	Controls N = 23	Cases N = 70	Controls N = 70
Age in months (SD)	14.8(12.6)	14.5(12.2)	29.0(15.7)	30.1(15.7)	28.5(15.9)	29.0(15.8)
No. of females (%)	57(41.6)	57(41.6)	9(39.1)	9(39.1)	37(52.9)	37(52.9)
Ethnic groups (%)						
Luo	114(83.2)	115(83.9)	22(95.7)	19(82.6)	3(4.3)	4(5.7)
Abagusii	1(0.7)	0	1(4.3)	4 (17.4)	66(94.3)	65(92.9)
Luhya	15(10.9)	19(13.9)	0	0	1(1.4)	1(1.4)
Other	7(5.1)	3(2.2)	0	0	0	0

Other: Kalenjin (3), Kikuyu (3), Turkana (1), Teso (1), Nandi (1), Bukusu (1)

4.1.2 *Sl2* and *McC^b* African Knops blood group antigen alleles

The frequencies of African alleles (*Sl2* and *McC^b*) from this study show that whereas these alleles are rare in non-African populations, they occur more frequently in populations of African descent (*Figure 5*) and were comparable with allelic frequencies from other African countries (*Table 14a*)

Figure 5: Swain Langley and McCoy genotypes in study population

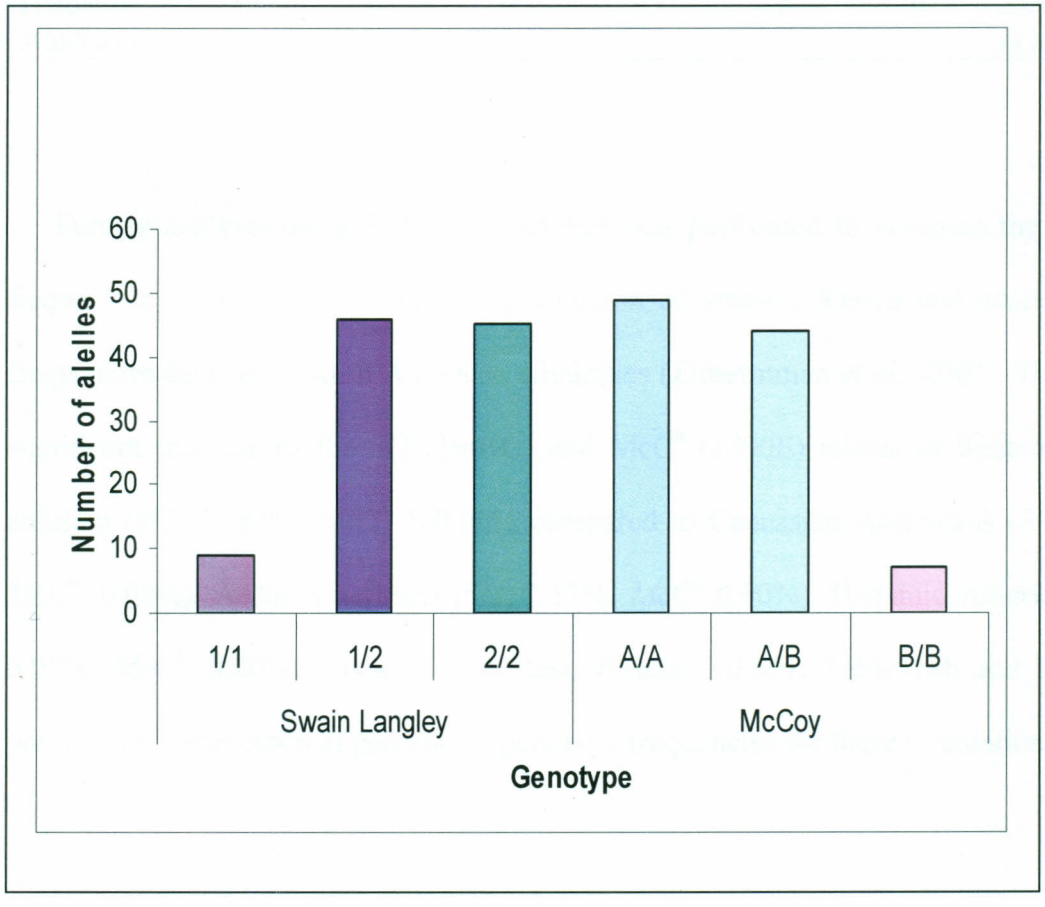


Table 14a: Overall prevalence (%) Knops blood group antigen polymorphisms in various populations

Populations	Swain-Langley			McCoy			Reference
	1/1	1/2	2/2	a/a	a/b	b/b	
Western Kenya	**9**	**46**	**45**	**49**	**44**	**7**	Thathy *et al.*, 2005
The Gambia	5	31	65	38	47	15	Zimmerman *et al.*, 2003
Mali	9	30	60	49	40	10	Moulds *et al.*, 2001
Caucasian Americans	99	1	0	100	0	0	Zimmerman *et al.*, 2003
Asian Americans	95	4	1	96	4	0	Zimmerman *et al.*, 2003
Hispanic Americans	94	6	0	95	5	0	Zimmerman *et al.*, 2003

Further analysis using Fisher's exact test was performed to compare the genotype frequencies obtained in this studied population of western Kenya and available gene frequencies data from North American ethnicities (Zimermman *et al.*, 2003). There was a significant increase of the *Sl2* (1601G) and McC^b (1590E) alleles in western Kenyan children (*Sl2*, 97.83%, McC^b 100.00% compared to Caucasian Americans (*Sl2*, 0.00%, McC^b, 0.00%), Asian Americans (*Sl2*, 2.17%, McC^b 0.00%), Hispanic Americans (*Sl2*, 0.00%, McC^b, 0.00%). Fisher's exact test, *P-value* <0.001. Table 14b and Table 14c provide the comparison of percentage genotype frequencies for these populations.

Table 14b: Comparing *McC* genotype frequencies: western Kenya vs other ethnicities

	McCoy (K1690E)			
Geographic group	***K/K***	***K/E***	***E/E***	Total
Western Kenya	**49**	**44**	**7**	**100**
Asian Americans	96	4	0	100
Caucassian Americnas	100	0	0	100
Hispanic Americans	95	5	0	100
Total	340	53	7	400

Fisher's exact test *P*-value < 0.001
Single letter amino acid sequence nomenclature used to identify CR1 CCP25 alleles (K=lysine, E=glutamic acid)

Table 14c: Comparing *Sl* genotype frequencies: western Kenya vs other ethnicities

Same comments as above

	Swain Langley (R1601G)			
Geographic group	***R/R***	***R/G***	***G/G***	Total
Western Kenya	**9**	**46**	**45**	**100**
Asian Americans	94	4	1	100
Caucassian Americnas	99	1	0	100
Hispanic Americans	94	6	0	100
Total	297	57	46	400

Fisher's exact test, *P*-value < 0.001
Single letter amino acid sequence nomenclature used to identify CR1 CCP25 alleles (R=arginine, G=glycine)

4.1.3. *SI2/2* Genotype And Association With Cerebral Malaria

In an attempt to explore whether *Sl2* or MC^b alleles confer resistance or susceptibility to severe malaria compared to *Sl1* and MC^a alleles, conditional logistic regression of the individual genotypes at each locus was performed using *Sl1* and MC^a as reference alleles. The results for cerebral malaria cases and their controls are tabulated in Table 15. The results showed that individuals with *Sl2* were less likely to develop cerebral malaria than their Sl1 counterparts (OR = 0.17, 95% CI 0.04 to 0.72, $P = 0.02$).

Table 15: Conditional logistic regression based on matching to compare individual genotypes and cerebral malaria using *SI1/1* and $McC^{a/a}$ as reference

Genotype	No. of CM cases (%)	No. of controls (%)	OR	95% CI	*P value*
Sl1/1	17(18)	10(11)	Ref	-	-
Sl1/2	44(47)	40(43)	0.49	0.12 to 2.05	0.32
Sl2/2	32(34)	43(46)	0.17	0.04 to 0.72	0.02
$McC^{a/a}$	51(55)	49(53)	Ref	-	-
$McC^{a/b}$	37(40)	40(43)	1.39	0.55 to 3.52	0.49
$McC^{b/b}$	5(5)	4(4)	3.52	0.43 to 29.06	0.24

OR = Odds Ratio, CI = Confidence Interval, Ref = Reference Category

4.1.4 *SI2/2* Genotype And Resistance To Severe Malarial Anaemia

In order to test the association between the individual CR1 genotypes and severe malarial anaemia, a conditional logistic regression was performed using *Sl1/1* and $McC^{a/a}$ as the reference genotypes (Table 16). The results showed no significant association between any of the individual genotypes (*Sl1/2* or *Sl2/2*) and either susceptibility or resistance to severe malarial naemia. However, there was a trend towards protection

against severe malarial anaemia among children with *Sl2/2* genotype. This association was not significant (OR = 0.65, 95% CI 0.14 to 3.06, $P = 0.59$).

Table 16: Conditional logistic regression based on matching to compare individual genotypes and severe malarial anaemia using *Sl1/1* and $McC^{a/a}$ as reference alleles

Genotype	No. of SA cases (%)	No. of controls (%)	OR	95% CI	*P value*
Sl1/1	11(8)	10(7)	Ref	-	-
Sl1/2	63(46)	57(42)	1.08	0.24 to 4.93	0.92
Sl2/2	63(46)	70(51)	0.65	0.14 to 3.06	0.59
$McC^{a/a}$	61(45)	61(45)	Ref	-	-
$McC^{a/b}$	65(47)	66(48)	0.93	0.41 to 2.11	0.86
$McC^{b/b}$	11(8)	10(7)	1.38	0.29 to 6.57	0.68

OR = Odds Ratio, CI = Confidence Interval, Ref = Reference Category

4.1.5 *SI2/2* genotype And Association with resistance to severe malaria as a whole

After performing statistical analysis to establish the relationship between individual genotypes and either severe malaria anaemia or cerebral malaria cases, the same analysis were done with all cases combined. That is, CM and SMA cases were combined and a conditional logistic regression analysis performed with individual genotypes combination, again taking *Sl1/1* and $McC^{a/a}$ as the reference genotypes. The result for this analysis showed a much stronger association between children with *Sl2/2* genotype and protection against severe malaria in general, (OR = 0.3, 95% CI 0.1 to 0.7, $P = 0.01$. However, there was no an association between $McC^{a/b}$ or $McC^{b/b}$ and severe malaria (Table 17).

Table 17: Conditional logistic regression based on matching to compare individual genotypes and severe malaria using *SI1/1* and $McC^{a/a}$ as reference

Genotype	No. of total cases (SA & CM) (%)	No. of Controls (%)	OR	95% CI	*P*
SI1/1	26 (12.0)	19 (8.8)	Ref.	-	-
SI1/2	102 (47.2)	90 (41.7)	0.6	0.2-1.7	0.34
SI2/2	88 (40.7)	107 (49.5)	0.3	0.1-0.7	0.01*
$McC^{a/a}$	106 (49.1)	105 (48.6)	Ref.	-	-
$McC^{a/b}$	95 (44.0)	97 (44.9)	1.34	0.7-2.3	0.41
$McC^{b/b}$	15 (6.9)	14 (6.5)	2.1	0.6-7.1	0.21

OR = Odds Ratio, CI = Confidence Interval, Ref = Reference Category

4.1.6 SI2/2 $Mcc^{a/b}$ Genotype And Associated With Decreased Susceptibility To CM

In an attempt to determine the effect of the different allele combinations at the Swain-Langley and McCoy loci on susceptibility to severe malaria, a conditional logistic regression was carried out using the *Sl1/1* $McC^{a/a}$ genotype as reference allelic combination. First, conditional logistic regression was done with cerebral malaria separately (Table 18). The results for this analysis showed that individuals with *Sl2/2* $McC^{a/b}$ genotype had increased resistance to CM (OR = 0.18, 95% CI 0.04 to 0.77, *P* = 0.02).

There were only six of the ten possible *Sl/McC* genotype combinations found.

Table 18: Conditional logistic regression based on matching to compare individual genotype combinations and cerebral malaria using *Sl1/1 McC$^{a/a}$* as reference

Genotype	No. of CM cases (%)	No. of controls (%)	OR	95% CI	*P*
Sl1/1McC$^{a/a}$	17(18)	10(11)	Ref	-	-
Sl1/2 McC$^{a/a}$	23(25)	25(27)	0.4	0.09 to 1.77	0.23
Sl2/2McC$^{a/a}$	11(12)	14(15)	0.24	0.05 to 1.19	0.08
Sl2/2McC$^{a/b}$	16(17)	25(27)	0.18	0.04 to 0.77	0.02*
Sl1/2McC$^{a/b}$	21(23)	15(16)	0.93	0.19 to 4.60	0.93
Sl2/2McC$^{b/b}$	5(5)	4(4)	0.65	0.07 to 0.07	0.71

OR = Odds Ratio, CI = Confidence interval, Ref = Reference Category * significance

4.1.7 *SI2/2 Mcc$^{a/b}$* Genotype Combination And Association With Resistance To Severe Malarial Anaemia

When a conditional regression analysis was carried out to determine the effects of the different allelic combinations on severe malarial anaemia, the outcome was different from the one seen in cerebral malaria cases. There was no association between *Sl2/2 McC$^{a/b}$* genotype and SMA (OR = 0.53, 95% CI 0.12 to 2.34, *P* = 0.40) (Table 19).

Table 19: Conditional logistic regression based on matching to compare individual genotype combinations and severe anaemia using *SI1/1 McC$^{a/a}$* as reference alleles

Genotype	No. of SA cases (%)	No. of controls (%)	OR	95% CI	*P*
Sl1/1 McC$^{a/a}$	11(8)	10(7)	Ref	-	-
Sl1/2 McC$^{a/a}$	33(24)	34(25)	0.93	0.2 to 4.37	0.93
Sl2/2 McC$^{a/a}$	17(12)	17(12)	0.86	0.17 to 4.43	0.86
Sl2/2 McC$^{a/b}$	35(26)	43(31)	0.53	0.12 to 2.34	0.40
Sl1/2 McC$^{a/b}$	30(22)	23(17)	0.19	0.26 to 5.4	0.82
Sl2/2 McC$^{b/b}$	11(8)	10(7)	0.87	0.15 to 5.14	0.88

OR = Odds Ratio, CI = Confidence Interval, Ref = Reference Category

4.1.8 *SI2/2 Mcc$^{a/b}$* Genotype Combination And Association With Resistance To Severe Malaria In General

Having performed regression analyses to establish association between different allele combinations and either SMA or CM, the same analyses were done, this time combining all severe malaria cases. There was an increased resistance to severe forms of malaria (CM and SMA) among children harbouring *SI2/2 McC$^{a/b}$* genotype (OR = 0.2, 95% CI 0.1 to 0.7, $P = 0.01$). The results are shown in Table 20.

Table 20: Conditional logistic regression based on matching to compare individual genotype combinations and severe malaria as a whole using *Sl1/1 McC$^{a/a}$* as reference

Genotype	No. of total cases (SA&CM)(%)	No. of Controls (%)	OR	95% CI	*P*
Sl1/1McC$^{a/a}$	26(12.0)	19(8.8)	Ref	-	-
Sl1/2McC$^{a/a}$	54(25.0)	56(25.9)	0.5	0.2 to 1.4	0.20
Sl1/2McC$^{a/b}$	48(22.2)	34(15.7)	1.0	0.3 to 3.0	0.98
Sl2/2McC$^{a/a}$	26(12.0)	30(13.9)	0.4	0.1 to 1.1	0.09
Sl2/2McC$^{a/b}$	47(21.8)	63(29.2)	0.3	0.1 to 0.8	0.01*
Sl2/2McC$^{b/b}$	15(6.9)	14(6.5)	0.6	0.1 to 2.1	0.40

OR = Odds Ratio, CI = Confidence interval, Ref = Reference Category, * significance

4.1.9 The distribution of Swain Langley genotype and relationship with age

To better understand how age matching may affect the association between *Sl2/2* and severe malaria, the data set was divided into three age groups <1 yr, > 1 yr ≤ 2 yr and > 2 yr. All the cases and their controls were combined and the results summarized *in Figure 6*. When a 3x3 Chi-squared test analysis was performed on the data set, the results showed a decline in Sl2/2 with age (*seeTable 21a*). However, the similar proportion of pattern was not observed when the data from each of the cases (CM and SMA) were analyzed separately (data not shown. Although there was a trend towards decline in the frequency of *Sl2/2* with age in cerebral malaria cases and their control, this was not statistically significant (*P*= 0.08). This is shown in *Table 21b.*

Table 21a: Chi-square test analysis to determine changes in the distribution of Swain Langley genotypes with age

Age group	Cerebral malaria and Severe malarial anaemia cases and controls combined			
	SI1/1	*SI1/2*	*SI2/2*	***P* value**
NO. ≤1yr (%)	13 (8)	64 (42)	76 (50)	**0.02**
NO. >1 to ≤2yr (%)	5 (4)	51 (45)	58 (51)	
NO. >2yr	17 (14)	64 (51)	44 (35)	

Figure 6: Changes in the distribution of Swain Langley genotype with age

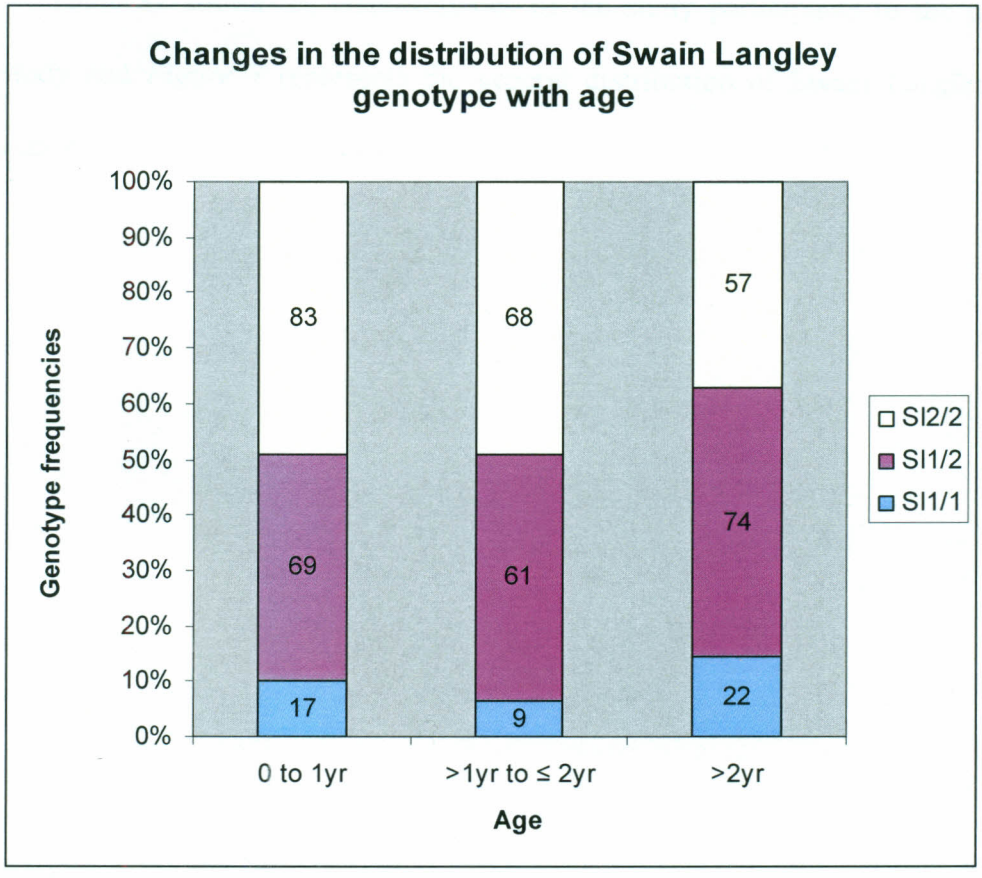


Table 21b: Chi-Square Test Of Sl2/2 And Age Group

Age group	*Severe anaemia cases and controls*				*Cerebral malaria cases and controls*			
	Sl1/1	Sl1/2	Sl2/2	*P value*	Sl1/1	Sl1/2	Sl2/2	*P value*
<1yr	9 (7)	57 (43)	67 (50)	0.25	4 (20)	7 (35)	9 (45)	0.08
>1-≤2yr	2 (3)	30 (47)	32 (50)		3 (6)	21 (42)	26 (52)	
>2yr	3 (8)	16 (46)	16 (46)		14 (16)	48 (53)	28 (31)	

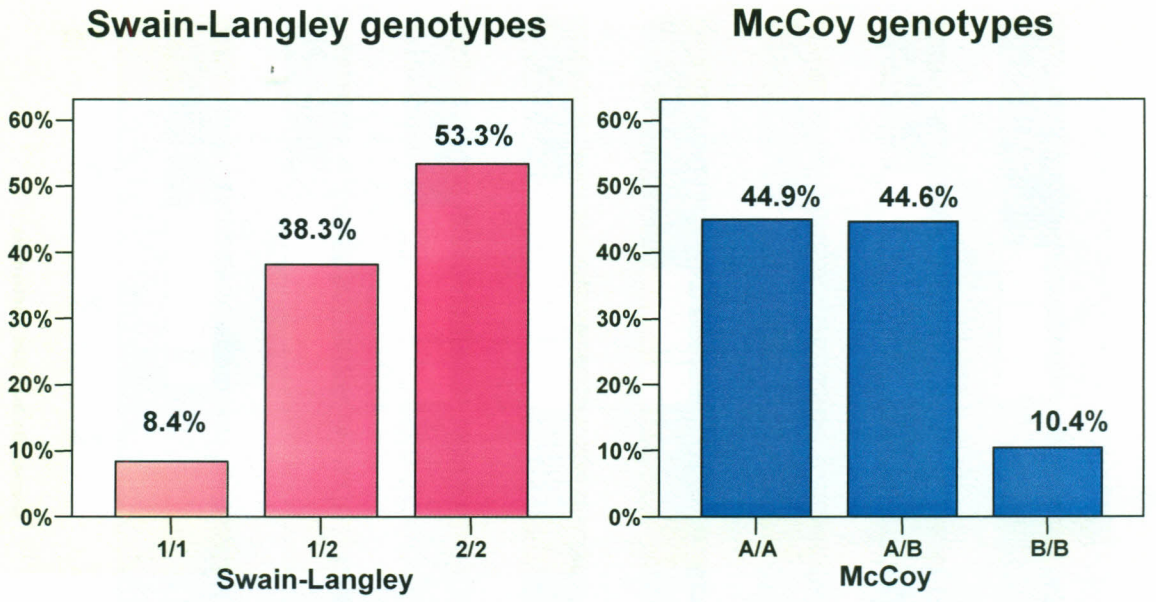
4.2 Age-Related Changes In The Prevalence Of Swain Langley And Mccoy Genotypes

Table 22 shows the characteristics of the study participants in the cross sectional study and *Figure 7* represents the general distribution of Swain Langley and McCoy genotypes in the study population.

Table 22: Group demographics of the Cross-sectional study participants

	Age Group (Months)							
	>0-6	>6-12	>12-24	>24-48	>48-96	>96-192	>192-384	>384
N	30	60	61	59	32	31	44	28
Mean age in months (SD)	3.0 (1.5)	9.1 (1.8)	17.5 (3.7)	34.4 (6.4)	70.5 (12.9)	128.8 (28.4)	305.4 (45.4)	453.4 (44.7)
No. of females (%)	17 (56.7)	29 (48.3)	27 (44.3)	29 (49.2)	14 (43.8)	13 (41.9)	25 56.8)	16 (57.1)
Ethnic groups (%)								
Luo	28 (93.3)	59 (98.3)	60 (98.4)	59 (100.0)	32 (100.0)	31 (100.0)	43 (97.7)	28 (100.0)
Luhya	1 (3.3)	1 (1.7)	1 (1.6)	0	0	0	0	0
Other	1 (3.3)	0	0	0	0	0	1 (2.3)	0

Figure 7: Percent of Swain Langley and McCoy genotypes in study population (N = 345)



4.2.1 Age-Related Frequencies Of *Sl2/2* Genotype

When the data set was analyzed for genotype frequencies among the eight age groups, it was observed that age group >46-96 months had the highest frequency of *Sl2/2* genotype (*Figure* 8 and 9). However, the same trend was not observed with $McC^{b/b}$ and $McC^{a/b}$ (see Figure 10 and 11 respectively). When a regression analysis to predict *Sl2/2* from each age group was carried out, it was observed that those individuals in age group >46-96 months showed a trend of being more likely to be *Sl2/2* than they were *Sl1/1* or *Sl1/2* (OR = 1.42, 95% CI 0.48-4.23) (*Table* 23).

Figure 8: Percent of Swain Langley and McCoy genotypes among age groups

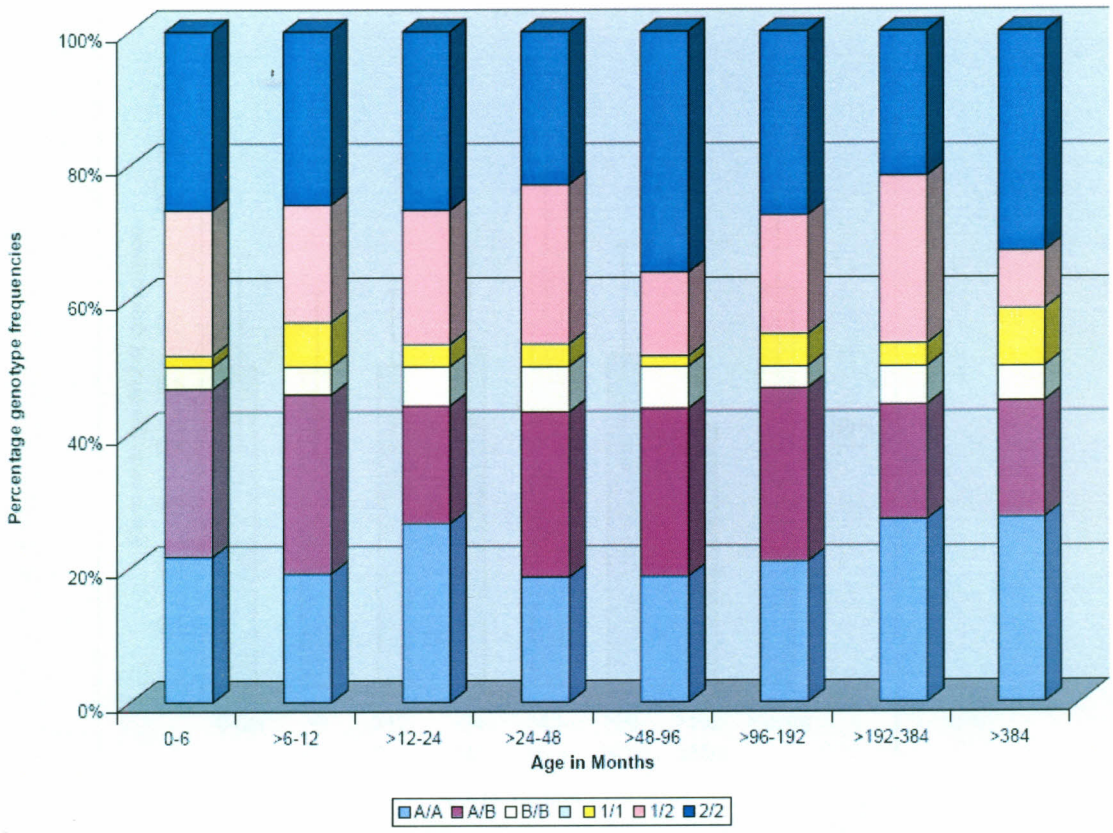


Figure 9: Changes in *SI2/2* genotype frequencies with age

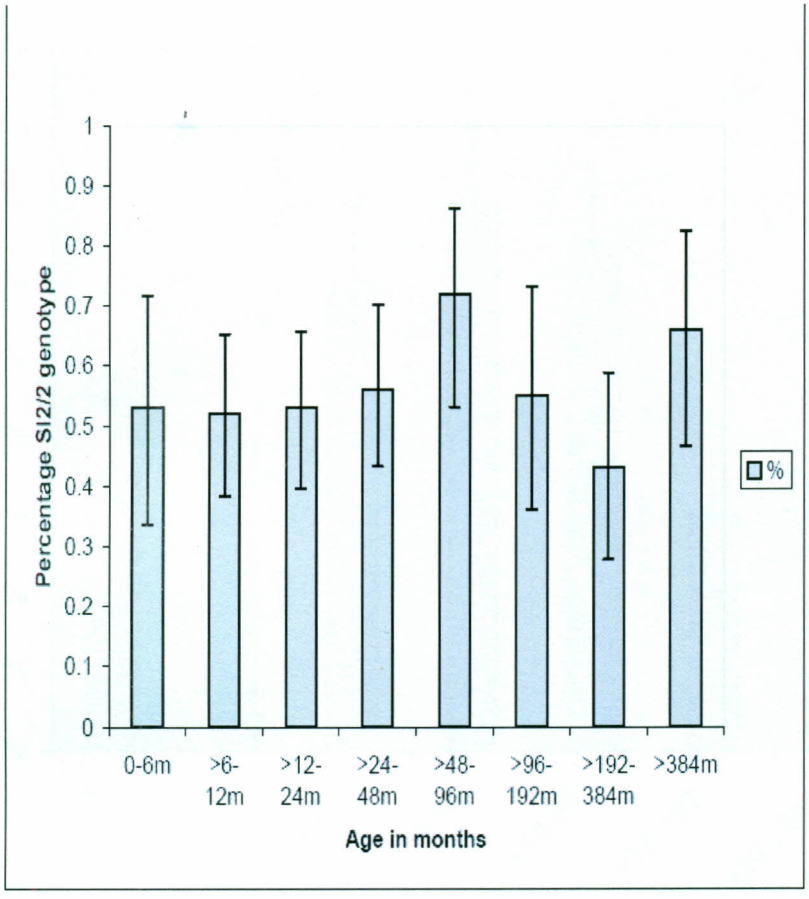


Figure 10: Changes in *McC*$^{b/b}$ genotype frequencies with age

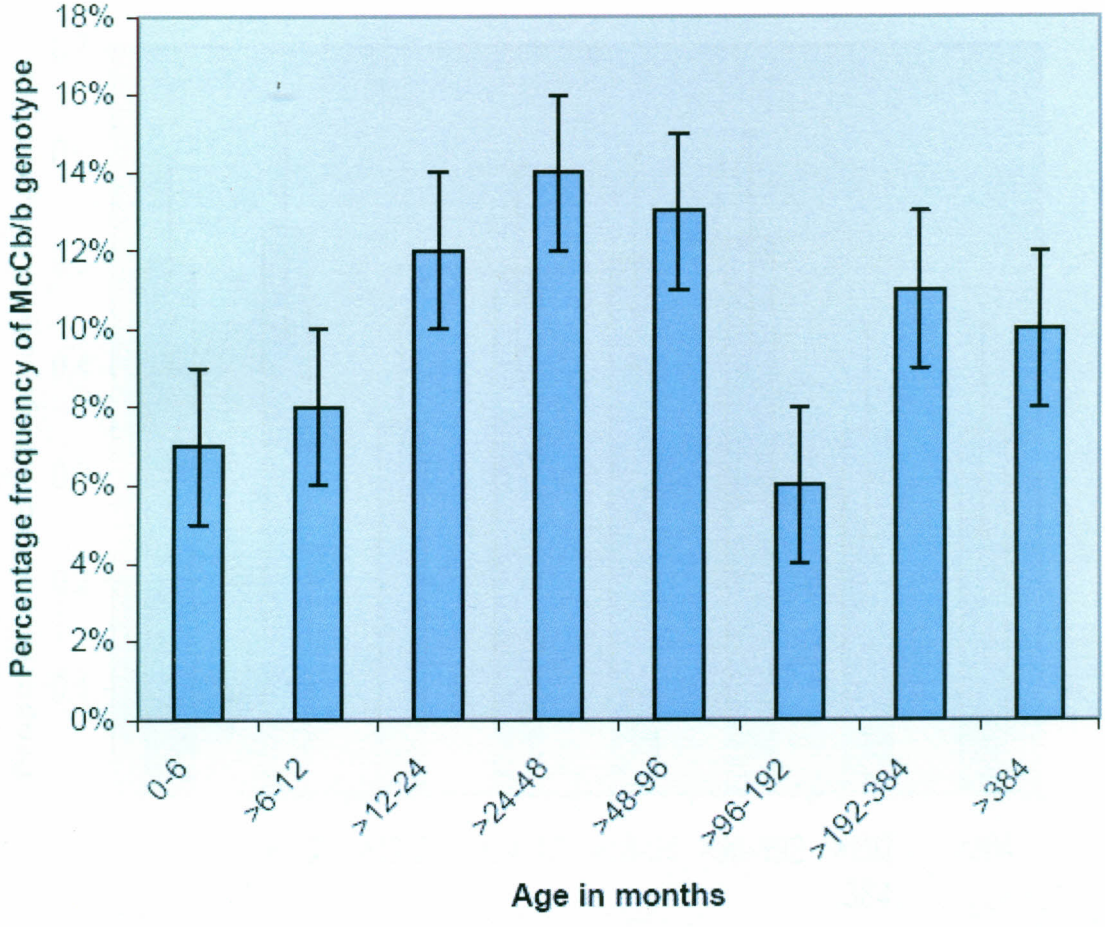


Figure 11: Changes of $McC^{a/b}$ genotype frequencies with age

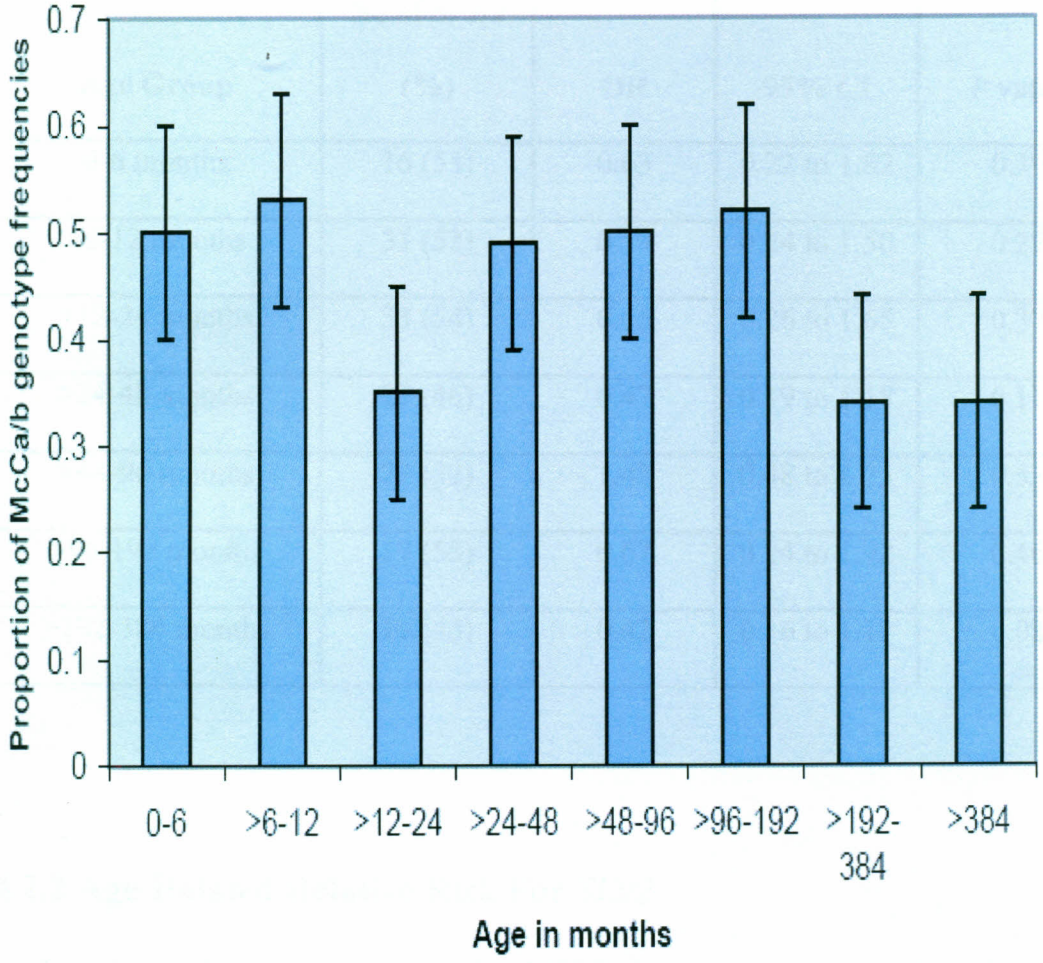


Table 23: Logistic regression analysis predicting *SI2/2* among age group

Age Group	No of *SI 2/2* (%)	OR	95% CI	*P* value
0-6 months	16 (53)	0.63	0.22 to 1.82	0.39
>6-12 months	31 (52)	0.59	0.24 to 1.50	0.27
>12-24 months	33 (54)	0.65	0.26 to 1.65	0.37
>24-48 months	27 (46)	0.47	0.19 to 1.18	0.11
>48-96 months	23 (72)	1.42	0.48 to 4.23	0.53
>96-192 months	17 (55)	0.67	0.24 to 1.92	0.46
>192-384 months	19 (43)	0.42	0.16 to 1.12	0.08

4.2.2 Age-Related Relative Risk For *SI2/2*

In order to determine relative risk of *SI2/2* among age groups, a logistic regression analysis was performed using <48 months in one hand and >96 months in another as reference age groups. The results showed that individuals within the age group >48-96 had higher relative risk of S2/2 genotypes compared to other age groups (*Table* 24).

Table 24: Logistic regression analysis for the presence of *Sl2/2* using the age group <48 months and >96 months as reference

Age Group (months)	N	No. of *Sl2/2* (%)	OR	95% CI	*P* value
<48	210	107(51)	Ref	Ref	Ref
>48-96	32	23 (72)	3.36	1.36 to 8.30	0.01*
>96	103	54 (52)	1.21	0.69 to 2.13	0.50
<48	210	107(51)	0.82	0.47 to 1.45	0.50
>48-96	32	23 (72)	2.77	1.09 to 7.00	0.03*
>96	103	54 (52)	Ref	Ref	Ref

* denotes significance

4.2.3 Relationship Between *Sl2/2* And Clinical Malaria

In an attempt to establish any existing associations between Swain Langley genotypes and clinical malaria, a regression analysis was conducted on the data set and the results showed that individuals having *Sl2/2* genotype had lower risk of developing clinical malaria than their *Sl1/1* and *Sl1/2* counterparts. However, this association was not significant (OR = 0.76, 95% CI 0.50-1.18, *P* = 0.22) (Table 25)

Table 25: Logistic regression analysis predicting malaria status from *Sl* genotype

Genotype	No. of each genotype (%)	OR	95% CI	*P value*
Sl1/1	28 (8)	1.67	0.73 to 3.81	0.23
Sl1/2	129 (38)	1.25	0.79 to 1.96	0.34
Sl2/2	181 (54)	0.76	0.50 to 1.18	0.22

4.2.4 Rare Genotype Combinations At The Mccoy And Sl Loci

When different genotype combinations at the Swain Langley and McCoy loci were tabulated, only six out of the ten possible *Sl/McC* genotype combinations were found. This is shown in Table 26. In the table, genotypes $Sl1/1McC^{a/b}$, $Sl1/1McC^{b/b}$ and $Sl1/2McC^{b/b}$ were not found in the study population.

Table 26: Tabulation of Swain Langley and McCoy genotype combinations

McCoy	Swain Langley			Total
	1/1	1/2	2/2	
A/A	29	66	60	155
A/B	**0**	66	88	154
B/B	**0**	**0**	36	36
Total	29	132	184	345

4.2.5 Hardy-Weinberg equilibrium Of Study Population

In order to test if the population was in Hardy-Weinberg equilibrium, Chi square was performed and the result showed a significant difference between the observed and predicted genotype frequencies only for the age group >32 years ($P = 0.02$). This observation indicated that Sl genotype in among individuals in this age group were no longer in Hardy-Weinberg equilibrium. Overal the study population was in Hardy-Weinberg equilibrium.

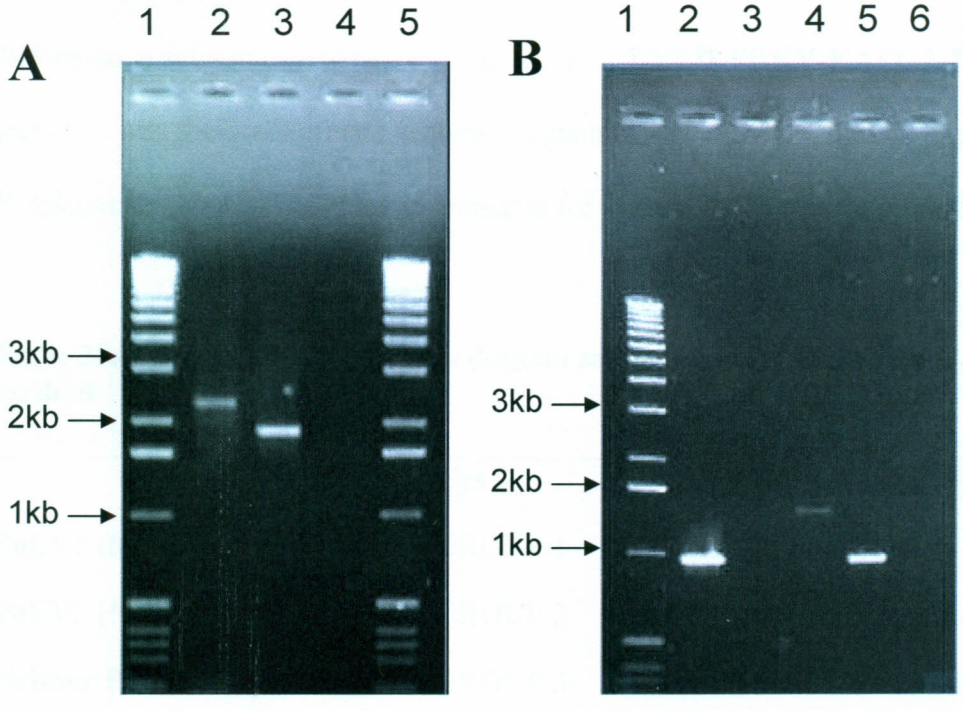
Table 27: Chi square analysis to compare the observed and predicted genotype frequencies for each age group

Age Group	N	Genotype	Observed frequency	Predicted frequency	P
0-6 mo	30	Sl1/1	1	2	0.63
		Sl1/2	13	11	
		Sl2/2	16	17	
>6-12 mo	60	Sl1/1	8	6	0.41
		Sl1/2	21	26	
		Sl2/2	31	29	
>12-24 mo	61	Sl1/1	4	4	1
		Sl1/2	24	24	
		Sl2/2	33	33	
>24-48 mo	59	Sl1/1	4	5	0.74
		Sl1/2	28	25	
		Sl2/2	27	28	
>48-96 mo	32	Sl1/1	1	1	1
		Sl1/2	8	8	
		Sl2/2	23	23	
>8-16 yr	31	Sl1/1	3	2	0.72
		Sl1/2	11	12	
		Sl2/2	17	16	
>16-32 yr	44	Sl1/1	3	4	0.68
		Sl1/2	22	19	
		Sl2/2	19	20	
>32 yr	28	Sl1/1	5	2	0.02
		Sl1/2	5	11	
		Sl2/2	18	15	

4.3 Generation of DBL1α products

In order to eliminate the possibility of amplifying any genomic DNA from the cDNA sample, it was necessary to rule out any contamination of the template used in RT-PCR by DNA. This was done with use of primers for house-keeping gene, histitine-rich protein (HRP) and Knob-associated histidine-rich protein (KAHRP) primers. There was no genomic DNA contamination since an expected 0.9 kb was generated from cDNA as compared to 1.3 kb product yielded from total RNA which had not been treated with DNase1. The results are shown with the gel analysis in *Figure* 12. Again KAHRP was shown to be expressed on both R+ and R- parasite portions. Fragments of 2.41 kb product were produced from total RNA (untretated with DNase1) and 1.97 kb from cDNA using this primer.

Figure 12: KAHRP and HRP control PCR



Panel A KAHRP PCR: Lanes 1 and 5 represent 1 kb ladder. Lane 2, total RNA used as template, lane 3, cDNA used as template while lane 4 is mock cDNA (DNase-treated total RNA but without reverse transcriptase).

Panel B HRP PCR: The first and lane represents a 1 kb ladder. Lane 2, cDNA used as template, lane 3 DNase-treated total RNA without reverse transcriptase, lane 4, total RNA without DNase1 treatment, lane 5, cDNA prepared from an old RNA while a no template (negative control) is shown in lane 6.

4.3.2 Predominant DBLα Sequence Obtained With AFBR clone Of SA075

The predominant sequence that was analyzed in AFBR clones was the same as the Sig2 sequence found by Bull and his colleagues (2005) except for one amino acid difference, a substitution for a "V" with an "F", "**LYLD-FREY-KAIT-2-PTNL**". This sequence was the predominant sequence signature of the DBLα domain expressed in the *P. falciparum* strain, SA075R+ responsible for the rosetting phenotype of this parasite.

Table 28a: Classification of DBLα domain sequences of SA075 using *Bull et al.*, 2005 method

	Cys 2	Cys 4	Cys X
PoLV1 (MFK*)	GROUP 1		
PoLV2 (*REY)	GROUP 2	GROUP 5	
Neither PoLV1 or 2	GROUP 3		
Without PoLV2		GROUP 4	
-			GROUP 6

Table 28b: Summary of dominantly expressed DBL1α tag sequences of SA075 as classified by Bull *et al.*, (2005)

	Experiment 1	Experiment 2
R+ "Sig2"	1/10	5/11
R- "Sig2"	5/8	6/8
Total "Sig2"	6/18	11/19

The sequences generated were used to search for similar sequences in the database employing protein BLAST (BlastP) as the search engine. The first six most significant hits are presented in the *Figure* 13. The full sequence alignment is shown in *Appendix* 1 and summarized in *Appendix* 2.

Figure 13: Dominant DBL1α tag from SA075R+ (BlastP)

```
>emb|CAJ40433.1|  erythrocyte membrane protein [Plasmodium falciparum]
 emb|CAJ40434.1|  erythrocyte membrane protein [Plasmodium falciparum]
 emb|CAJ39510.1|  erythrocyte membrane protein [Plasmodium falciparum]
 emb|CAJ39511.1|  erythrocyte membrane protein [Plasmodium falciparum]
 emb|CAJ39512.1|  erythrocyte membrane protein [Plasmodium falciparum]
 emb|CAJ39513.1|  erythrocyte membrane protein [Plasmodium falciparum]
Length=111

 Score =  231 bits (589),  Expect = 1e-59, Method: Compositional matrix adjust
 Identities = 111/111 (100%), Positives = 111/111 (100%), Gaps = 0/111 (0%)

Query  5    DIGDIIRGKDLYLDHEPGKQHLEERLERIFENIKKKNNNNELNNLSLDKFREYWWALNRD  64
            DIGDIIRGKDLYLDHEPGKQHLEERLERIFENIKKKNNNNELNNLSLDKFREYWWALNRD
Sbjct  1    DIGDIIRGKDLYLDHEPGKQHLEERLERIFENIKKKNNNNELNNLSLDKFREYWWALNRD  60

Query  65   QVWKAITCKAPEEDHYFKPAQNRKREFTDGHCGHRQGNVPTNLDYVPQFLR  115
            QVWKAITCKAPEEDHYFKPAQNRKREFTDGHCGHRQGNVPTNLDYVPQFLR
Sbjct  61   QVWKAITCKAPEEDHYFKPAQNRKREFTDGHCGHRQGNVPTNLDYVPQFLR  111
```

4.3.3 Sequences producing significant alignments to SA075R+ DBL1α tag (BlastP)

Further analysis of the most significant hits generated with BlastP using SA075R+ sequences as the query sequence was performed. The search results showed that the DBL1α tag from SA075+ was 100% similar to clones isolated from patients in Kilifi, Kenya (Bull *et al.*, 2005). The dominant sequence was identical to a block-sharing group 2, cys/PoLV group 2 sequence isolated in Kilifi (Bull *et al*, 2005 & 2008). This is shown in Table 28c.

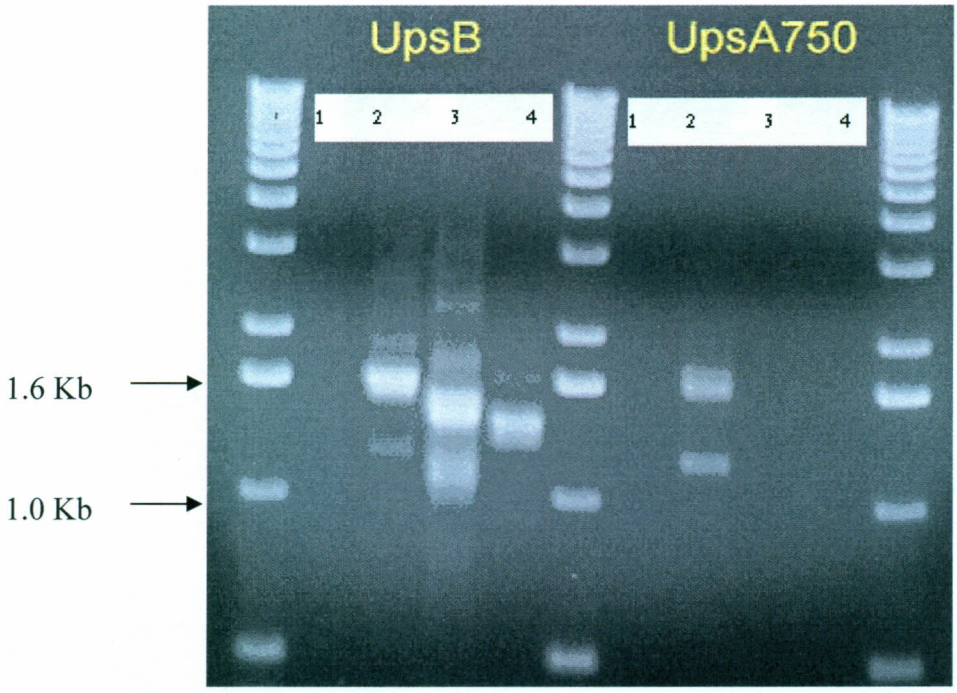
Table 28c: Sequences producing significant alignments to SA075 DBL1α tag (BlastP)

Sequence	Score	E value	% identity	Source
embICAJ40433.1	231	1e-59	100	**Kilifi-Bull (2005) clone 0263, patient 4180 (+5 more clones)**
gbIAA41640.1	199	3e-50	87	**Brazil-Kirchgatter, K and del Pertillo, H.A (isolate G2, clone 7**
embICAJ3973.1	172	5e-42	73	**Kilifi-Bull (2005) clone 0988, Patient 4130**
embICAJ39779.1	171	1e-41	72	**Kilifi- Bull (2005) clone1664, Patient 4130**
embICAJ39780.1	169	4e-41	71	**Kilifi- Bull (2005), clone 1665, Patient 4130**
gbIAAF36623.1I AF221783.1	165	1e-39	71	**Kenya-Taylor, H.M and Newbold C.I, wild isolate 19**
bgIABC95865.1	**160**	**2e-38**	**69**	**Mali-Kyriancou, H.M and Rowe, J.A (CM7f)**

4.3.4 Amplification Of Full Length DBL1α Domain

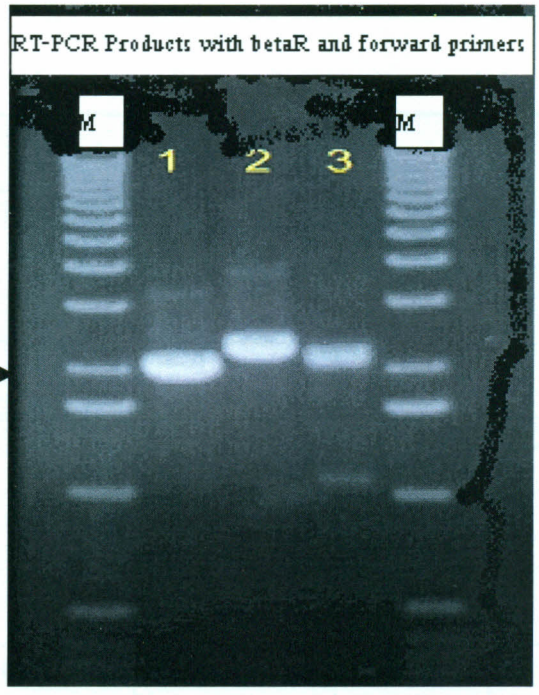
The applied strategy produced products of the correct sizes using the upsB primer. The products were sequenced to confirm the identity of the DBLα tag region. These products were 1.4 kb and 1.6 kb for the PoLV1 (LYLD) and PoLV4 (PTNL) primers respectively.The second strategy to amplify the full length DBL1α domain was to design primers that would yield products both from upstream and downstream of DBL1α tag. The primers were designed from the clones that contained DBL1α tag sequences. These

Figure 14a: RT-PCR products for amplification of upstream of DBL1α region



The RT-PCR was set with primers shown in *Table* 4. The lanes labeled 1, either UpsA or UpsB as forward primer and several reverse primers as described in the methods. The sizes of the generated products are summarized in *Table* 29. There were no products generated for the sequence upstream of DBL1α tag using an upsA specific forward primer. However, products of the correct sizes were obtained with upsB specific forward primer. The PfEMP1 promoter domains are shown in *Appendix* 3.

Figure 14b: RT-PCR products for amplification of downstream of DBL1α region



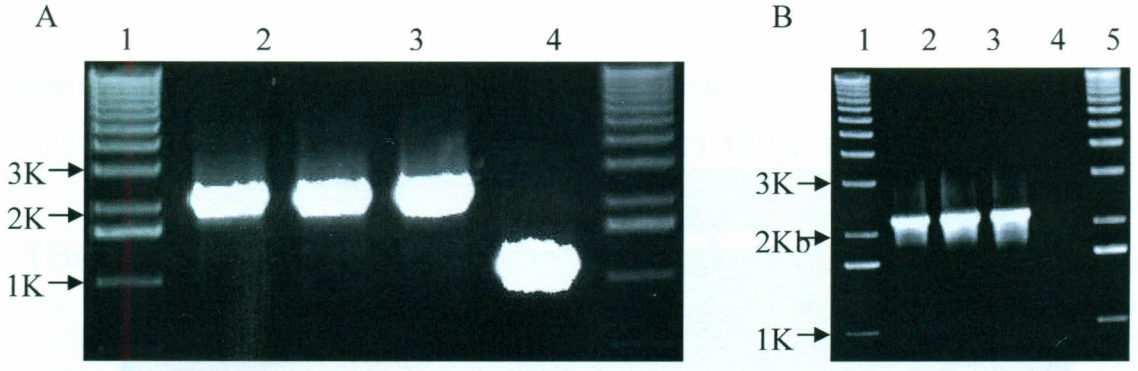
The first and last lanes marked M, show the molecular weight marker for sizing the RT-PCR products. The second lane labeled 1 shows the size of the RT-PCR product generated with betaR and LYLD5' primer set, lane 3 marked 2 betaR and PTYF5', lane 4 marked 3 betaR and PTNL5'. The RT-PCR was performed as described in the methods and the products resolved on 1.2% agarose gel. The sizes of the products are summarized in *Table* 29. Other primer sets did not yield any products with either cDNA from R+ or R-.

Table 29: Summary of RT-PCR products generated for upstream and downstream of DBLα domain

Primer	UpsA750		UpsB		BetaR	
	R+	R-	R+	R-	R+	R-
LYLD5'					**+/2.3**	**nd**
LYLD3'	**-**	**nd**	**+/~1.4**	**+/~1.4**		
PTNL5'					**+/2.0**	**nd**
PTNL3'	**+/1.2,1.6**	**nd**	**+/1.6**	**nd**		
MFLP5'					**-**	**nd**
MFLP3'	**-**	**nd**	**-**	**nd**		
PTYF5'					**+/2.2**	**nd**
PTYF3'	**-**	**nd**	**+/multiple**	**nd**		

- No product generated, $^{R+}$ cDNA used as template, $^{R-}$ cDNA used as template nd not done.

Figure 15: RT-PCR for DBL1α and DBL1α-CIDR domains



Panel A represents RT-PCR products for the amplification of DBL1α-CIDR domain and DBL1α region. Lanes 1 and 6 show DNA ladder, Lane 2 and 3 show a 2.1 Kb product for DBL1α-CIDR while Lane 4 is a DBL1α-CIDR-FLAG product. Lane 5 shows a 1.1Kb product for DBL1α-FLAG. Panel B represents RT-PCR products for the amplification of DBL1α-CIDR domain at different annealing temperature. Lane 2, 3 and 4 are products yieldedat 68°C, 70°C and 72°C respectively while Lane 5 is no cDNA (negative) control. Lanes 1 and 6 show 1Kb DNA ladder.

4.3.5 TOPO XL Sub Clones And Endonucleases Analysis

The products were purified using PCR Purification Kit (QIAGENE) and cloned into TOPO XL Vector as already described in the methods. The TOPO XL clones were screened using appropriate restriction enzymes and the right clones picked for sequencing. *Figure* 16a shows a representative minipreps for TOPO XL clone while *Figure* 16b shows restricted clones to find the ones with DBL1α domain sequences

inserted in the right orientation. More of the used endonucleases in this study are shown in Appendix 12.

Figure 16a: Minipreps for DBL1α domain cloned into TOPO XL vector

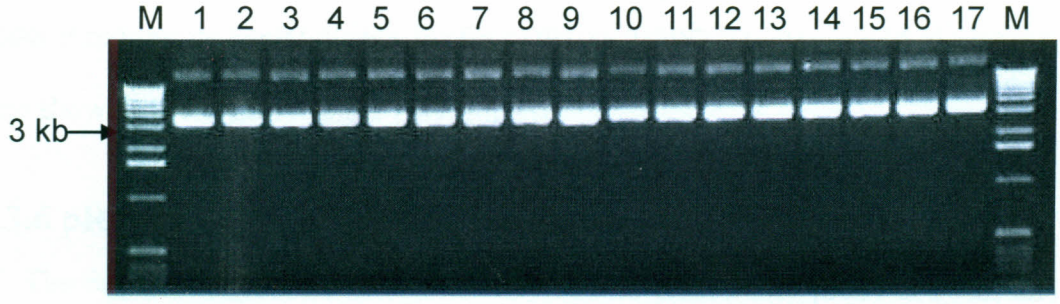
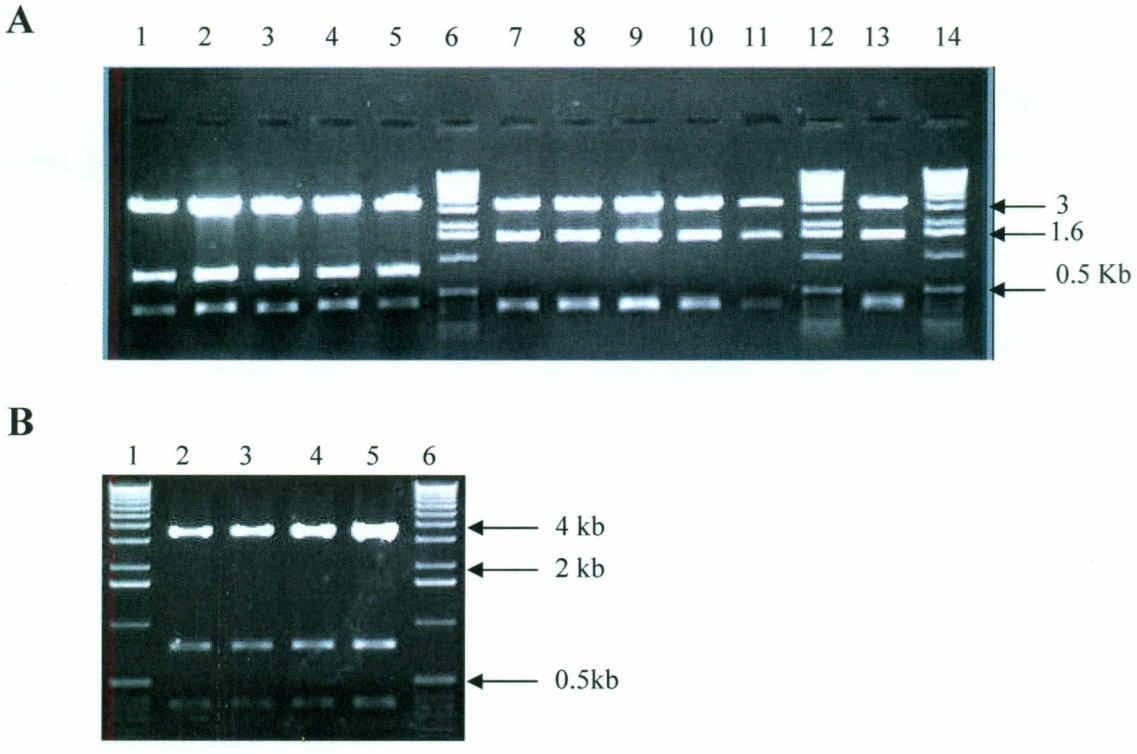


Figure 16b: Restriction analysis of TOPO XL clones



Panel A represents EcoRI digests. Lanes 1 to 5 represent 5 DBL1α-FLAG TOPO XL clones whereas lanes 7 to 11 show the restriction fragments for DBL1α-CIDR TOPO XL

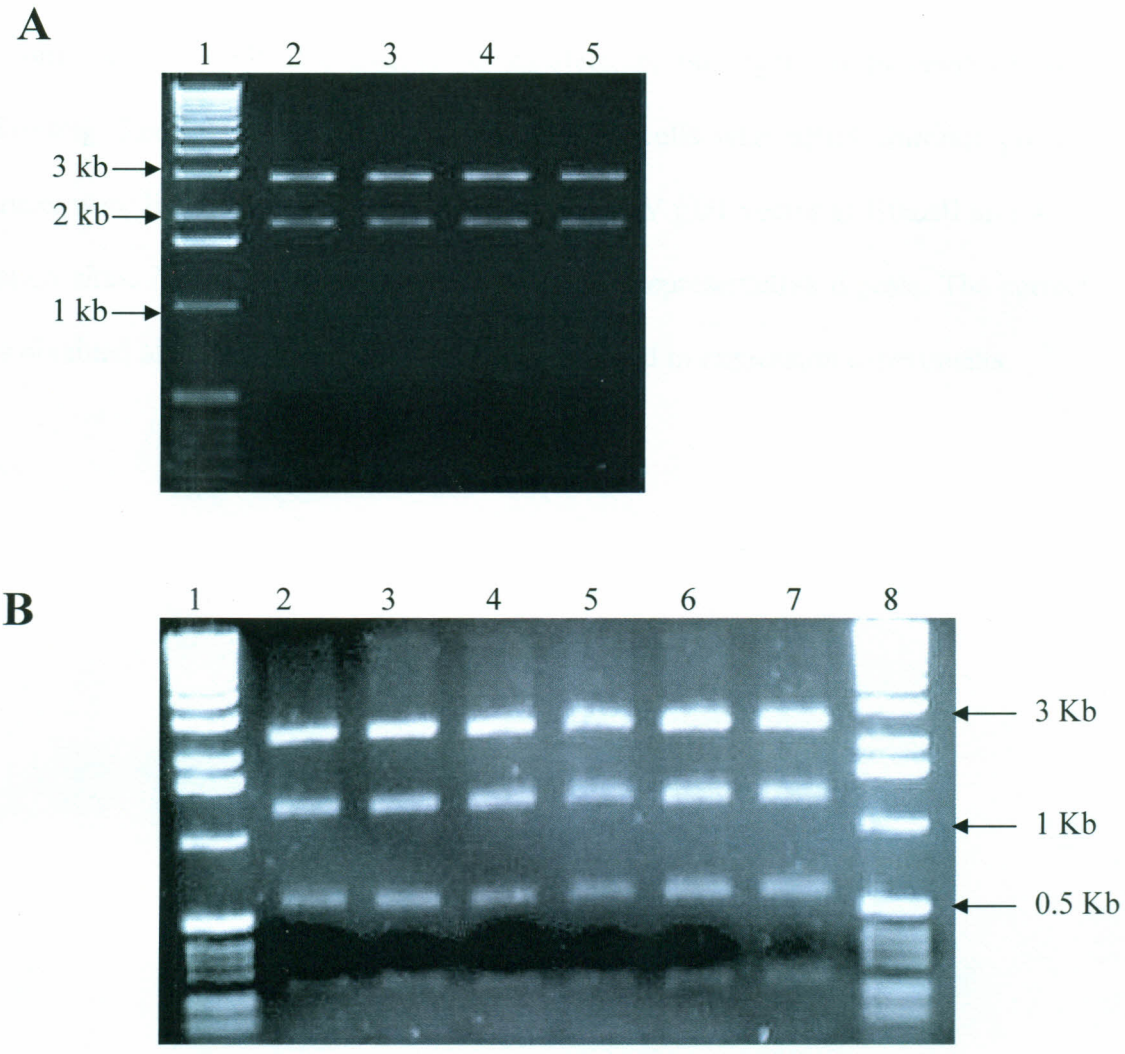
Panel A represents EcoRI digests. Lanes 1 to 5 represent 5 DBL1α-FLAG TOPO XL clones whereas lanes 7 to 11 show the restriction fragments for DBL1α-CIDR TOPO XL clones. Lane 13 shows the restricted products for DBL1α-CIDR-FLAG TOPO clone. Lanes 6, 12 and 14 represent 1Kb DNA ladder (Invitrogen).

Panel B represents EcoRI digests for four DBL1α TOPO XL clones with first and last lane showing 1 Kb DNA ladder (Invitrogen).

4.3.6 pRE4 clones And endonucleases analysis

The correct TOPO XL clone was selected and cloned into pRE4 at PvuII and ApaI restriction sites. The clones obtained by this ligation were again screened using appropriate restriction enzymes. *Figure* 17 shows an example of the representative digests. The map of pRE4 vector is shown in *Appendix* 4. DBL1α sequence tag domain cloned in pRE4 vector is also shown (see *Appendix* 5)

Figure 17: Gel analysis of DBL1α construct in pRE4 vector

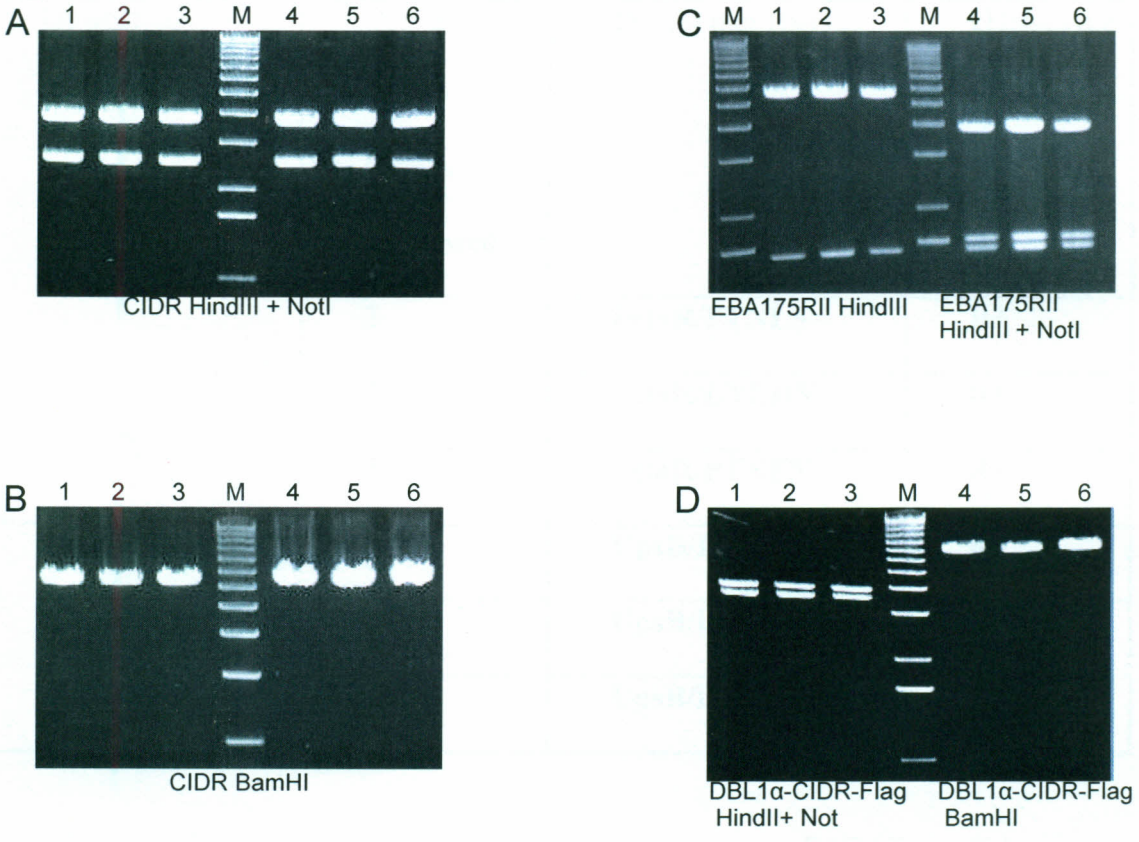


Panel **A** represents four pRE4-DBL1α clones digested with HindIII with 1 Kb DNA ladder in the first lane while Panel **B** shows double digestion (HindIII and EcoRI) of pRE4-DBL1α clones with 1 Kb DNA ladder marking the edges of 0.9% ethidium bromide-strained the gel. The expected products for HindIII digest for this construct were a 1.7 kb and 3 kb while for double digestion with HindIII plus EcoRI several fragments were obtained.

4.3.7 pEGFP-NI clones And Endonucleases Analysis

In order to use GFP as a marker of transfection, the right clones obtained after transforming *E.coli* TOP10F' chemically competent cells with pRE4 chimeric protein constructs were ligated into pEGFPN1-EBA175rII/HSV gD1 vector at HindIII and ApaI restriction sites. *Figure* 18 shows an example of the representative digests. The correct clones obtained after this screening procedure were used in expression experiments.

Figure 18: Restriction digests of different final constructs in pEGFPN1 vector



Panel A shows CIDR miniprep digests. Lanes 1, 2, 3, 4, 5 and 6 are the representative clones with the correctly expected fragment sizes (2.4 kb and 4 kb) while Panel B shows the same clones digested with BamHI which linearizes the construct at 6.4 kb.

Panel C shows minipreps for pEGFPN1-EBA175RII plasmid construct. Lane 1, 2 and 3 are clone digested with HindIII (1.5 kb and 5.7 kb) while 4, 5 and 6 are same clones double digested with HindIII and NotI which yields a 1.5 kb, 1.7 kb and 4 kb fragments.

Panel D shows BDL1α-CIDR-FLAG miniprep digests. Lane 1, 2 and 3 yielded products of 3.5 kb and 4 kb while lanes 4, 5 and 6 produced linearized fragments at 7.5 kb.

Table 30: Summary of RT-PCR products generated for downstream of DBLα domain

Downstream clones	No. of TOPO Clones sequenced	Primer pair (pcr)	cDNA type
	2	**betaR/PTNL5'**	**R+**
	5	**betaR/LYLD5'**	**R+**
	4	**betaR/PTYF5'**	**R+**
Upstream clones	**5**	**UpsB/LYLD3'**	**R+**
	5	**UpsB/LYLD3'**	**R-**
	5	**UpsB/PTNL3'**	**R+**

After sequencing the clones containing the upstream (upsB/PTNL) and downstream (LYLD/betaR) of DBL1α tag domain, the sequences were aligned and primers designed to amplify the whole (full length) domain. These were gene-specific primers that would amplify full-length DBL1α and DBL1α-CIDR regions. Table 30 above summarizes the number of TOPO clones sequenced. The products were sub-cloned into TOPO XL vector and cloned into expression vector. The annotated form of the final DBL1α domain transiently expressed on CO7 cells is presented in *Appendix* 6 while the map of the expression vector pEGFPNI-EBA175RII shown in *Appendix* 7. *Appendix* 8 present the map of the final construct of DBL1α as cloned into pEGFPNI expression vector. More of gel analysis of vectors and synthetic test constructs are shown in *Appendix* 9.

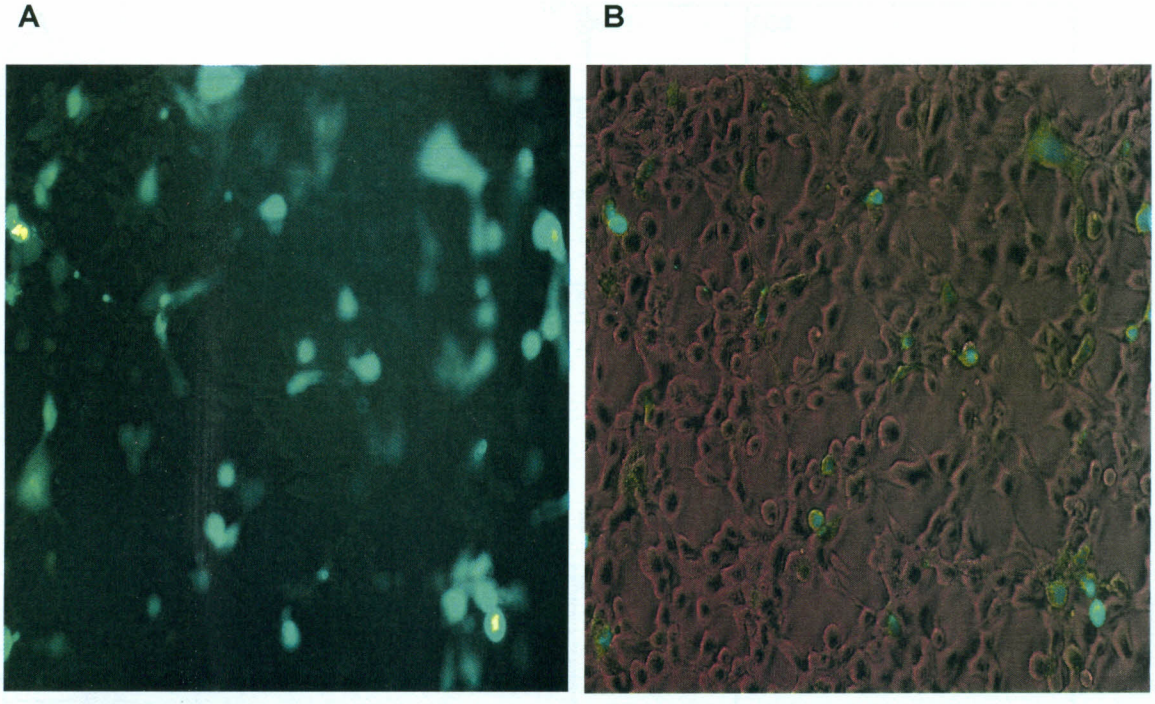
4.4 Expression Of DBL1α Domains In Mammalian Cells

To follow expression, COS7 cells were grown on cover slips in a 24-well culture plates. The cells were transfected with constructs designed to express DBL1α domains and the expression levels detected using various techniques.

4.4.1 Immunofluorescence Assay Showed Expression Of DBL1α Domain Constructs

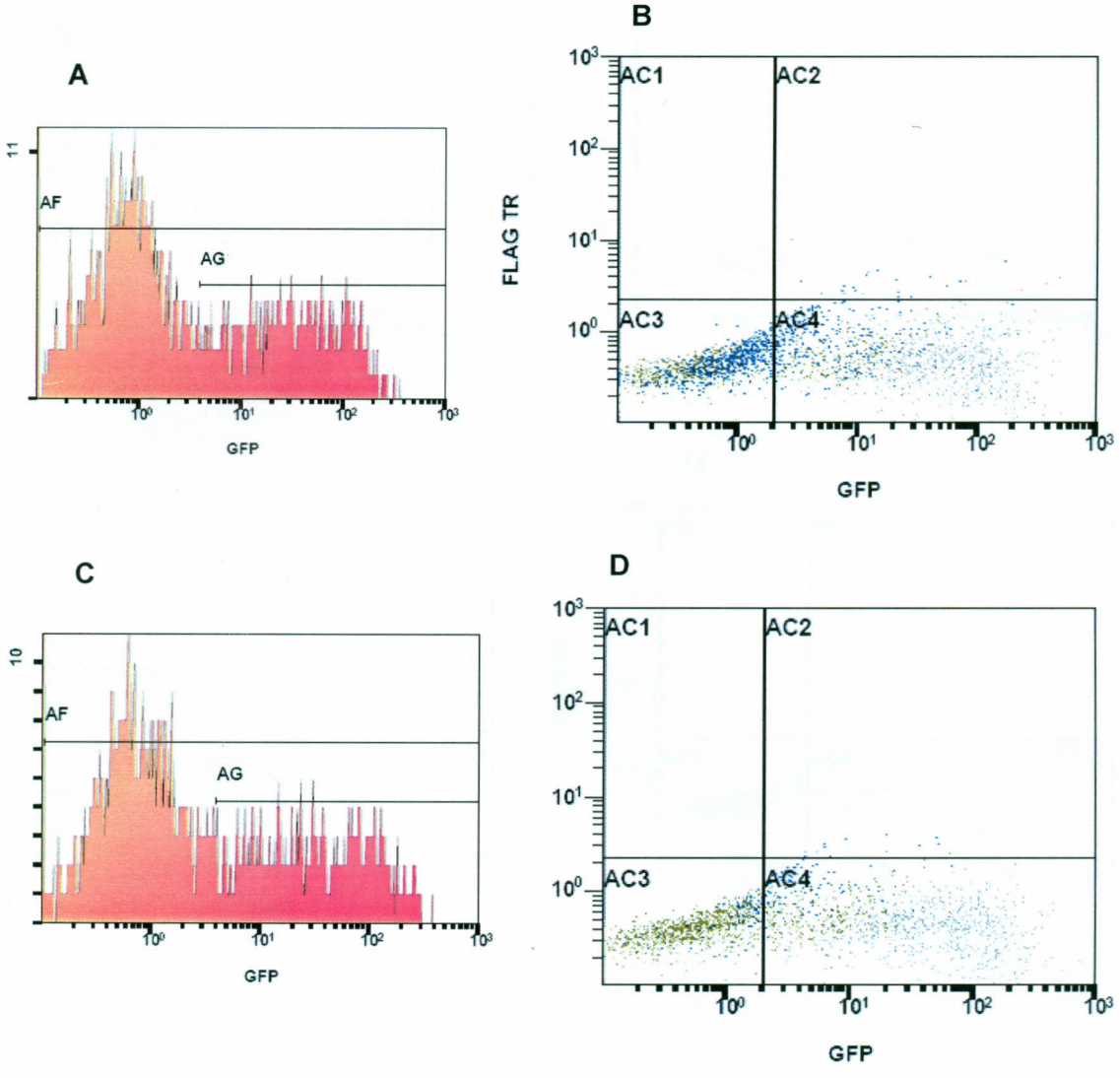
Transfected COS7 cells were observed by fluorescence microscopy to detect the positive transfectants and all the constructs showed positive transfection using GFP as a marker of transfection. The transfection efficiency was varied for different constructs. The result for positive transfection with DBL1α construct is shown in *Figure* 19 while *Figure* 20 shows flow cytometry analysis for constructs using GFP as a marker of transfection. More fluorescence analysis for transfection efficiency is shown in *Appendix* 10.

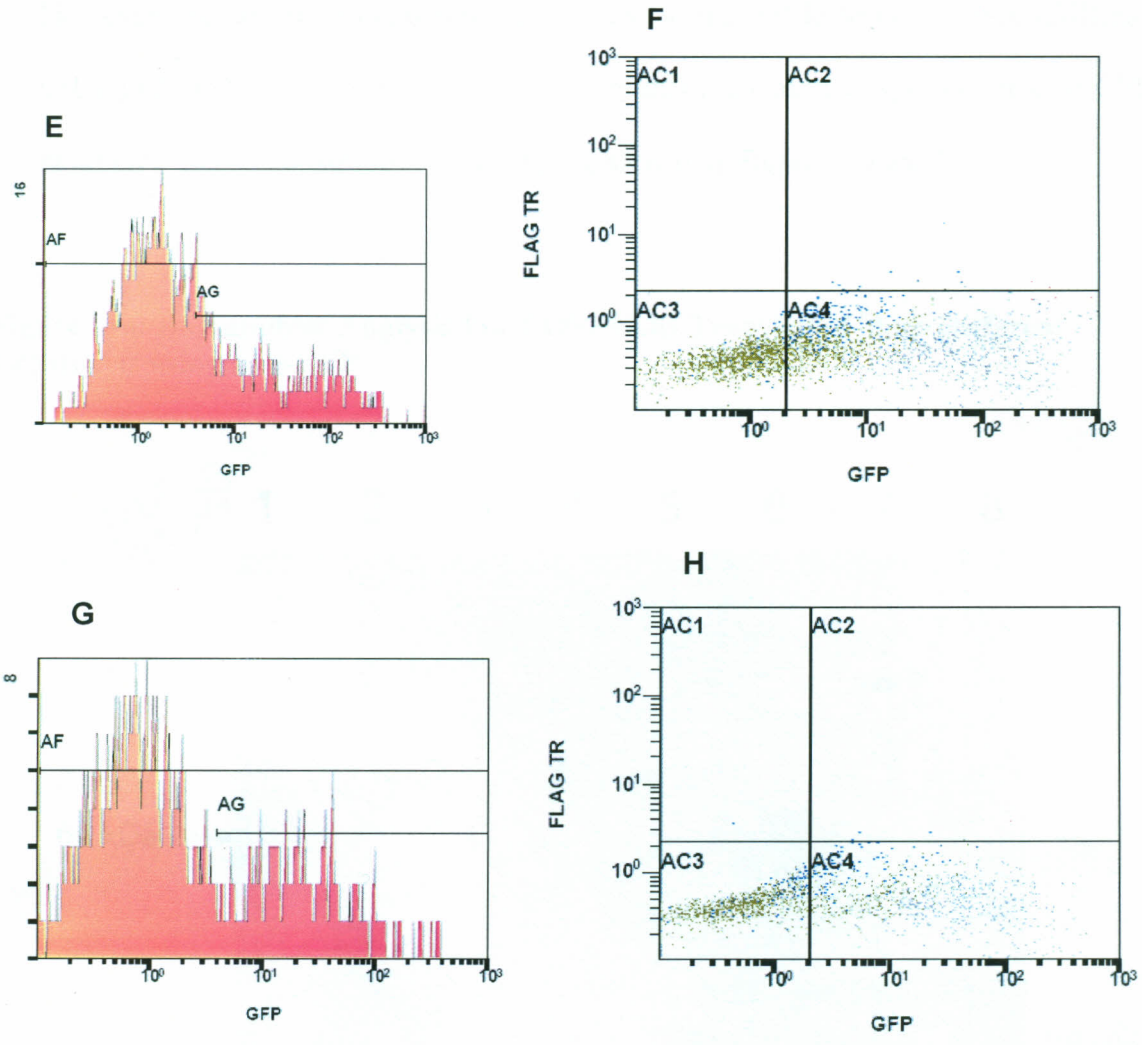
Figure 19: Fluorescence Analysis Of Pegfpn1-HSV Gd1-DBL1α Domain Construct Using GFP As A Transfection Marker



Panel A shows GFP positive cells under fluorescent light (Gfp) at 200X magnification while panel B represents the same field at same magnification with both fluorescent light and bright light (GFP-Phase)

Figure 20: Flow Cytometry Analysis for DBL1α domain construct expression on COS7 using GFP as a transfection marker



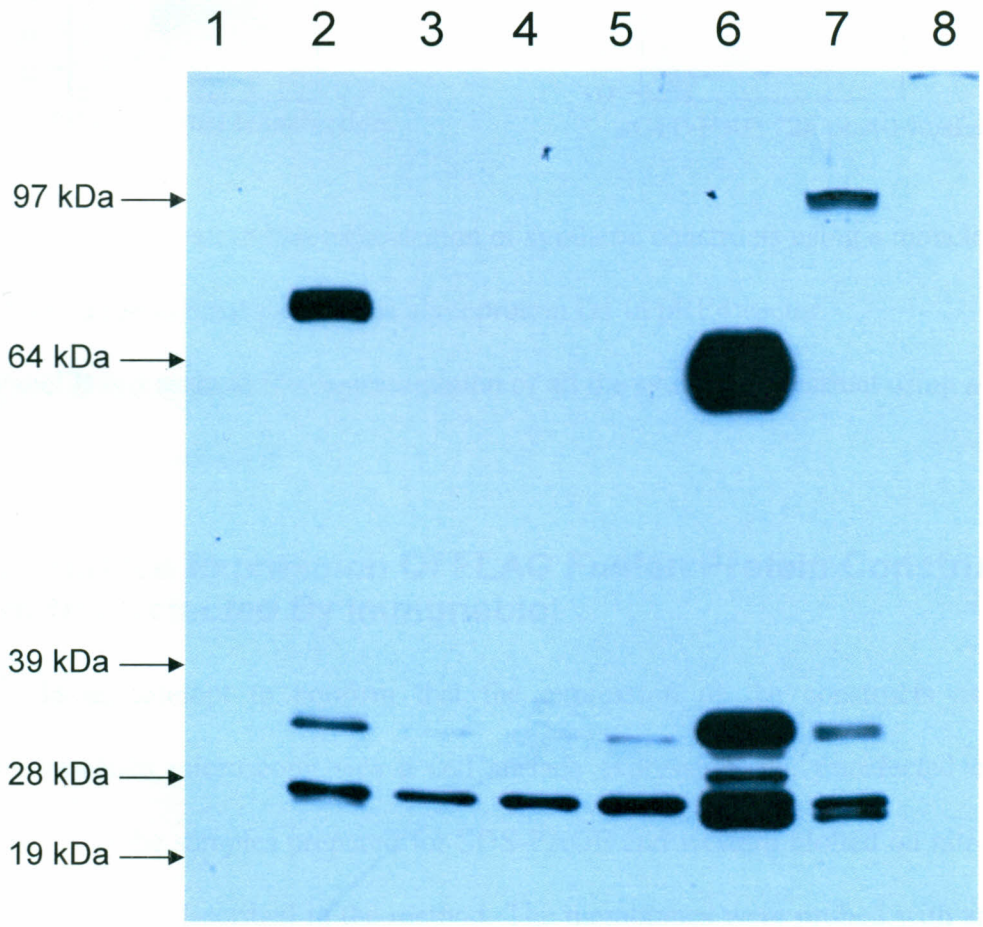


4.4.2 The Expression Of DBL1α Domain Was Detected By Western Blot Analysis

In order to establish the expression of DBL1α domain construct, cell lysates were prepared as already been described in the methods. The presence of the construct as a GFP-fusion protein was probed by anti-GFP antibody conjugated with horse radish peroxidase. The analysis showed as expected the presence of a 70 kD DBL1α protein. HSV gD1 and EBA175RII were also shown to be expressed by immunoblot analysis.

However, the construct containing sequences for the whole head structure (DBL1α-CIDR) of PfEMP1 gene was not detected by this method. The system detected GFP in all GFP fusion constructs.The results are shown in *Figure* 21a and b.

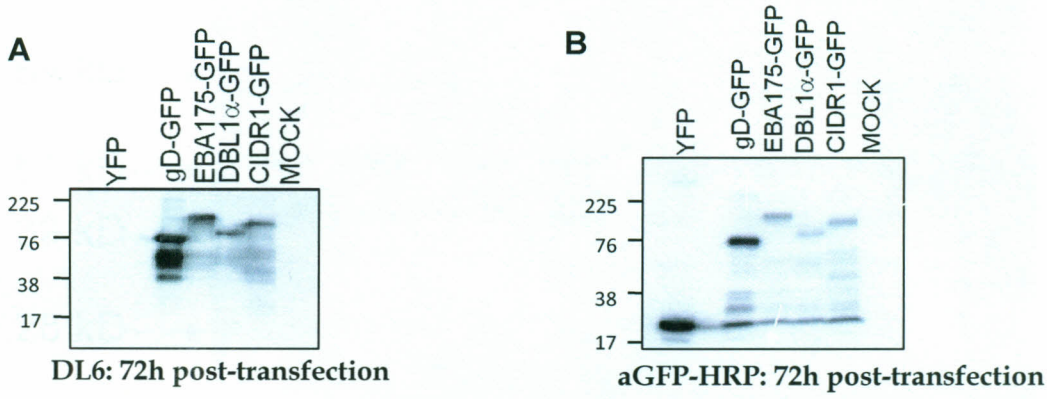
Figure 21a: Immunoblot Analysis For COS7 Cells Transfected With Different Constructs using anti-GFP



Lane 1 represents lyasate obtained from mock transfected CO7 cells, lanes 2, 3, 4, 5, 6, and 7 represent lysates obtained from CO7 cells transfected with DBL1α, DBL1α-FLAG, DBL1α-CIDR, DBL1α-CIDR-FLAG, HSV gD1, and EBA-175RII chimeric protein constructs respectively. Last lane represents lysate obtained from COS7 cells

transfected with pFLAG-CMV-4-BAP control plasmid. The membrane was probed with anti-GFP-HRP.

Figure 21b: Immunoblot Analysis using Anti-GFP and anti-gD1



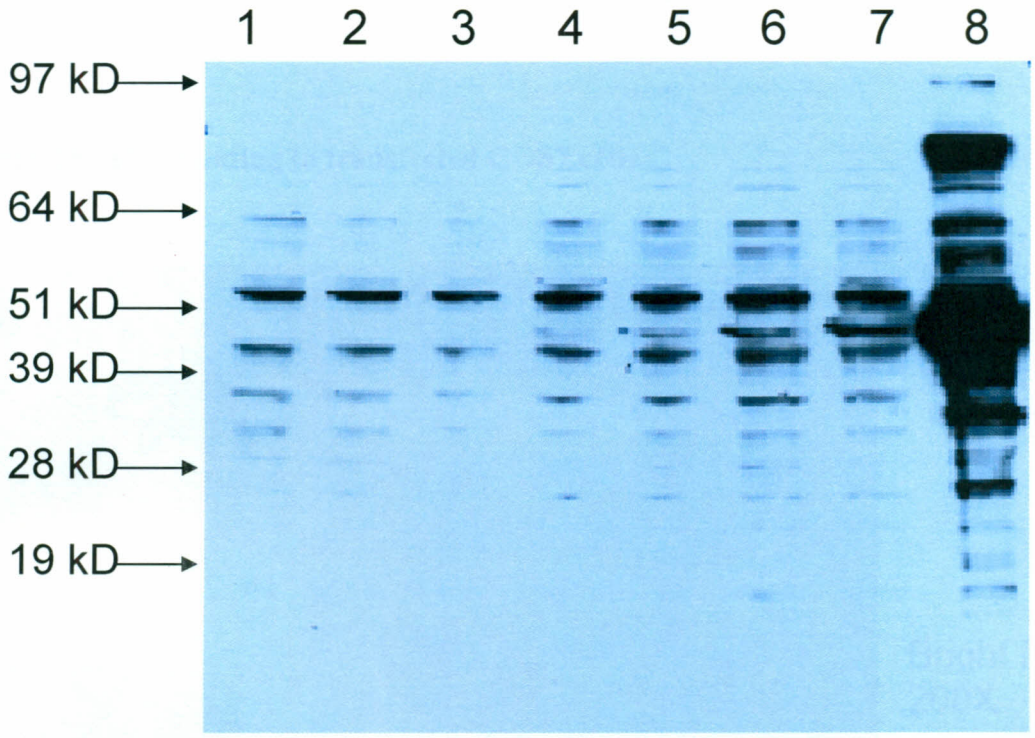
Panel **A** is a western blot represention of synthetic constructs using a monclonal (DL6) antibody that recognizes glycoprotein D1 in pRE4 vector.

Panel **B** is a western blot representation of all the synthetic construct using a monoclonal, anti-GFP.

4.4.3 Surface Expression Of FLAG Fusion Protein Constructs Were Not Detected By Immunoblot

In an attempt to confirm that the expression of the constructs as seen by fluorescence microscopy was a cell surface expression, the transfected cells were lysed and the samples prepared for SDS-PAGE and western blotted on nitrocellulose membrane as described in the method. The membranes were probed with anti-FLAG (clone M2) monoclonal antibody conjugated with horse radish peroxidase. No FLAG-fusion protein for the construct was detected with the antibody. The positive control on the other hand produced the expected product. These results are shown in *Figure* 22.

Figure 22: Western blot detection of FLAG fusion protein with ANTI-FLAG HRP antibody



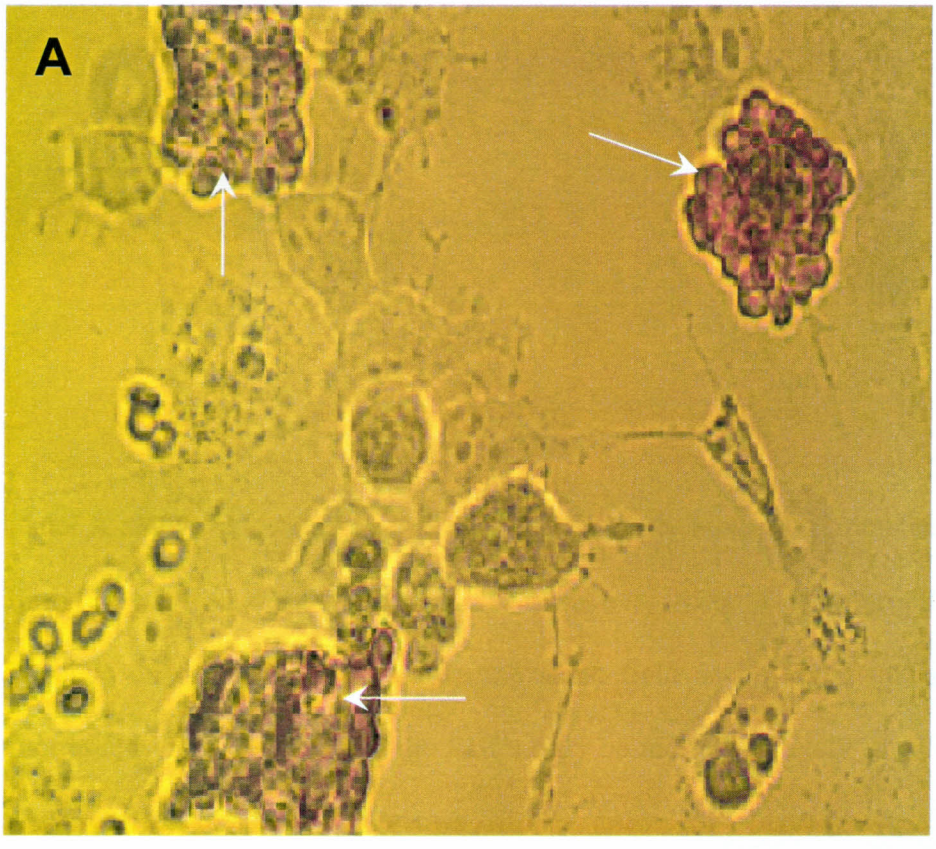
The first lane represents lysate obtained from mock transfected CO7 cells while lanes 2, 3, 4, 5, 6 and 7 represent Lysates obtained from COS7 cells transfected with DBL1α, DBL1α-FLAG, DBL1α-CIDR, DBL1α-CIDR-FLAG, HSV gD1 and EBA175RII chimeric protein constructs. The last lane represents lysate from COS7 cells transfected with positive control plasmid, pFLAG-CMV-4-BAP

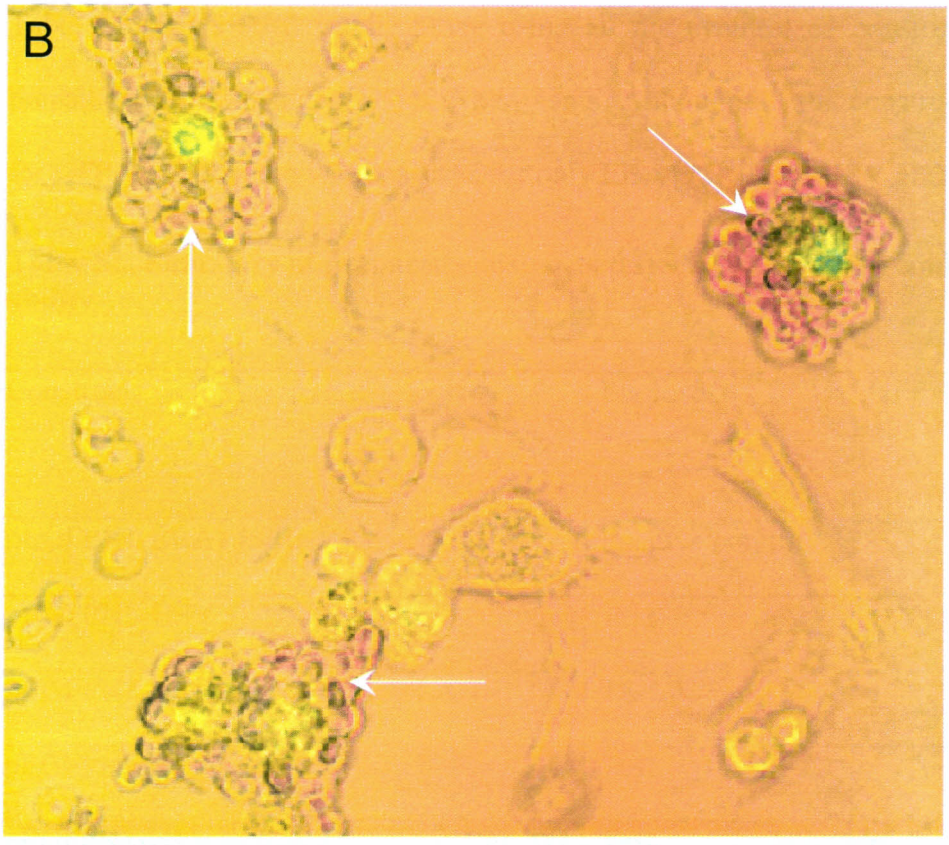
4.4.3 Analysis Of DBL1α Domain Constructs Did Not Show Binding To Rbcs

After transfection with the various constructs designed to express different domains of DBL1α, the positively transfected cells were tested for their ability to bind normal RBCs already genotyped for Knops antigen (Sl and McC). There was no observed binding of RBCs to any of the test constructs. However, RBCs bound to the construct

containing EBA175RII antigen. This was shown by fluorescence microscopy and FACs analysis. The results are shown in Figure 25 panels A and B.

Figure 23: RBC binding to transfected COS7 cells





Panel A shows an example of several RBCs tightly binding to transfected COS7 cells to form "rosettes" as observed under fluorescence microscope using bright light.

Panel B on the other hand shows the same field as observed under both bright and fluorescent light to show that the RBCs only bound to positively transfected COS7 cells. The "rosettes" are shown by arrows.

Table 31 shows RBC bindig assay using all the synthesized constructs. The table is prepared from four independent experiments. Although all the constructs were positive for GFP, RBC binding was observed for only EBA175RII and HSV gD1 chimeras.

Table 31: Summary of prepared constructs tested for expression and RBC binding ability

Construct	Purpose	GFP	RBC binding
pEGFPN1-EBA175RII-HSV gD1	Positive control	+	+++
pEGFPN1-HSV gD1-DBL1α	Test	+	-
pEGFPN1-HSV gD1-DBL1α-FLAG	Test	+	-
pEGFPN1-HSV gD1-DBL1α-CIDR	Test	+	-
pEGFPN1-HSV gD1-DBL1α-CIDR-FLAG	Test	+	-
pEGFPN1-HSV gD1	+ve control	+	+
pEGFPN1-HSV gD1-CIDR	Negative control	+	-

CHAPTER 5: DISCUSSION

The general observation that *Sl2/McC*^{b} haplotype is almost exclusively found distributed within African population is an interesting one. It has been suggested that this allele may have emerged as a result of an adaptive selective pressure exerted by the burden of numerous infectious diseases including *P. falciparum* malaria. The allele has been designated 'African allele' and is thought to offer some advantage to the host.

5.1 *Sl2/2* And *McC*$^{a/b}$ Genotypes And Association With Resistance To Severe Malaria

In this case-control study it has been demonstrated that individuals with the *Sl2/2* genotype that confers the Sl:2 phenotype are more resistant to severe malaria, and in particular cerebral malaria, than individuals with the *Sl1/1* genotype that confers Sl:1. Interestingly, individuals with *McC*$^{b/b}$ did not show a significant protective advantage against severe malaria. In fact, the presence of *McC*$^{a/a}$ or *McC*$^{b/b}$ seemed to diminish some of the advantages of the *Sl2/2* genotype. However, since the association between *Sl2/2* and resistance to severe malaria appeared to be strongest in individuals who had the genotype *Sl2/2 McC*$^{a/b}$, individuals who had the genotype *McC*$^{a/b}$ did have an advantage only in the background of a *Sl2/2* genotype. These results support the conclusion that both the *Sl2* and the *McC*$^{a/b}$ alleles have evolved in the context of malaria transmission and probably confer a survival advantage to these populations.

The mechanism by which the *Sl2* and *McC*$^{a/b}$ alleles may confer protection from severe malaria is not clear especially since these polymorphisms are located near the C-

terminus of the CR1 molecule in an area that has no known function and is outside the binding sites for C3b, C4b, and PfEMP-1(Smith et al., 2001). Nonetheless, based on knowledge of CR1 and the nature of amino acid substitutions that result in these mutations, a number of potential explanations come to mind. The *Sl2* allele is the result of the substitution of glycine, a neutral amino acid, for the basic amino acid arginine at position 1601 (R1601G) whereas the McC^b allele is the result of the substitution of the acidic amino acid glutamic acid for the basic amino acid lysine at position 1590 (K1590E). These changes may inflict significant conformational or charge changes to the rest of the molecule and may consequently impact on the conformation and function of the C3b and C4b binding sites, and/or the PfEMP-1 binding site. Accordingly, *Sl:2* erythrocytes have been reported to rosette less than *Sl:1* erythrocytes. In addition, it is known that CR1 molecules are aggregated on the erythrocyte surface and this aggregation is felt to be critical to the binding affinity of CR1 for C3b and C4b. (Paccaud *et al.*, 1990). Therefore, it is possible that the amino acid substitutions not only induce conformational changes to the individual CR1 molecules but also affect the ability of functional aggregates to form due to conformational incompatibility or charge interference between different CR1 molecules.

Although the current belief is that CR1 may influence the development of severe malaria by virtue of its function as a regulator of the complement cascade on RBCs or by its direct interaction with the infected RBCs leading to rosette formation, CR1 may exert its effect on malaria in other ways that we do not yet understand. In addition to being present on RBCs, CR1 is also present on most leukocytes including T cells and,

terminus of the CR1 molecule in an area that has no known function and is outside the binding sites for C3b, C4b, and PfEMP-1(Smith et al., 2001). Nonetheless, based on knowledge of CR1 and the nature of amino acid substitutions that result in these mutations, a number of potential explanations come to mind. The *Sl2* allele is the result of the substitution of glycine, a neutral amino acid, for the basic amino acid arginine at position 1601 (R1601G) whereas the McC^b allele is the result of the substitution of the acidic amino acid glutamic acid for the basic amino acid lysine at position 1590 (K1590E). These changes may inflict significant conformational or charge changes to the rest of the molecule and may consequently impact on the conformation and function of the C3b and C4b binding sites, and/or the PfEMP-1 binding site. Accordingly, *Sl:2* erythrocytes have been reported to rosette less than *Sl:1* erythrocytes. In addition, it is known that CR1 molecules are aggregated on the erythrocyte surface and this aggregation is felt to be critical to the binding affinity of CR1 for C3b and C4b. (Paccaud *et al.*, 1990). Therefore, it is possible that the amino acid substitutions not only induce conformational changes to the individual CR1 molecules but also affect the ability of functional aggregates to form due to conformational incompatibility or charge interference between different CR1 molecules.

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therefore, may also have immunoregulatory functions (Aheam and Fearon 1989). Data from another finding suggesting that the role of CR1 in the pathogenesis of severe malaria may not be as expected is found in a case-control study in Papua New Guinea. This investigation aimed at determining whether there was an association between individuals who had the low CR1 expression allele (L) and resistance to severe malaria (Cockburn *et al.*, 2004). Contrary to expectation, the association was found only in heterozygotes (HL), and not in homozygotes (LL), which presumably had the lowest CR1 levels, and, although there was an association with resistance to severe malaria as a whole, there was no apparent association between these polymorphisms and CM. These results suggest that the relationship between CR1 copy number and severe malaria is not a clear cut one.

In contrast to this study, a previous case-control study in The Gambia (Zimmerman *et al.*, 2003) failed to demonstrate any significant association between *Sl2/2* and resistance to severe malaria. Despite the striking increased frequency of *Sl2* and McC^b in the Gambian study population, the study did not observe an association between these African CR1 alleles and protection from severe malaria phenotypes. In fact, the results showed a greater proportion of *Sl2/2* genotype in cases than in the controls. The divergence in findings between this and the previous study could be explained by important methodological differences. In The Gambian study controls did not have malaria. This may have led to an overrepresentation of non-protective genotypes in the controls because these children did not have the most important risk factor for severe malaria. Further, cases and controls were not matched by age in The Gambian study, and in fact cases were younger than controls by an average of almost one year, a difference

genotypes is greater in younger than older children with malaria, then comparing cases of severe malaria to older controls may have resulted in the overrepresentation of *Sl2/2* among the cases.

It is interesting that *Sl2* and *Sl2/2* $McC^{a/b}$ were more strongly associated with resistance to CM than to SMA. These differences probably are a reflection of the distinct pathogenic mechanisms between the two diseases, but they do not exclude a role for CR1 in the pathogenesis of SMA. The results obtained from this study have biological significance and offer further supportive evidence of the importance of CR1 in the pathogenesis of severe malaria. Whatever the mechanism by which CR1 is involved, knowledge of which alleles are associated with resistance or susceptibility to severe malaria will allow the opportunity to make observations on the relationship between structure and function and susceptibility to severe malaria.

5.2. Uncommon Genotypes In Western Kenya

In the study population, only six out of the ten possible *Sl/McC* genotype combinations were found. Although it was known whether the haplotypes for the group with genotype *Sl1/2 McCa/b* are 1a/2b or 1b/2a, it was assumed these individuals were 1a/2b because genotype $Sl1/1McC^{a/b}$, $Sl1/1McC^{b/b}$ and $Sl1/2McC^{b/b}$ were not found in the population. These observations suggested that haplotype 1b is extremely rare or does not exist in this population.

The possible explanation that could be given for the absence of these genotype combinations is mortality selection. This kind of selection suggests that certain genotypes are less successful than others in surviving through to the end of their reproductive period. Since the fitness of a genotype is measured by its reproduction rate relative to

other genotypes, fitter genotypes are those that have enough members surviving to reproduce substantial quantities. Perhaps individuals which had these genotype combinations were not found in the population because the combinations are too lethal and people who possess them do not survive long enough to reproduce. If an organism dies without reproducing, its genes disappear from the gene pool without being replicated.

The genotype combinations could occur in very low frequencies that would require huge sample size to detect in the study population. This could be another possible reason given to explain the absence of the genotype combinations that were never found in this study population of western Kenya.

5.3. Age-Related Prevalence Of *Sl2/2* Genotype

Different populations have evolved different genetic variants to protect against severe malaria. Genetic polymorphisms in CR1 may influence the susceptibility to severe malaria (Rowe et al., 1997). Since results from the age and gender matched case-control study demonstrated that Sl2 allele of the Sl blood group of CR1 may confer decreased susceptibility to cerebral malaria in an age-dependent manner, a follow up to this observation was made using samples from cross sectional study. These data were generated to describe the age-dependent prevalence of Sl and McC blood antigens of CR1.

The results showed an increase in the prevalence of *Sl2/2* genotype in individuals in the age groups >48-96 months and >32 years, although this did not reach statistical significance. The increase in the frequencies of *Sl2/2* particularly in this age group (>48-96 months) may suggest how age as a risk factor may influence the distribution of Sl

96 months) may suggest how age as a risk factor may influence the distribution of Sl alleles in an area with intense transmission of *P. falciparum* malaria. However, the role of other factors can not be excluded since various genetic variables may determine the risk of an exposed individual for developing severe malaria.

Moreover, there was a significant increase in the relative risk of *Sl2/2* genotype in the age group >48-96 months. These results are interesting in light of the fact that cerebral malaria complications tend to occur in children of this age group. These observations may suggest that *Sl2/2* could play some role in the protection against severe conditions of the disease, and that this protection may vary with age in exposed populations. In contrast, no age-related changes in the prevalence of the McC genotypes were observed.

Although it may be premature to make any strong conclusions based on these observations with regards to immunity to malaria, plausible hypotheses can be put forward that would explain these findings. The first one is that frequencies of *Sl2/2* would be expected to decline with an increase in age if immune mechanisms were involved in the observed malaria protective effect of *Sl2/2*. This could be explained by the fact that genotypes that are involved in immune mechanisms would increase with age up until when children generally become functionally immune to malaria. At this time, when immune system is fully developed and functions optimally, any additional immunological advantage should be lost. The other suggestion is that, if malaria protection by Sl2 was predominantly innate, it should not show any significant variation with age. It should remain constant with age.

From the perspective of malaria pathogenesis, the marked increase of Sl2 frequencies in the age group >4 to ≤8 years could underscore the protective function of this genotype

against malaria particularly cerebral malaria that predominantly manifests in this age group. It could be speculated that the other genotypes are not beneficial to individuals at this particular age group and are substantially wiped out of the population and majority do not stay in the population long enough to reach this age group. The forces that could lead to their decline may not necessarily be malaria infection. It could be due to other infectious diseases common in most developing country set ups.

Furthermore, the increase of *Sl2* in the age group >32 years could be due to the presence of a disease factor that would drive the upward trend of the genotype. Malaria may be involved. However these results are limited due to small sample size and the possibility of bias in the study design by exclusion of some malaria-positive individuals since the design was meant to answer other questions. A larger study is required to further explore the age-related changes in the prevalence of *Sl2/2*.

Although the protective effect of these alleles has been demonstrated, the mechanisms of this protection are still unclear and thus open to speculation. The study further sought to establish the effect of these protective polymorphic alleles on rosette formation. Several clinical and field studies have demonstrated the association between rosette formation and severe malaria (Carlson *et al.*, 1990; Kun *et al.*, 1998; Rowe *et al.*, 1995; Treutiger *et al.*, 1992). These studies have shown that the ability of *P. falciparum*-infected red cells to form rosettes correlates with an increase in disease severity. This has led to the suggestion that the high mortality due to *P .falciparum* malaria may be related to rosette formation which is positively associated with cerebral malaria. Rosetting has been demonstrated to involve the interaction between CR1 and PfEMP1. Rowe *et al.*

The exact role of Sl and McC alleles in conferring protection against severe forms of *P. falciparum* malaria is still not known. Numerous possible suggestions have however been given, beginning with the secondary and tertiary folding of CR1 protein that is thought to may bring together the SCR 25 in the vicinity of the active sites that directly interact with PfEMP1 (Krych-Goldberg *et. al.*, 2002). The other postulation is the role of SCR 25 in the CR1 clustering which is the process required for clearance of immune complexes by erythrocytes. Moreover, it has been hypothesised that changes in SCR 25 may affect the binding of c1q or Mannan binding lectin to LHR-D, proteins that might possibly be interacting with PfEMP1 (Krych-Goldberg *et. al.*, 2002).

5.4 Expression Of DBL1α Domain As A Chimeric Protein

Previous studies have demonstrated the relationship between group A *var* transtription and rosetting frequencies where group A transcripts have been shown to be more frequent in rosetting parasites compared to group B which are commonly transcribed in parasites obtained from children with severe malaria (Bull *et al.*, 2005; Kaestli *et al.*, 2006; Rottmann *et al.*, 2006). However, in this study, cDNA prepared from a predominantly rosetting portion of *P. falciparum* laboratory strain did not produce the expected products with UpsA primers. Instead, there were products of correct sizes with UpsB primers. This observation suggests that var genes in some groups would share certain functional features with var genes in another group leading to what is described as block-sharing characteristics (Bull *et al.*, 2005, 2008).

The observation that RBC bound to EBA175RII construct and never adhered to any of the DBL1α constructs could be explained by their lack of surface expression. Morever, experiments performed to acertain the surface expression did not detect these DBL1α

The observation that RBC bound to EBA175RII construct and never adhered to any of the DBL1α constructs could be explained by their lack of surface expression. Morever, experiments performed to acertain the surface expression did not detect these DBL1α constructs on the surface of mammalian cells (COS7 cells). Since binding assays would require that the ligand is located on the surface to effectively interact with its corresponding receptor, it was not possible for these chimeric proteins to bind RBCs.

The other explanation why the proteins were not detected on the surface of transfected mammalian cells would be their structural conformation. Proteins need to fold properly to function normally and misfolding leads to changes in the protein properties and functions. Since protein folding is related to amino acid sequences, alteration in these sequences would result into totally different proteins that would exhibit unique properties from the expected ones.

Other expression systems would not just work for some protein constructs. This could be another reason why DBL1α chimeric proteins did not express on the surface of COS7 cells. Expression systems where protein secretion and/or transport pathways are interfered with are difficult to work with. It is not clear at this stage to strongly single out any of these factors to be responsible for lack of surface expression of DBL1α domain constructs.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The results of this study have demonstrated that malaria has exerted selective pressure in different ways on CR1 gene. These findings support the general idea that CR1 plays significant role in the pathogenesis of severe malaria.

The observation that *Sl2* and *Sl2* McC$^{a/b}$ are more associated with protection against CM than SMA may possibly explain the different mechanisms of pathogenecity of these severe forms of malaria.

The absence of certain genotype combinations in the study population could suggest that these genotypes have been selected against because of their harmful effects in the population. The observation provides an indication that specific genotypes have been positively selected due to their advantageous effects against severe *P. falciparum* malaria.

The elevation of *Sl2/2* genotype frequencies in the age group >48 to ≤96 and >382 months may mean that the proportion of this genotype varies with age. This could suggest that its role in protection from malaria or its relationship to other diseases may vary with age.

Dominantly expressed DBL1α in SA075 is a group 2 sequence had a sequence signature **"LYLD-FREY-KAIT-2C-PTNL ("sig2")"**. "Sig2" signature was found among dominantly expressed sequences in isolates from children with severe malaria as also previously reported by Bull *et al.*, 2005.

DBL1α chimeric constructs were positive for GFP as a transfection marker. However, the protens were not detected on the surface and hence did not bind RBCs to form "rosettes".

The capacity of expressed sequences containing DBL1α domains was not tested because this required surface expression of these constructs. The system that was used to test for surface expression of recombinant proteins did not detect these proteins.

6.2 Study Recommendations

PfEMP1 has been shown to bind to active sites on CR1. It is therefore plausible that it may inhibit the functions of CR1. It would be important to study CR1 functions such as cofactor activity as well as decay-accelerating activity in order to better understand the CR1-PfEMP1 interaction and its role in malaria pathogenesis.

Larger population studies should be conducted in order to shed more light in the potential role of $McC^{a/b}$ and *Sl2/2* in conferring protection against severe *P. falciparum* malaria.

Since it is not possible to give much stronger conclusions about how age could influence the distribution of *Sl2/2* in a population exposed to malaria using the current study design and sample size, a larger and well designed study is required to further explore the relationship between *Sl2/2* prevalence and age.

The chimeric proteins containing DBL1α domain sequences already prepared in this study should be sequenced and their surface expression tested before they could be used in binding assays using RBCs genotyped for Sl and McC CR1 variant alleles. The RBCs should have their CR1 copy numbers determined besides determination of their ABO blood group antigens. These experiments could provide insights into the mechanisms of

protection of *Sl2/2* and *Sl2/2McC*$^{a/b}$ against malaria as observed in one of the sections of this study.

The genetic basis of resistance to malaria is an intricate one and involves many different genes that interact with environmental variables and parasite genetic factors. It would be important to study other human genes encoding proteins that have been shown to interact with the parasite to either positively or negatively influence the outcome of the disease.

6.3 Suggestion For Future Research

a) Studies involving larger populations should be conducted in order to understand how different genotypes influence malaria outcome.

b) The role of complement receptor 1 polymorphic variants in malaria co-infections should be established to better understand the clinical significance of such diseases on malaria severity.

c) The other host receptors should be studied to establish their roles in malaria and malaria co-infections.

d) The roles of other parasite ligands should be studied in order to have a broader picture of malaria pathogenesis. This will enhance the development of better management tools for the disease.

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APPENDICES

Appendix 1: Alignment of sequences analysed against sequences in the Plasmodium *spp database*

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                                ....|....|  ....|....|  ....|....|  ....|....|
....|....|
                                        10          20          30          40
50
R+400Clone#1 M13For     HEFADIGDII RGKDLYLDHE PGKQHLEERL ERIFENIKKK NNNN-
----E
R+400Clone#8 M13Rev     HEFADIGDII RGKDLYLDHE PGKQHLEERL ERIFENIKKK NNNN-
----E
R+400Clone#7 M13Rev     HEFADIGDII RGKDLYLDHE PGKQHLEERL ERIFENIKKK NNNN-
----E
R+400Clone#14 M13Fo     TSVADIGDII RGKDLYLDHE PGKQHLEERL ERIFENIKKK NNNN-
----E
R+400Clone#15 M13 f     AEFADIGDII RGKDLYLDHE PGKQHLEERL ERIFENIKKK NNNN-
----E
R-400Clone#1 M13Rev     AEFADIGDII RGKRFIP--W IMNQGKQHLE ERLERIFENI
KKKNNNN---
R-400Clone#2 M13Rev     AEFADIGDII RGKDLYLDHE PGKQHLEERL ERIFENIKKK NNNN-
----E
R-400Clone#3 Patche     AEFADIGDII RGIDLYLDHE PGKQHLEERL ERIFENIKKK NNNN-
----E
R-400Clone#5 M13Rev     AEFADIGDII RGKDLYLDHE PGKQHLEERL ERIFENIKKK NNNN-
----E
R-400Clone#12 M13Re     HEFADIGDII RGKDLYLDHE PGKQHLEERL ERIFENIKKK NNNN-
----E
R-400Clone#13 M13Re     HEFADIGDII RGKDLYLDHE PGKQHLEERL ERIFENIKKK NNNN-
----E
R+400Clone#4 M13For     HEFADIGDIV RGRDMFLP-- --NKDDK--V QKGLQVVFEK
INNGLKKI--
R+400Clone#10 M13Re     AEFADIGDIV RGRDMFLP-- --NKDDK--V QKGLQVVFEK
INNGLKKI--
R+400Clone#12 M13Re     HEFADIGDIV RGRDMFLP-- --NKDDK--V QKGLQVVFEK
INNGLKKI--
R+400Clone#9 M13For     AEFADIGDIV RGRDLYSG-- --NRKEKEKL QNTLKQIFGK
IYEELISTSG
R-400Clone#9 M13For     HEFADIGDII RGKDLFLGTT QEKKSLEENL KNIFRKLYNE LTKE-
----E
R-400Clone#10 M13Re     AEFADIGDII RGKDLFIGYN EKDKEEKKQL QDSLKKIFEK
IYNDVTSS--


                                ....|....|  ....|....|  ....|....|  ....|....|
....|....|
                                        60          70          80          90
100
R+400Clone#1 M13For     LNNLSLD--- --------KF REYWWALNRD QVWKAITCKA
PEEDHYFKPA
R+400Clone#8 M13Rev     LNNLSLD--- --------KF REYWWALNRD QVWKAITCKA
PEEDHYFKPA
R+400Clone#7 M13Rev     LNNLSLD--- --------KF REYWWALNRD QVWKAITCKA
PEEDHYFKPA
```

```
R+400Clone#14 M13Fo   LNNLSLD--- --------KF REYWWALNRD QVWKAITCKA
PEEDHYFKPA
R+400Clone#15 M13 f   LNNLSLD--- --------KF REYWWALNRD QVWKAITCKA
PEEDHYFKPA
R-400Clone#1 M13Rev   --ELNNLSLD ---------- -KFREYWWAL NRDQVWKAIT
CKAPEEDHYF
R-400Clone#2 M13Rev   LNNLSLD--- --------KF REYWWALNRD QVWKAITCKA
PEEDHYFKPA
R-400Clone#3 Patche   LNNLSLD--- --------KF REYWWALNRD QVWKAITCKA
PEEDHYFKPA
R-400Clone#5 M13Rev   LNNLSLD--- --------KF REYWWALNRD QVWKAITCKA
PEEDHYFKPA
R-400Clone#12 M13Re   LNNLSLD--- --------KF REYWWALNRD QVWKAITCKA
PEEDHYFKPA
R-400Clone#13 M13Re   LNNLSLD--- --------KF REYWWALNRD QVWKAITCKA
PEEDHYFKPA
R+400Clone#4 M13For   ---GINA--- ---YNDGSGN YSKLREVWWN VNRDQVWRAI
TCSAPGDVNY
R+400Clone#10 M13Re   ---GINA--- ---YNDGSGN YSKLREVWWN VNRDQVWRAI
TCSAPGDVNY
R+400Clone#12 M13Re   ---GINA--- ---YNDGSGN YSKLREVWWN VNRDQVWRAI
TCSAPGDVNY
R+400Clone#9 M13For   KTNGTNVDKA KARYGSDKEN YFQLREDWWE ANRKEVWKAI
TCSAPRDAEY
R-400Clone#9 M13For   ENGTAIKSRY ENDGPNYYQL REDWWALNRK EIRKAITCDT
EESDTYFKQS
R-400Clone#10 M13Re   ---GNNKDTL KERYQNDGDN YYKLREDWWD ANRKDVWRAI
TCGAGEGDRY

                      ....|....| ....|....| ....|....| ....|....|
....|.
                              110        120        130        140
R+400Clone#1 M13For   QN-----RKR EFTDGHC-GH RQGNVPTNLD YVPQFLRWSR R-
....
R+400Clone#8 M13Rev   QN-----RKR EFTDGHC-GH RQGNVPTNLD YVPQFLRWFE
NG....
R+400Clone#7 M13Rev   QN-----RKR EFTDGHC-GH RQGNVPTNLD YVPQFLRLVR
EG....
R+400Clone#14 M13Fo   QN-----RKR EFTDGHC-GH RQGNVPTNLD YVPQFLRRFE
NG....
R+400Clone#15 M13 f   QN-----RKR EFTDGHC-GH RQGNVPTNLD YVPQFLRWFE
KG....
R-400Clone#1 M13Rev   KPAQN----- RKREFTDGHC -GHRQGNVPT NLDYVPQFLR
WFEKG.
R-400Clone#2 M13Rev   QN-----RKR EFTDGHC-GH RQGNVPTNLD YVPQFLRWFE
NG....
R-400Clone#3 Patche   QN-----RKR EFTDGHC-GH RQGNVPTNLD YVPQFLRWFE
KG....
R-400Clone#5 M13Rev   QN-----RKR EFTDGHC-GH RQGNVPTNLD YVPQFLRWFE
NG....
R-400Clone#12 M13Re   QN-----RKR EFTDGHC-GH RQGNVPTNLD YVPQFLRWFE
KG....
R-400Clone#13 M13Re   QN-----RKR EFTDGHC-GH RQGNVPTNLD YVPQFLRWFE
NG....
R+400Clone#4 M13For   FRKISG---- DTRTFENAGK CRRHDN-KVP TNLDYVPQFL
RWFENG
```

```
R+400Clone#10 M13Re    FRKISG---- DTRTFENAGK CRRHDN-KVP TNLDYVPQFL
RWFEKG
R+400Clone#12 M13Re    FRKISG---- DTRTFENAGK CRRHDN-KVP TNLDYVPQFL
RWFENG
R+400Clone#9 M13For    FHATCGDDGK TGTLAKNNCR CDGSNADQVP TYFDYVPQYL
RWFENG
R-400Clone#9 M13For    SE-----GKY SFTNGQC-GH NEENVLTNLD YVPQFLRWFE
NG....
R-400Clone#10 M13Re    SKTIT----- YGTTTTSNSK C-GHDDQDVQ TYLDYVPQYL
RWFENG
```

Appendix 2: Summary of sequences analyzed for both R+ and R- clones

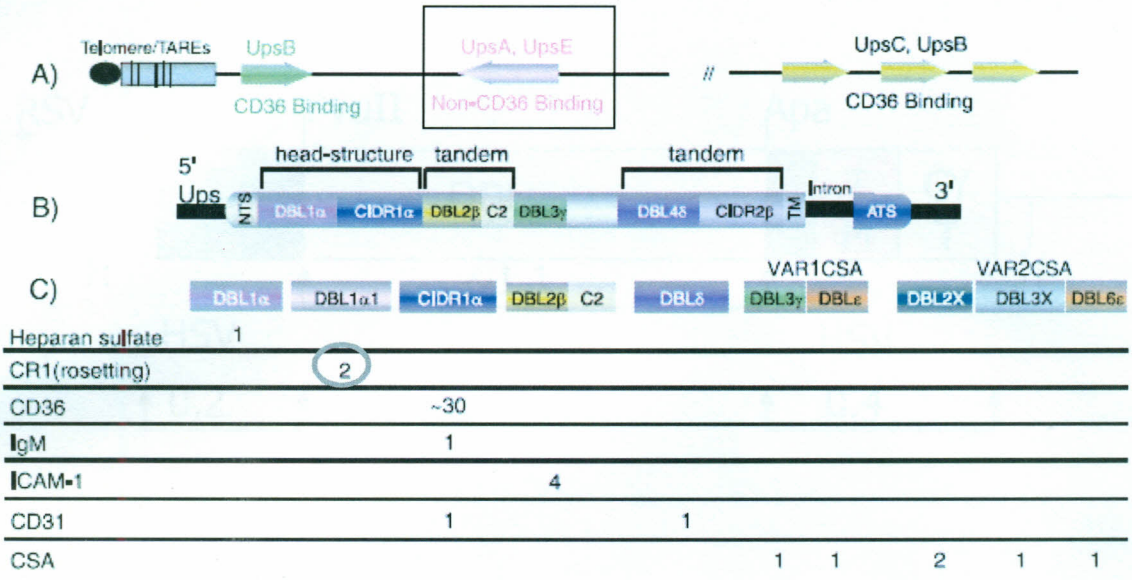
R+ (10 SEQUENCES analyzed; 1 clone failed; 1 incomplete)

*PoLV1 PoLV2 PoLV3 PoLV4	
DLYLD-FREYWW-WKAIT-2C-PTNLD (5 SEQUENCES)	GROUP 2(*REY)
DMFLP-LREVWW-WRAIT-2C-PTNLD (3 SEQUENCES)	GROUP 1/3?(MFL*)
DMFKP-LREVWW-WEAIT-2C-PTNID (1 SEQUENCE)	GROUP 1(MFK*)
DLYSG-LREDWW-WKAIT-4C-PTYFD (1 SEQUENCE)	GROUP 4

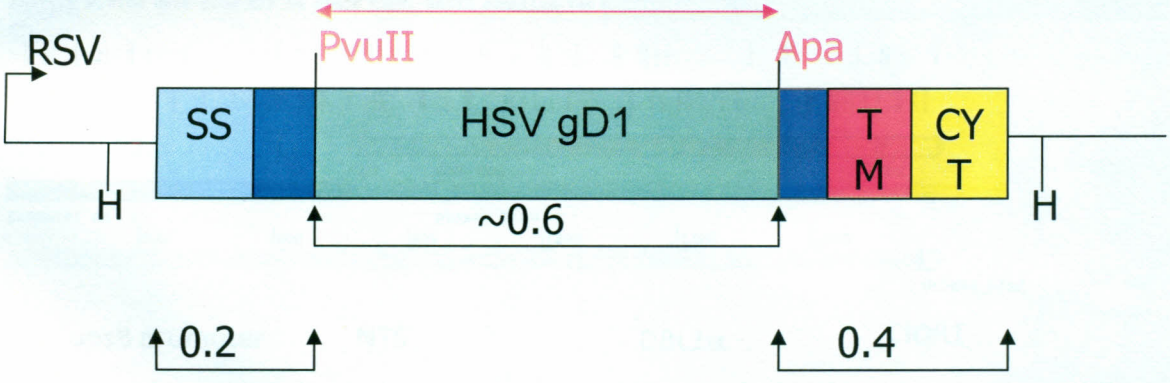
R- (8 SEQUENCES analyzed; 1 cloned failed completely/ 3 clones had M13R sequence only)

*PoLV1 PoLV2 PoLV3 PoLV4	
DLYLD-FREYWW-WKAIT-2C-PTNLD (6 SEQUENCES)	GROUP 2(*REY)
DLFLG-LREDWW-RKAIT-2C-LTNLD (1 SEQUENCE)	GROUP 3
DLFIG-LREDWW-WRAIT-2C-QTYLD (1 SEQUENCE)	GROUP 3

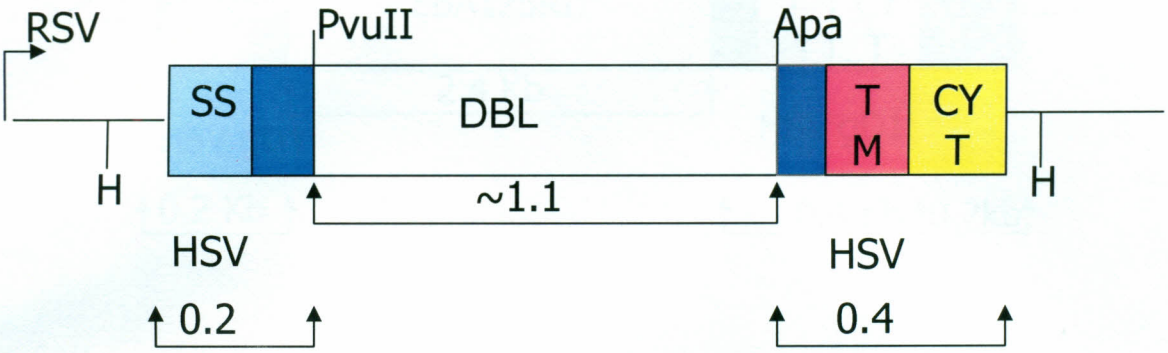
Appendix 3: Host receptor-PfEMP1 domain interactions (Kyes, Kraemer & Smith, Eukaryote Cell 2007)



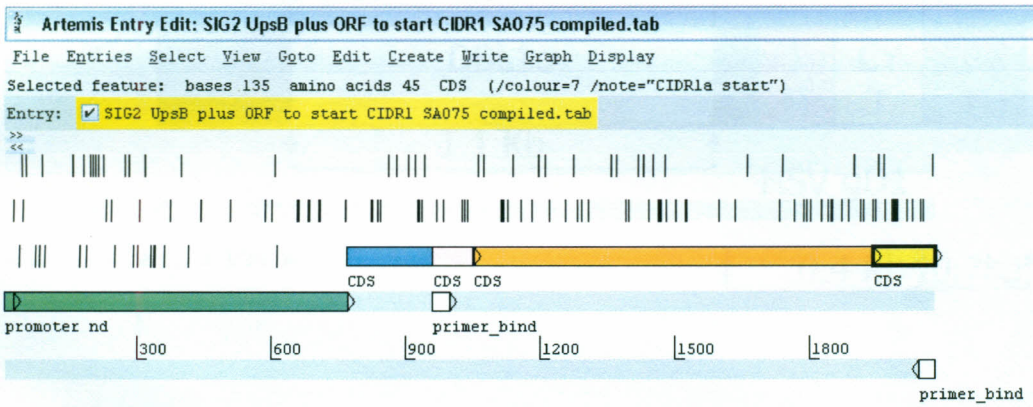
Appendix 4: pRE4 Vector



Appendix 5: pRE4-DBL1α construct

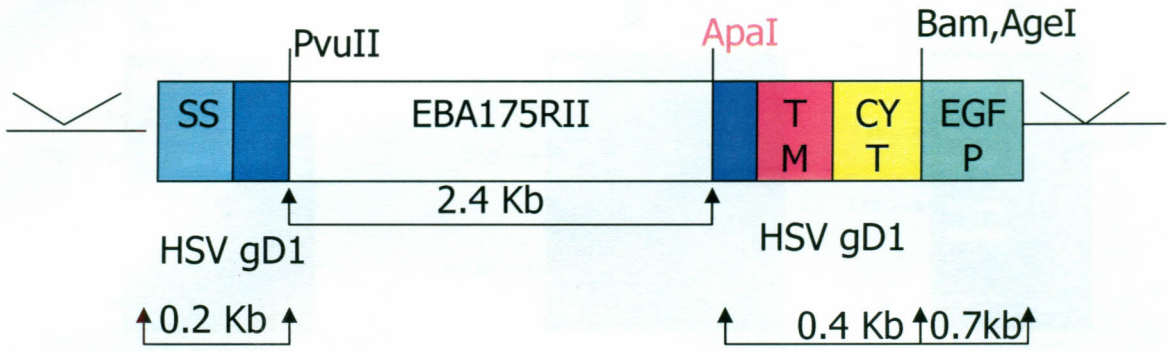


Appendix 6 DBL1α domain expression coding sequences

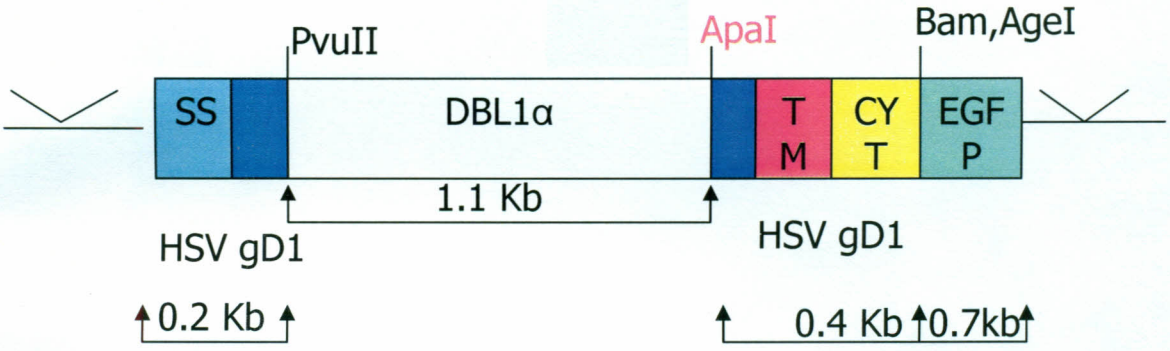


upsB promoter NTS DBL1α CIDR1

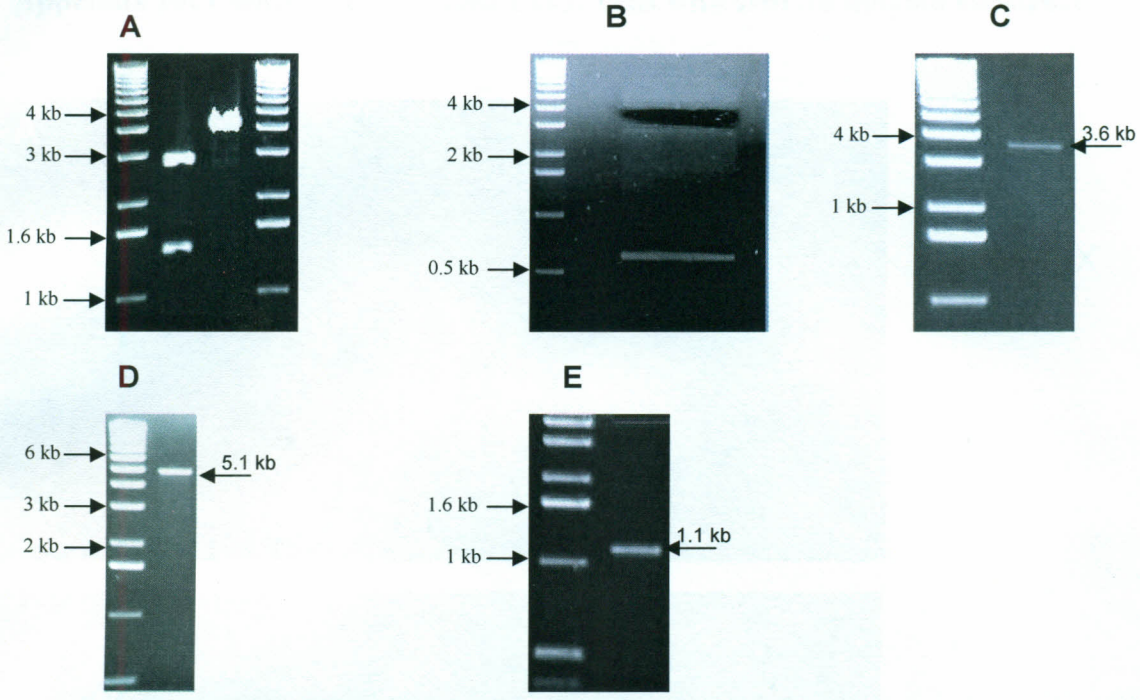
Appendix 7: Expression Vector; pEGFPNI-EBA175RII/HSV gD1



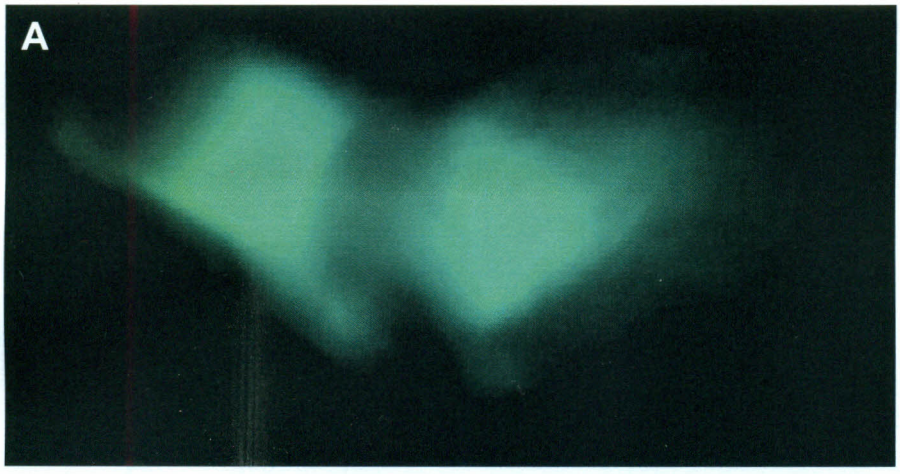
Appendix 8: Expression construct: pEGFPN1-HSV-DBL1α



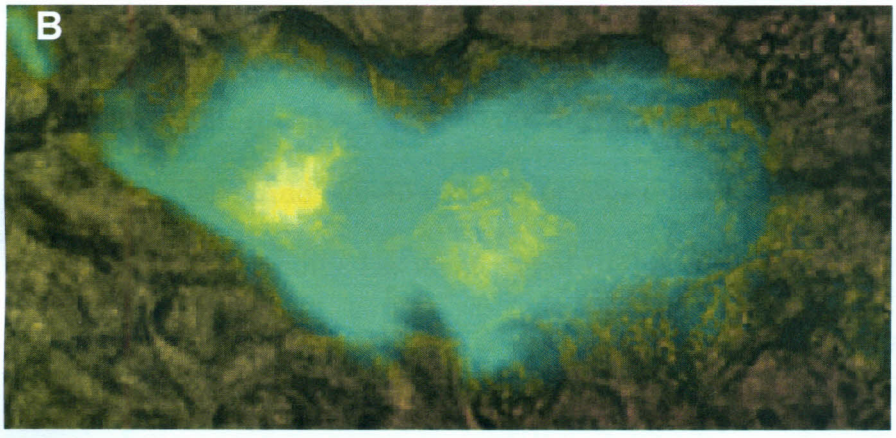
Appendix 9: Gel analysis of vector and representative contruct preparation



Appendix 10: Positively transfected COS7 cells with DBL1α domain construct



GFP 40X



GFP-Phase 40X

Appendix 11: Preparation Of Buffers And Media

To prepare 1L, 1X phosphate buffered saline (PBS) pH 7.4

Weigh the following into a 1 litre beaker: 8 g of NaCl, 0.2 g of KCl, 1.44 g of NaHPO4 and 0.24 g of KH_2PO4 and star in nearly 1 litre of distilled water. Bring the pH to 7.4 by adding HCl and adjust the volume to 1 liter with additional dH2O. Filter-sterile or autoclave.

To prepare 1L, 50X TAE Gel Electrophoresis buffer

Weigh the following into a 1 litre beaker: 242 g Tris base and stir in nearly 1 litre of distilled water. Add 57.1 ml Glacial acetic acid, 100 ml, 0.5M EDTA pH 8.0. Check the pH and if necessary adjust to 8.5 with glacial acetic acid. Bring to final volume of 1 litre with dH_2O.

To prepare 1L, 1X TAE buffer

Measure 20 ml of 50X TAE stock in a 1 litre measuring sylinder and top with dH_2O.

To prepare 1L, 0.5M EDTA pH 8.0

Weigh 148 g EDTA and add to ~800 ml ddH2O and stir vigorously on a magnetic stirrer with minimal heat. Add ~20-30 g NaOH to adjust the pH to 8.0. NaOH is added to adjust pH and enhance solubility. Disodium salt of EDTA will not dissolve without the addition of NaOH. Bring volume to 1L with ddH_2O. Can be autoclaved if needed sterilized. Store at RT.

To prepare 10 mL, 10X DNA loading dye

Weigh 1.5 g Ficoll, 0.02 g bromophenol blue, 0.02 g xylene cyanol FF and dissolve in 10 mL ddH_2O. Aliquot in 1.5 ml tubes and store at room temperature.

To prepare 0.5L, 3M NaAc pH 5.2

Weigh 204g NaAc.$3H_2O$ (F136) and dissolve in ~400 ml distilled H_2O. Adjust the pH by glacial acetic acid to 5.2. Bring the final volume to 0.5L with distilled H_2O. Autoclave and store at RT.

To prepare 10mL, 100mg/ml ampicilin

Weigh 1g of ampicilin and dissolve in 8mL of ddH_2O by vortexing. Adjust the volume to 10mL with ddH_2O. Filter sterilize the solution into a 15 ml falcon tube using a 20 ml syringe and a 0.22 μm filter. Make 500 μl aliquots into 1.5 ml eppendorf tubes and store at -20°C.

To prepare 10mL, 50mg/ml kanamycin

Weigh 0.5g of kanamycin and dissolve in 8ml of ddH_2O by vortexing. Adjust the volume to 10mL with ddH_2O. Filter sterilize the solution into a 15 ml falcon tube using a 20 ml syringe and a 0.22 μm filter. Make 500 μl aliquots into 1.5 ml eppendorf tubes and store at -20°C.

To prepare 10mL, 20mg/ml isopropylthio-b-D-galactoside (IPTG)

Weigh 0.2g of IPTG and dissolve in 8mL ddH_2O. Adjust the volume of the solution to 10mL with ddH_2O and sterilize by passing through a 0.22um disposable filter. Dispense the solution into 1 mL aliquots and store them at -20°C.

To prepare 10mL, 20mg/mL 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal)

Weigh 0.2g of X-gal and dissolve in 8mL dimethylformamide. Adjust the volume to 10mL with dimethylformamide. Dispense the solution into polypropylene tubes in 1ml aliquots. Wrap the tubes in aluminum foil to prevent damage by light and store them at -20°C. It is not necessary to sterilize X-gal solutions.

To prepare 1L, LB medium

Weigh 25g of LB powder and dissolve in ~800ml of ddH_2O by mixing until the powder goes into solution. Adjust the volume to 1L with ddH_2O. Sterilize by autoclaving and store at RT. LB is stable at RT for several months. However, if there are any signs of contamination or the medium turns cloudy, discard and prepare fresh.

To prepare 1L, LB agar

Dispense 15g per litre of agar directly into final vessel and autoclave. Agar will not go into solution until autoclaved or boiled. If adding antibiotic(s), allow to cool until warm to the touch with bare hand then add the antibiotic(s). Swirl to mix and dispense ~20ml per plate. Allow plates to dry and store in original Petri plate bags, inverted, at 4°C for a maximum of 2 weeks.

To prepare 1L, 10% sodium lauryl (or dodecyl) sulphate (SDS)

Dissolve 100g of electrophoresis-grade SDS in ~900ml of ddH_2O. Heat to 68°C and stir with a magnetic stirrer to enhance dissolution. Adjust the pH 7.2 by adding a few drops

of concentrated HCl if it is necessary. Adjust the volume to 1L with ddH_2O and store at RT. Sterilization is not necessary. Do not autoclave.

Appendix 12: Restriction enzymes used and their recognition sites

Enzyme	Source	Recognition sequence	Cut
BsmI	***Bacilus stearothermophilus***	**5'...GAATGCN$^{\vee}$...3'**	**5'...GAATGCN...3'**
		3'...CTTAC$^{\wedge}$GN...5'	**3'...CTTAC GN...5'**
MfeI	***Mycoplasma fermentas***	**5'...C$^{\vee}$AATTG...3'**	**5'....C AATTG...3'**
		3'...GTTAA$^{\wedge}$C...5'	**3'....GTTAA C...5'**
ApaI		**5'...GGGCC$^{\vee}$C...3'**	**5...GGGCC C...3'**
		3'...C$^{\wedge}$CCGGG...5'	**3'...C CCGGG...5'**
PvuII		**5'...CAG$^{\vee}$CTG...3'**	**5'...CAG CTG...3'**
		3'...GTC$^{\wedge}$GAC...5'	**3'...GTC GAC...5'**
HindIII		**5'...A$^{\vee}$AGCTT...3'**	**5'...A AGCTT...3'**
		3'...TTCGA$^{\wedge}$A...5'	**3'...TTCGA A...5'**
BamHI		**5'...G$^{\vee}$GATCC...3'**	**5'...G GATCC...3'**
		3'...CCTAG$^{\wedge}$G...5'	**3'...CCTAG G...5'**

Appendix 13 Abstracts for conferences/seminars/ workshops/ publications

Age-related Changes in Prevalence of The Swain-Langley and McCoy Blood Group Polymorphisms of Complement Receptor 1 in Western Kenya

Bernard Guyah[1,3], Aloys S.S Orago[3], Michael F. Otieno[3], Vandana Thathy[1], Walter Otieno[1], and Jose A. Stoute[2].

1 Kenya Medical Research Institute, Nairobi, Kenya and 2 The US Army Medical Research Unit-Kenya, 3 Kenyatta University, Nairobi Kenya

Abstract

The complement receptor 1 is a 200 kD protein found on the surface of erythrocytes. It mediates the binding of *Plasmodium falciparum*-infected red blood cells (RBCs) to uninfected RBCs to form rosettes, a phenomenon that has been associated with severe malaria in Africa. The *CR1* gene contains Swain-Langley and McCoy Knops blood group antigens with respective alleles *SI1/2* and *McCa/McCb. McCb and SI2* are nearly exclusively found in persons of African descent. Preliminary studies by our research team suggest that *SI2/2* may confer decreased susceptibility to malaria in an age-dependent manner. In order to study the age-dependent *prevalence* of the African alleles, we carried out a cross-sectional survey of 345 inhabitants of Kombewa Division, western Kenya, an area of intense malaria transmission. The data was stratified by age-group as follows: 0-6 months, >6-12 months, >12-24 months, >24-48 months, >48-96 months, >8-16 years, >16-32 years, and 32 years. Crosstabulation was carried out to determine whether the proportion of individuals with Sl2 differ among age-group. Logistic regression was performed to investigate the relative risk

of *Sl2/2* genotype for each age group taking into account other potential confounding variables.

Malaria Journal

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Research

Open Ac

Complement receptor 1 polymorphisms associated with resistance to severe malaria in Kenya

Vandana Thathy[1], JoAnn M Moulds[2,4], Bernard Guyah[1], Walter Otieno[1] and José A Stoute*[3]

Address: [1]The US Army Medical Research Unit and the Kenya Medical Research Institute, Nairobi, Kenya, [2]Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA, USA, [3]Department of Cellular Injury, Walter Reed Army Institute of Research, Silver Spring, MD and Department of Medicine, Uniformed Services University of Health Sciences, Bethesda, MD, USA and [4]LifeShare Blood Centers, Shreveport, LA, USA

Email: Vandana Thathy - vthathy@wrp-ksm.org; JoAnn M Moulds - jmmoulds@lifeshare.org; Bernard Guyah - Bguyah@wrp-ksm.org; Walter Otieno - Wotieno@wrp-ksm.org; José A Stoute* - jose.stoute@us.army.mil

* Corresponding author

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Abstract

Background: It has been hypothesized that the African alleles *Sl2* and *McC*^{b} of the Swain-Langley (Sl) and McCoy (McC) blood group antigens of the complement receptor 1 (CR1) may confer a survival advantage in the setting of *Plasmodium falciparum* malaria, but this has not been demonstrated.

Methods: To test this hypothesis, children in western Kenya with severe malaria-associated anaemia or cerebral malaria were matched to symptomatic uncomplicated malaria controls by age and gender. Swain-Langley and McCoy blood group alleles were determined by restriction fragment length polymorphism and conditional logistic regression was carried out.

Results: No significant association was found between the African alleles and severe malaria-associated anaemia. However, children with *Sl2/2* genotype were less likely to have cerebral malaria (OR = 0.17, 95% CI 0.04 to 0.72, P = 0.02) than children with *Sl1/1*. In particular, individuals with *Sl2/2 McC*$^{a/b}$ genotype were less likely to have cerebral malaria (OR = 0.18, 95% CI 0.04 to 0.77, P = 0.02) than individuals with *Sl1/1 McC*$^{a/a}$.

Conclusion: These results support the hypothesis that the *Sl2* allele and, possibly, the *McC*^{b} allele evolved in the context of malaria transmission and that in certain combinations probably confer a survival advantage on these populations.

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Plasmodium falciparum antigenic variation. Mapping mosaic *var* gene sequences onto a network of shared, highly polymorphic sequence blocks

Peter C. Bull,[1,2]* Caroline O. Buckee,[1,3] Sue Kyes,[2] Moses M. Kortok,[1] Vandana Thathy,[1,4] Bernard Guyah,[4] José A. Stoute,[4] Chris I. Newbold[2] and Kevin Marsh[1,2]

[1]*Kenya Medical Research Institute Centre for Geographic Medicine Research, Coast, Kilifi, Kenya.*
[2]*Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford, UK.*
[3]*Department of Zoology, University of Oxford, Oxford, UK.*
[4]*US Army Medical Research Unit-Kenya, Kisumu, Kenya.*

Summary

***Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) is a potentially important family of immune targets, encoded by an extremely diverse gene family called *var*. Understanding of the genetic organization of *var* genes is hampered by sequence mosaicism that results from a long history of non-homologous recombination. Here we have used software designed to analyse social networks to visualize the relationships between large collections of short *var* sequences tags sampled from clinical parasite isolates. In this approach, two sequences are connected if they share one or more highly polymorphic sequence blocks. The results show that the majority of analysed sequences including several *var*-like sequences from the chimpanzee parasite *Plasmodium reichenowi* can be either directly or indirectly linked together in a single unbroken network. However, the network is highly structured and contains putative subgroups of recombining sequences. The major subgroup contains the previously described group A *var* genes, previously proposed to be genetically distinct. Another subgroup contains sequences found to be associated with rosetting, a parasite virulence phenotype. The mosaic structure of the sequences and their division into subgroups may reflect the conflicting problems of maximizing antigenic diversity and minimizing epitope sharing between variants while maintaining their host cell binding functions.**

Accepted 6 April, 2008. *For correspondence. E-mail pbull@kilifi.kemri-wellcome.org; Tel. (+254) 41522063; Fax (+254) 41522390.

Introduction

Children living in malaria endemic areas develop significant naturally acquired immunity to severe malaria during the first 5 years of life (Marsh, 1992). The variant surface antigens (VSA) expressed on malaria-infected erythrocytes are strong candidate targets of naturally acquired immunity as they are exposed to host antibodies for long periods while the parasite is still alive. The major component of VSA, called PfEMP1 (*P. falciparum* erythrocyte membrane protein 1) is encoded by a family of approximately 60 *var* genes per genome (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995). These molecules are implicated as virulence factors. Through interactions with host molecules such as ICAM-1, CD36, CR1 and CD31, PfEMP1 plays a central role in mediating cytoadherence of infected erythrocytes to host cells. Cytoadherence is believed to be responsible for the severe pathology associated with *P. falciparum* malaria (Craig and Scherf, 2001; Kyes *et al.*, 2001; Baruch *et al.*, 2002; Flick and Chen, 2004). PfEMP1 molecules undergo clonal antigenic variation meaning that a single genotype can evade host antibodies by switching between *var* genes (Roberts *et al.*, 1992). After repeated exposure to infection, a repertoire of antibodies build up that can recognize most VSA circulating in the parasite population. The gradual restriction of the PfEMP1 molecules capable of sustaining infection as the host antibody repertoire develops could potentially explain the observed modification of the host parasite relationship that occurs during the development of naturally acquired immunity to malaria (Bull *et al.*, 1998; Giha *et al.*, 2000).

var genes have a modular organization (Smith *et al.*, 2000; Gardner *et al.*, 2002; Lavstsen *et al.*, 2003) consisting of various numbers and combinations of duffy binding-like (DBL) domains of different types (α, β, δ, ε, γ and x) and cysteine rich interdomain regions (CIDR), again of