PREVALENCE OF *Plasmodium falciparum* ERYTHROCYTE BINDING LIGAND -1 AND HUMAN GLYCOPHORIN B NULL GENOTYPES IN KISUMU AND MOMBASA DISTRICTS OF KENYA

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DECLARATION AND RECOMMENDATION

Declaration

I hereby declare that this research thesis is my original work and has not been presented wholly or in part for any award in any other institution of learning.

Evalyne Muthoni Karanja Signed: …… Date: ……

Recommendation

We confirm that this research thesis was prepared under our supervision and has our approval to be presented for examination as per the Egerton University regulations.

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DEDICATION

This thesis is dedicated to my loving mum, dad, brother and sister for their support throughout the period of this research project.

The Lord recompenses you for what you have done, and a full reward will be given to you by the Lord….

Ruth 2: 12

ACKNOWLEDGEMENT

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ABSTRACT

Plasmodium, the causative agent, invades red blood cells on the basis of receptor- ligand interaction. Glycophorin B (GYP B), a transmembrane protein, is among identified red blood cell receptor that binds to *P. falciparum* Erythrocyte Binding Ligand-1(EBL-1). A mutation in GYP B gene impairs EBL-1 affinity, hence reducing malaria infection in the process. A community in the Democratic Republic of Congo (Efe pygmies) has eliminated glycophorin B gene from their gene pool through gene deletion, allowing them to be partially protected from malaria. Malaria is endemic in both Kisumu and Mombasa, which would cause human hosts to eliminate receptor genes that make them susceptible to infection. Prevalence of GYP B null genotypes and EBL-1 was to be established in Kisumu and Mombasa Districts. Parasite and human DNA was extracted from infected blood samples from children (5 years and older) and adults attending district hospitals in Kisumu and Mombasa, using standard phenol chloroform method. The GYP B and EBL-1 genotypes were detected through amplification using gene specific primers coupled to sequencing of the fragments. Five and 20 percent of the twenty samples each from Kisumu and Mombasa respectively were negative of the amplification (GYP B null genotype), whereas all the samples amplified for EBL-1. Sequenced glycophorin B fragments were aligned to the GYP B sequence in the NCBI database using routines in MEGA 5 genetic analysis software. There was presence of deletion in the GYP B gene, while the parasites have maintained their ligands. This study provides a direction into studying the highly polymorphic GYP B gene which could also be playing part in the recently observed decline in malaria in Kenya and Africa in general. These findings also putatively indicate the susceptibility of people living in Kisumu and Mombasa based on the maintained genes responsible for the malaria parasite receptors on the red blood cells. Hence they may influence public health approaches to malaria control in the regions.

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LIST OF ABBREVIATIONS

dNTPs	deoxynucleotide triphosphates
ddNTPs	dideoxynucleotides triphosphates
EBL-1	Erythrocyte Binding Ligand - 1
EDTA	Ethylene diamine tetra acetate
GYP	Glycophorin
NMK	National Museum of Kenya
SDS	Sodium Dodecyl Sulphate
STE	Sodium Tris EDTA
TBE	Tris Boric EDTA
TBS	Tris buffered saline
TE	Tris EDTA

LIST OF FIGURES

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

WHO estimates that malaria is responsible for an annual morbidity and mortality of 500 and 1.5- 2.7 million people respectively, mostly in children under 5 yrs in sub- Saharan Africa (Swan *et al.,* 2005; UNICEF, 2008). *Plasmodium falciparum,* causative agent of the most severe form of human malaria, has the most debilitating erythrocytic stage among causative agents (Mayer *et al.,* 2001; Moritz *et al.,* 2006). *Plasmodium falciparum* invasion is contingent to specific merozoites ligand - erythrocytes receptor interactions (Curtidor *et al.,* 2004; Lantos *et al.,* 2008). These receptors include glycophorins (GYP), specific for (DBL-EBP) Duffy binding like erythrocytes binding proteins (Goel *et al.,* 2002). Among GYPs, GYP B receptors bind to erythrocyte binding ligand -1 (EBL-1) protein, facilitating successful infection of the erythrocyte by the parasite in some but not all cases (Lobo *et al.,* 2004; Deans *et al.,* 2007; Mayer *et al.,* 2009).

Erythrocyte binding ligand – 1, a merozoite protein, is encoded by the *ebl- 1* gene, a member of the erythrocyte binding- like family (Peterson *et al.,* 1995; Curtidor *et al.,* 2004). It is among multiple *Plasmodium* ligands that enhance merozoite invasion (Dolan *et al.,* 1994; Chitnis and Blackman, 2000). As opposed to other members of its family which have eight conserved regions, the *ebl- 1* gene has four and they mediate binding to glycophorins on the RBC's surface by the parasite (Smith *et al.,* 2000; Adams *et al.,* 2001).

Plasmodium prevalence in endemic regions has driven the host into adapting genetic mutations that have attributed to protection against malaria (Baird *et al.,* 1991; Kwiatkowski, 2005). Diseases such as the sickle cell trait, thalassemias, ovalocytosis, hemoglobin C and E and deficiencies in glucose-6-phosphate dehydrogenase, are examples of such a phenomenon (Holding *et al.,* 2001; Carter and Mendis, 2002). Similarly, glycophorin B is highly polymorphic, with partial gene knock outs specifically leading to loss of the GYP B phenotype (GYP B null) in inhabitants of malaria endemic regions (Huang *et al.,* 1991; Rahuel *et al.,* 1991; Mayer *et al.,* 2009). The erythrocytes with GYP B null phenotype are

known as S-s-U- erythrocytes and give rise to S-s-U- blood type, which is refractory to *Plasmodium* infection (Pasvol, 2003). This phenotype arises from two gene alterations with the well understood alterations being a large deletion in the gene (Rahuel *et al.,* 1991). Correlations between frequency of GYP B null allele and resistance to malaria infection in humans have been established in nature (Fraser *et al.,* 1966; Mayer *et al.,* 2009; Heathcote *et al.,* 2011). This thesis project study, estimates the frequency of the S-s-U- blood type, as proxy for GYP B allele in residents of Kisumu and Mombasa, malaria endemic districts in Kenya.

1.2 Statement of the problem

Plasmodium falciparum malaria parasite has been in Sub- Saharan Africa for approximately 400 million years and causes the most severe form of malaria. The impact is felt particularly among the young children and expectant women. Combined strategies of controlling malaria are becoming increasingly successful due improved drug interventions and use of treated bed nets. However insecticide resistance by mosquitoes compounded by elevated poverty levels is still derailing the malaria control process. The ability of the parasite to invade the human erythrocyte protects it from the immune system. Invasion of the parasite is facilitated by a receptor-ligand interaction of the host's red blood cells (RBCs) and *P. falciparum* merozoites respectively. These receptors include RBC sialoglycoproiteins known as glycophorins that bind to specific parasite merozoite proteins.

Among the glycophorins, GYP B is polymorphic with a gene deletion that causes silencing of the gene being most common which prevents expression of the respective proteins on the RBC surface. This polymorphism has been shown in Efe pygmies of the *Ituri* forest in the Democratic Republic of Congo where *Plasmodium falciparum* is endemic. This allows this community to have some partial resistance to malaria which may lead to a variation of malaria prevalence. Studies have been conducted to establish prevalence of *GYP A*, *GYP B* and *GYP E* in several ethnic groups in Africa.

1.3 Objectives

1.3.1 General Objective

1. To determine the prevalence of a partially protective glycophorin B polymorphism and erythrocyte binding ligand (*ebl-1)* gene in Kisumu and Mombasa.

1.3.2 Specific Objectives

1. To determine the prevalence of glycophorin B null genotype in blood samples from malaria infected people in Kisumu and Mombasa.
2. To determine the prevalence of ebl-1 gene in blood samples from malaria infected people in Kisumu and Mombasa.

1.4 Hypothesis of the study

1. The prevalence of human glycophorin B null genotypes in blood samples from malaria infected people in Kisumu and Mombasa is similar.
2. The prevalence of *Plasmodium falciparum* ebl-1 genotype in blood samples from malaria infected people in Kisumu and Mombasa is similar.

1.5 Justification

Glycophorin B, an EBL-1 protein receptor, modulates susceptibility of individuals to *Plasmodium* infection in malaria endemic regions. The genome of people living in these regions is under evolutionary selection pressure to eliminate the genes responsible for the receptors and associated proteins from the local gene pool. Under these circumstances, it is expected that polymorphism of these genes will favor the null genotype, wherein this genotype will confer relative protection of the respective population from malaria infection. Knowledge of the prevalence of the genotypes (susceptible or resistant) in the population will aid in guiding the malaria intervention policy in the affected regions in terms of resource mobilization, including chemotherapy regiments to be adopted.

CHAPTER TWO

2.0 LITERATURE REVIEW

Malaria is one of the most life threatening infectious disease causing approximately one million deaths annually, of which ninety percent are in Africa. This calls for determination of the populations at risk to enhance resource allocation (Snow *et al.,* 2005; WHO 2008; Ko *et al.,* 2011). However there is substantial decline in malaria morbidity and mortality in African countries including Kenya, due to improved malaria intervention strategies (Zhou *et al.,* 2011). Unlike other *Plasmodium* species (*Plasmodium vivax, Plasmodium malariae, Plasmodium ovale*), *Plasmodium falciparum* is the most common in Sub- Saharan Africa and causes the severest form of disease as it invades both young and old erythrocytes (Mullen *et al.,* 2009). The infection mechanism uses cell adhesion of the parasite to multiple receptors in the human host cells (Okoyeh *et al.,* 1999; Barbin *et al*., 2004; Cavasini *et al.,* 2007; Lantos *et al.,* 2008; Maier *et al.,* 2009b).

The malaria parasite exhibits a complex life cycle (Figure 1) involving an insecvector (mosquito) and a human host (Fujioka and Aikawa, 2002). Sporozoites are transmitted through a mosquito bite on the human host and undergo asexual replication in liver hepatocytes. This results in production of merozoites which are released into the blood circulation and invade the erythrocytes. The invasion marks the beginning of an erythrocytic cycle that involves rupturing and invasion of new erythrocytes (Pasvol, 2003).

Four key steps in merozoite invasion are attachment, re-orientation, tight junction formation, and entry, all taking place in a span of about 20 seconds (Figure 2; Gratzer and Dluzewski 1993; Pasvol, 2003). The attachment to the RBC surface is mediated by specific ligand on the parasite and a receptor in the host interaction followed by reorientation of apical end of the parasite to erythrocyte surface. The apical end forms a tight junction with the erythrocyte surface followed by formation of a parasitophorous vacuole to facilitate parasite entry into the erythrocyte. (Adams *et al.,* 2001; Pasvol, 2003; Deans *et al.,* 2007; Singh *et al.,* 2010).

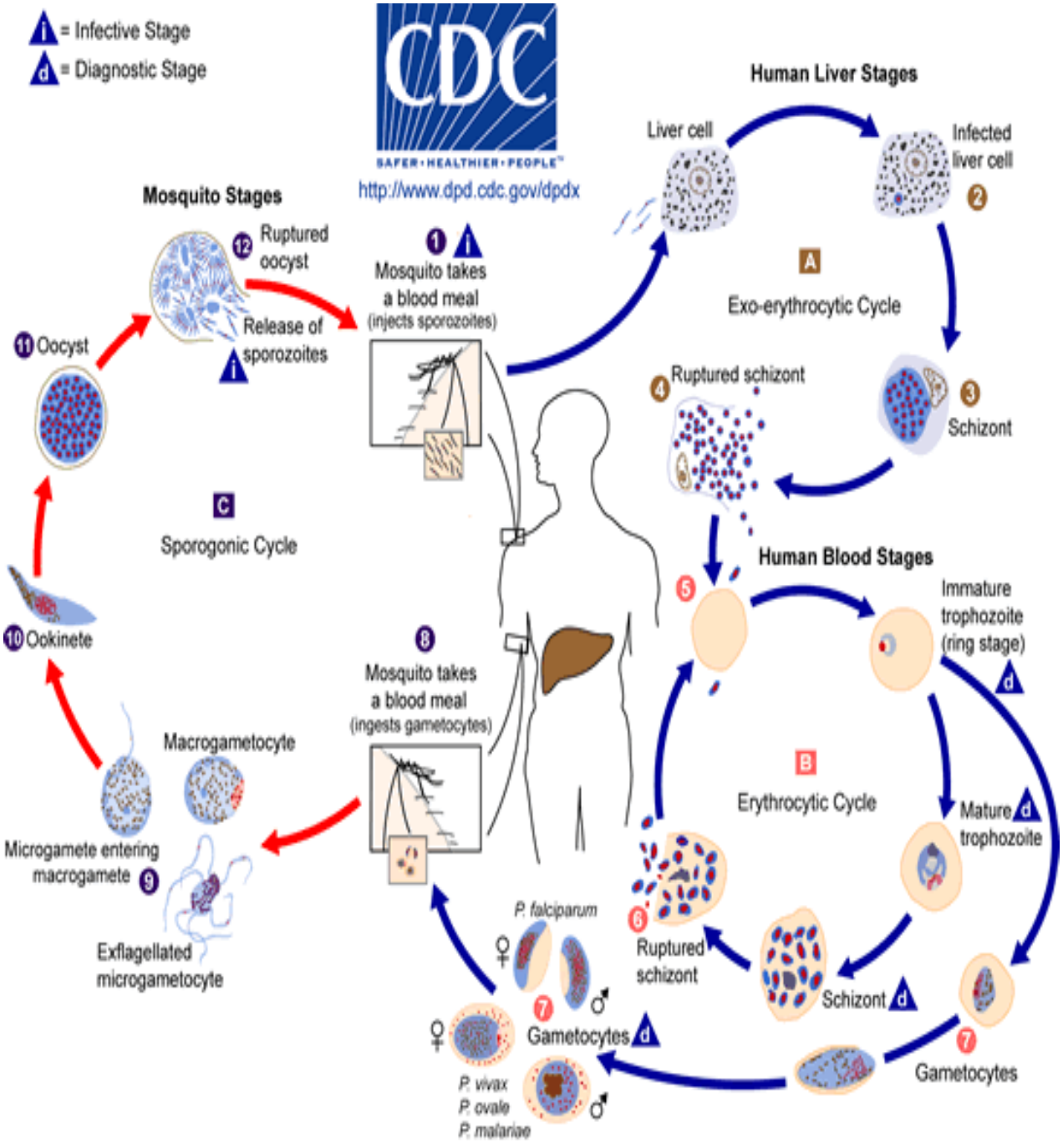

Figure 1: A schematic representation of *Plasmodium falciparum* lifecycle. Replicated in full as appears in: (http://www.dpd.cdc.gov/dpdx) Accessed on 30/06/2009.

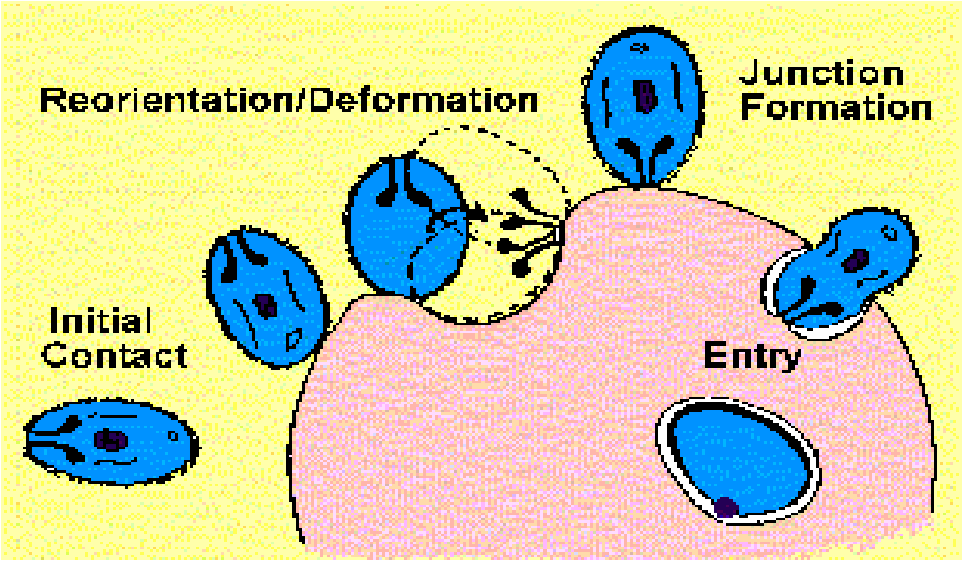

Figure 2: A schematic drawing of the malaria parasite invasion process. Reproduced in full as appears in (Gratzer and Dluzewski, 1993)

Compared to other parasites, *Plasmodium falciparum* has complex and specific multiple molecular invasion mechanisms. Merozoites invade red blood cell through reticulocyte binding like (PfRh) proteins and the duffy binding like erythrocyte binding proteins (DBL-EBP) families (Gunalan *et al.,* 2011; Lopaticki *et al.,* 2011; Rayner *et al.,* 2001). The PfRh proteins bind to band 3 on the RBC surface (DeSimone *et al.,* 2009). The DBL-EBP family is composed of six members sharing sequence homology in approximately eight regions, with which they bind to glycophorin receptors. They include JSEBL, PEBL, EBA-175, MAEBL, BAEBL (EBA-140) and EBL-1 (Peterson and Wellems, 2000; Adams *et al.,* 2001; Baum *et al.,* 2003; Pasvol, 2003; Curtidor *et al.,* 2004).

These ligands have specific glycoproteins on the red blood cells that act as their receptors. Specifically they interact with a group of O-linked sialoglycosylated proteins known as (GYP) glycophorins (Pasvol, 1984; Gaur *et al.,* 2003). Studies done using neuraminidase, trypsin and chymotrypsin proteases, which enzymatically cleave specific sites, have identified at least five glycophorin receptors (Baum *et al.,* 2002; Lobo *et al.,* 2004; Curtidor

et al., 2005). Among these, glycophorin A (GYPA) (most dominant), glycophorin B (GYPB), and glycophorin C (GYPC) molecular forms are well characterized while glycophorin D (GYPD) and glycophorin E (GYPE) are less characterized (Pasvol, 2003; Wang *et al.,* 2003; Lobo *et al.,* 2004; Githui *et al.,* 2010). The glycophorin C gene (*GYP C*) has been shown to code for both GYP C and GYP D through translation of the same mRNA transcript via two independent start codons (Figure 3). However the glycophorin C translation initiation site is conserved and unique to humans in comparison to other primate species, unlike the start codon in *GYP D* that is conserved across the primates (Wilder *et al.,* 2009).

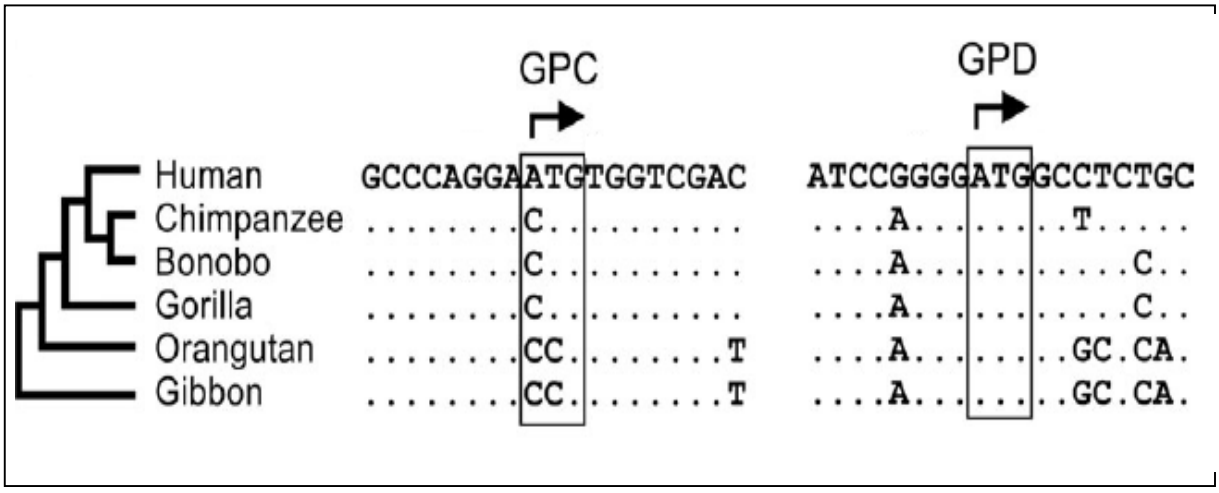

Figure 3: A schematic representation of the translation initiation sites for glycophorins C and D. The start codon (boxed) for GYP C is only present in humans whereas that of GYP D is conserved in humans and across all apes. (Adapted from Wilder *et al.,* 2009)

Glycophorins A and C bind to merozoites ligands EBA-175 and EBA-140 respectively and more recently an erythrocyte binding ligand-1 (EBL-1) was found to be specific for glycophorin B (Maier et al 2009a; Mayer *et al.,* 2009). The EBL-1 shares a similar genetic structure and amino acid sequence with the other members of the DBL-EBP family, whereas its coding gene is located on chromosome 13 (Adams *et al.,* 2001). Unlike other ligands with eight conserved regions, this protein contains four conserved 5´ and 3´ cysteine- rich regions (Figure 4). Regions II and VI of EBL-1 are important for recognition of sialic acid on erythrocyte receptors (Duraisingh *et al.,* 2003; Githui *et al.,* 2010). Moreover region II in

Plasmodium falciparum is duplicated for increased optimal binding to erythrocytes (Sim *et al.,* 1994).

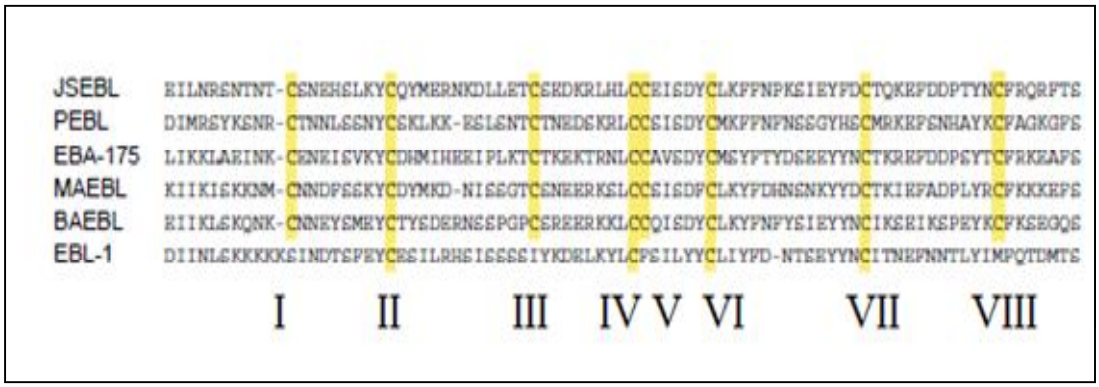

Figure 4: A schematic representation of the conserved cysteine rich regions in the *Plasmodium falciparum* merozoite ligands. Five of the six ligands posses eight cysteine rich conserved regions (I- VIII). These are important in recognition of sialic acid residues on RBCs, while EBL-1 has only regions II, IV, VI and VII. (Adapted from Adams *et al.,* 2001).

Glycophorins A, B and E resulting genes, *GYPA, GYPB and GYPE* are located on chromosome 4 at chromosomal region 4q28-31, where they show nucleotide sequence homology with a 95 percent identity, causing the exons to be more diverse than the introns. This may promote unequal cross over or gene conversions that may result in hybrids (Storry e*t al.,* 2003; Heathcote *et al.,* 2011; Ko *et al.,* 2011). Evolution studies have indicated that *GYPB* and *GYPE* arose from *GYPA* through gene duplications (Kudo and Fukuda, 1994).

Glycophorins A and B are type I membrane RBC proteins and have been identified as carrier proteins of blood group antigens forming the MNS blood group system (Huang *et al.* 1992; Halverson *et al.,* 2009). These proteins are anchored to the membrane lipid bilayer and have their N terminus located in the extracellular space where they may act as receptors (Almen *et al.,* 2009). The MNS system was the second blood group classification system, described in 1927 after the ABO blood group classification system. M and N antigens were named after the second and fifth letters of the word immune, while the S antigen was named after Sydney where the first anti-S was found (Hillyer, 2006).

GYPA contains M or N antigens defined by amino acid residues at positions one and five at the N terminus. M contains serine and glycine respectively, while N has leucine and glutamine respectively (Khalid and Green, 1990; Ko *et al.*, 2011). GYPA is identical to GYPB for the first 26 amino acids thus the latter also posses the N antigen at the N terminus (Akane *et al.*, 1997; Wang *et al.*, 2003). However there occurs a point mutation at exon 2 of GYPB that results in an amino acid change of (A/G) alanine to glutamine hence possession of the N antigen and not the M antigen (Brunner, 2000). Additionally at position 29, GYPB contains either a methionine residue hence the S antigen or a threonine residue for the s antigen (Storry and Reid, 1996).

Glycophorin B is 72 amino acids long, highly threonine and serine glycosylated with phenotypes expressed as either S-s+, S+s- or S+s+ on the RBC surface (Siebert and Fukuda, 1987; Storry and Reid, 1996; Halverson *et al.*, 2009; Santos *et al.*, 2011). Moreover, GYPB contains a U antigen defined by amino acids 33- 39 as identified by enzymatic studies using papain (Figure 5; Storry and Reid, 1996). Glycophorin B occurs on healthy erythrocytes in values ranging between 80,000 and 300,000 molecules per cell (Merry *et al.*, 1986; Dahr *et al.*, 1987).

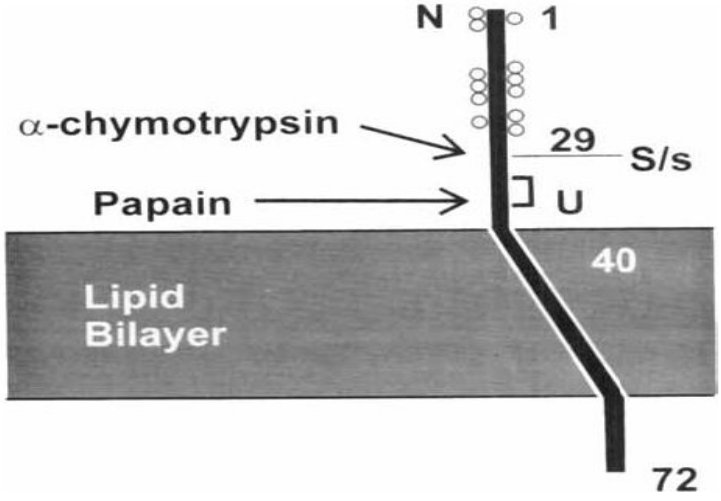

Figure 5: A schematic representation of Glycophorin B and the enzymatic cleavage sites. The small circles (o) represent O- linked oligosaccharides. Replicated in full as depicted in Storry and Reid, 1996.

Some host abnormalities impede the pre- and post- parasite invasion process. The pre- and post- invasion abnormalities prevent binding of the parasite to RBC receptors and proliferation of the parasite in the RBC respectively (Carter and Mendis, 2002). In the latter there exists several human hemoglobin variants that prevent utilization of hemoglobin hence impede proliferation of *Plasmodium falciparum* in the host cells. This confers protection against severe malaria (Pasvol, 2010). These variants include primarily the sickle cell trait, thalassemias, ovalocytosis, glucose-6-phosphate dehydrogenase deficiency, hemoglobin C and E (Holding *et al.,* 2001; Carter and Mendis, 2002).

The malaria hypothesis by J.B.S. Haldane suggests that as in other infectious diseases, malaria is major selective force in human evolution. Natural selection has driven presence of pre- invasion abnormalities among populations living in malaria endemic regions (Haldane, 1949; Lederberg, 1999; Sabeti, 2008). Absence of GYPC on red blood cells also known as gerbich negative phenotype due to a deletion in exon 3 on glycophorin C gene provides protection against cerebral malaria (Patel *et al.,* 2004). On the other hand a non-sense single nucleotide polymorphism with a base change of threonine to alanine (T to A) causes an immediate stop codon in the *FY* gene. This terminates synthesis and deprives RBCs the duffy antigen of the amino acid sequence required for invasion of *Plasmodium vivax* hence promoting resistance against this parasite particularly in West Africa (Storry and Olsson, 2004; Kwiatkowski, 2005; Howell *et al.*, 2006; Santos *et al.*, 2011).

Plasmodium falciparum pressure on several populations has also led to occurrence of rare null phenotypes of the MNS blood group system caused primarily by deletions. These include, En (a-) phenotype where the RBCs lack the M and N antigens hence have no glycophorin A and are highly refractory of *P. falciparum* invasion (Blumenfeld and Huang, 1997; Ratliff et al., 2007; Santos *et al.,* 2011). The M^KM^K phenotype is also rare where the cells lack both the MN and Ss antigens such that they are devoid of glycophorins A and B. However this does not change the RBC membrane architecture which suggests that glycophorins are not mainly involved in the membrane structure (Brunner, 2000; Reid, 2003). *GYPB* gene composed of six exons (B1-B6) including one pseudo exon, B3, which is also highly polymorphic, particularly in malaria endemic regions. The mutation results into a

U- phenotype represented as S-s-U-as it lacks the Ss antigens and thus no GYPB protein on red blood cells (Alain *et al.,* 1990; Rahuel *et al.,* 1991; Mayer *et al.,* 2009).

Glycophorin B gene polymorphisms occur due to various gene alterations including gene deletions and single nucleotide polymorphisms, where the latter may occur both in coding and non-coding sequences causing partial or complete exon skipping (Rahuel *et al.,* 1991). On the other hand, gene recombination between *GYPA* and *GYPB* gives rise to different hybrids mainly glycophorins A-B and B-A-B which may result to gene deletions. The former crossover events result in miltenberger variants in glycophorin B (Storry and Reid, 1996; Mayer *et al.,* 2009).

The S-s-U- phenotype results due to either a gene deletion of exons B2 through to B5 on the glycophorin B gene or presence of a 5´ deletion breakpoint between exon B1 and B2. (Rahuel *et al.,* 1991; Blumenfeld and Huang, 1997; Brunner, 2000). Individuals homozygous for glycophorin B coding region knock out lack this protein on the surface of their erythrocytes (Daniels, 2009). Single nucleotide polymorphism also plays part in developing this phenotype as it is the case in C to T change at the 3´ end of B5 exon and a G to T change at position five of intron five in the gene where both alter the splice sites causing complete exon skipping (Rahuel *et al.,* 1991; Brunner, 2000).

S-s-U- red blood cells provide a degree of protection against *Plasmodium falciparum* infection. However protection from *Plasmodium* infection by *GYPB* gene abnormality (S-s-U- phenotype) is not absolute since the parasite uses multiple invasion mechanisms (Gaur *et al.,* 2003; Pasvol, 2003; Storry *et al.,* 2003; Wang *et al.,* 2003; Santos *et al.,* 2011). In populations of African descent, this phenotype is common and is thought to be selected due to a long time of exposure to malaria (Mayer *et al.,* 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Area of Study

Kisumu and Mombasa districts are areas where malaria is most prevalent in Kenya. A contributing factor for malaria prevalence is that both regions are located near water bodies as well as having high temperatures (Noor *et al.,* 2009). This environment is excellent for flourishing of female *Anopheles* species mosquitoes carrying malaria causing parasites. On the other hand due to climate change, there have been temperature shifts making even the Kenyan highlands experience increased incidences of malaria as shown in Figure 6.

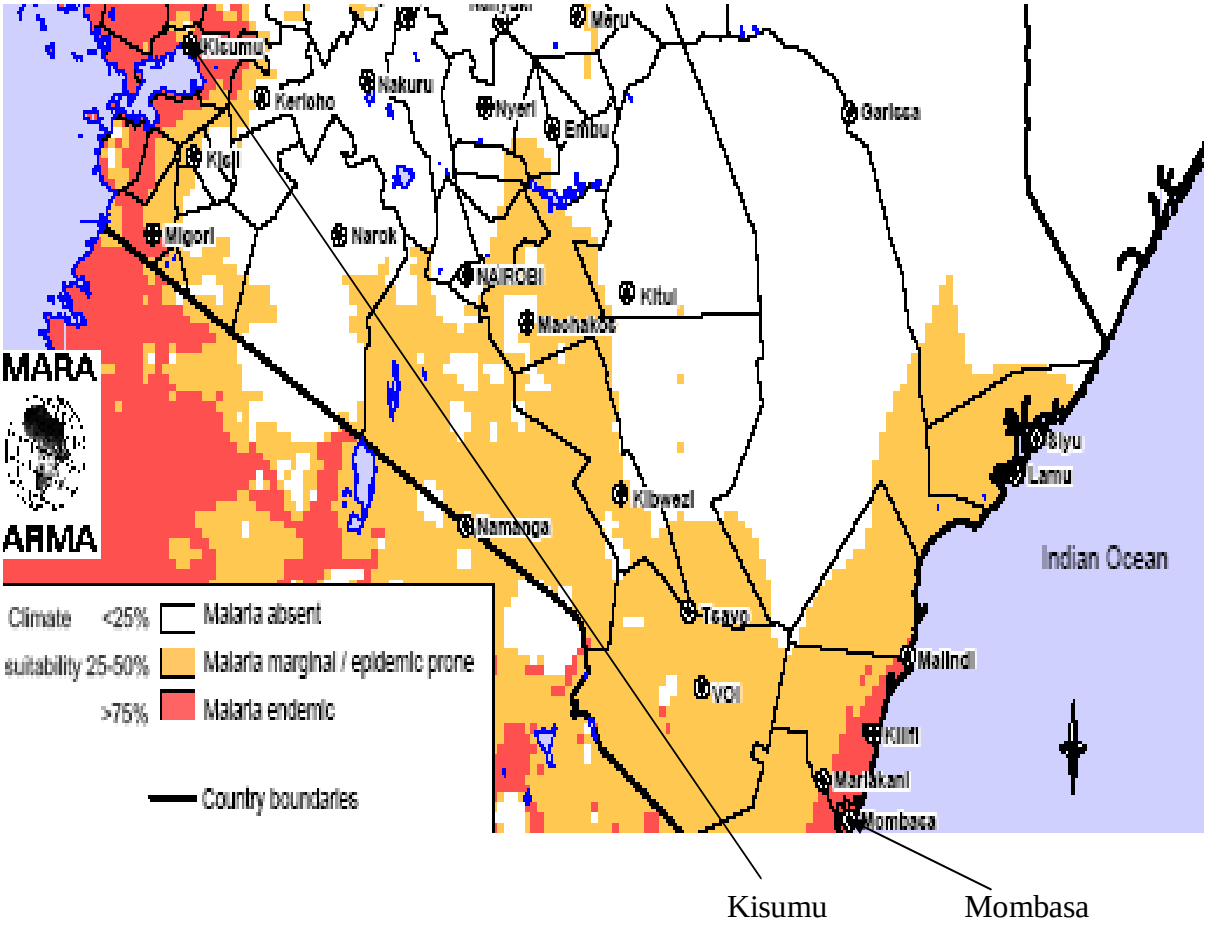

Figure 6: Study sites and distribution of malaria in Kenya. Areas in red indicate places where malaria is most prevalent. Those in brown show regions that are marginally malaria prone,

suggesting that malaria is present occasionally even in the Kenyan highlands for example in Meru and Kericho. Replicated in full as appears in (http://www.mara.org.za.) Accessed on 05/07/2009.

3.1.1 Kisumu

Kisumu district is situated in Nyanza province, southeast of Kenya ($0^0$5' 51'' South, 34^0 45' 16''East) and it is densely populated with 968,909 people (Kenya National Bureau of Statistics, 2009). It covers a surface area of 918.5sq km and its 1,146m (3759 feet) above sea level. Rainfall is seasonal with two rain seasons annually; long rains during March through to May, while short rains occur between September and January. The average annual rainfall is 1,050mm with average temperatures between 23.3^0 C and 32^0 C. Malaria transmission is higher in Kisumu compared to Mombasa and is encountered mostly in the rainy seasons, with *Plasmodium falciparum* infections being most common in the two regions.

3.1.2 Mombasa

Mombasa district situated in the Coast province, 4^0 3' 0'' South, 39^0 40' 0'' East, is densely populated with 939,370 people (Kenya national bureau of Statistics, 2009). It covers a surface area of 260 sq km and is 55m above sea level. Mombasa receives an average annual rainfall of 1,059mm with average annual temperatures ranging between 22.4^0C and 32.17^0 C. April and May are the two months that receive the most rain. The district is malaria endemic with a continuous transmission rate effective mostly during the two wet months.

3.2 Blood samples

Samples used in this study were collected from individuals aged five to 35 years, attending district hospitals located in Kisumu and Mombasa. Twenty samples, from each site were used in this study (Appendix 2). Informed consents of the participants or that of guardians for minors were obtained. The protocol was approved by Kenyatta National Hospital Ethical and Research Committee. One ml of blood collected (transferred into tubes with EDTA) and stored at 4^0C for transfer, then later at -20^0C until use.

3.3 DNA Extraction

DNA was extracted from all whole blood samples collected and S+s+U+ blood (positive control) using the phenol chloroform method as described in Sambrook *et al* (2000). Briefly, blood samples were washed with tris- buffered saline and centrifuged at 15,000rpm for 10 min.Sodium Tris EDTA (STE) buffer, proteinase K and sodium dodecyl sulphate (SDS) were added to digest cell membranes at 55^0C for 2 hours. Equal volumes of phenol and chloroform were added, mixed gently and allowed to stand at room temperature for 5 min. The tubes were centrifuged at 10,000g for 5min and supernatant aspirated into clean tubes. To remove the phenol, a 24:1 ratio of chloroform/ isoamyl alcohol was added mixed gently and incubated at room temperature for 3 min. Washes with sodium chloride were done followed by precipitation of DNA with ice-cold absolute alcohol at -20^0C overnight. The resultant pellet was air dried and re-suspended in 50 µl Tris-EDTA (TE) buffer. Samples were stored at -20^0C until needed.

3.4 Polymerase Chain Reaction (PCR)

DNA samples were amplified using two sets of primers. Set one sequences were forward 5´GTACCCCTGAATATAAAGTTCCTTTTTT3´, reverse5´ GGTTATCTGTACAAATCCATTTCATTTC 3´. Whereas for set two primers, the sequences were, forward 5´TGCAAAGTCCAATCTCTGCTC3´and reverse5´ CAGTTTGCATAAACAAGAGAA 3´. Set one was specific for region two of *Plasmodium falciparum* EBL-1 gene, whereas set two amplified exon five region of human glycophorin B gene. Each PCR tube contained 9.9µl deionized water, 4.0 µl Go Taq (Promega, UK) specific buffer with 1.5mM Mg^{2+}, 2.0 µl of 2.5mM dNTPs, 0.8 µl forward primer, 0.8 µl reverse primer, 0.1ng/ µl DNA template and 0.5 µl of Go Taq (Promega, UK) polymerase enzyme. Conditions for amplification of *ebl-1* gene using set one primers were: Initial denaturation at 94^0C for 3min, then 30 cycles of denaturation, 94^0C for 1min, annealing 55^0C for 1min, extension 70^0C for 2min and final extension at 70^0C for 5min. The conditions for amplification of *GYP B* gene using set two primers were: Initial denaturation, 94^0C for 7min,

then 30cycles of denaturation, 94^0C for 1min, annealing 54^0C for 1min, extension72^0C for 1.15min and final extension at 72^0C for 4min. The TECHNE TC-4000 thermo cycler was used in all the reactions.

3.5 Agarose Gel Electrophoresis

PCR products amplified using both set one and two primers, were electrophoresed on a 1% Agarose gel stained with ethidium bromide (0.5µg/ml) in x1 Tris acetate EDTA buffer at 100V for 1 hr. The size of the products was estimated by running a 1.5Kbp molecular marker alongside the samples. The bands were visualized under an ultraviolet light transilluminator and photographed.

3.6 Extraction of Glycophorin B and EBL-1 PCR product

The PCR products were purified using the glass milk method as described by Vogelstein and Gillespie (1979). The approximately 500bp GYP B and 650bp EBL-1 bands were excised from the gel with a sterile blade and placed into autoclaved eppendorf tubes. 200 µl /g of sodium iodide (NaI) was added to the gel and the mixture incubated at 56^0C for 1 hr with intermittent mixing until the gel dissolved completely. 5 µl of glassmilk solution (Appendix 1) was added to bind the DNA. Each tube was placed on ice for an hour with intermittent mixing. The tubes were then centrifuged at 14,000rpm for 5min and the supernatant discarded. Ice cold ethanol wash (50% ethanol, 20mM Tris-HCl (pH 7.6-8.0), 1mM EDTA (pH 8.0) and 100mM NaCl) was used. Samples were washed three times. In the final dry spin a micropipette was used to remove the remaining ethanol completely avoiding disturbing the pellet. The pellet was air dried and 25 µl of elution buffer AE (Qiagen, Inc., Valencia, CA) added. The tubes were incubated at 56^0C for 30min then 20 µl of the mixture was transferred into clean tubes and 3 µl of the gene clean PCR products were ran on a 1% agarose gel. The gel was visualized and photographed under a UV transilluminator.

3.7 Sequencing

Gene clean PCR products were sent to the International Livestock Research Institute (ILRI), Nairobi, Kenya, for sequencing.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Amplification of Glycophorin B gene

A total of 40 samples were collected from Kisumu and Mombasa district hospitals. The primers used for amplification of targeted exon five region of the glycophorin B gene to give a 500bp fragment. Amplicons generated using set two primers were separated using a 1% agarose gel electrophoresis are presented in Figure 7 (Kisumu) and Figure 8 (Mombasa). Lack of amplification indicated the absence of exon five.

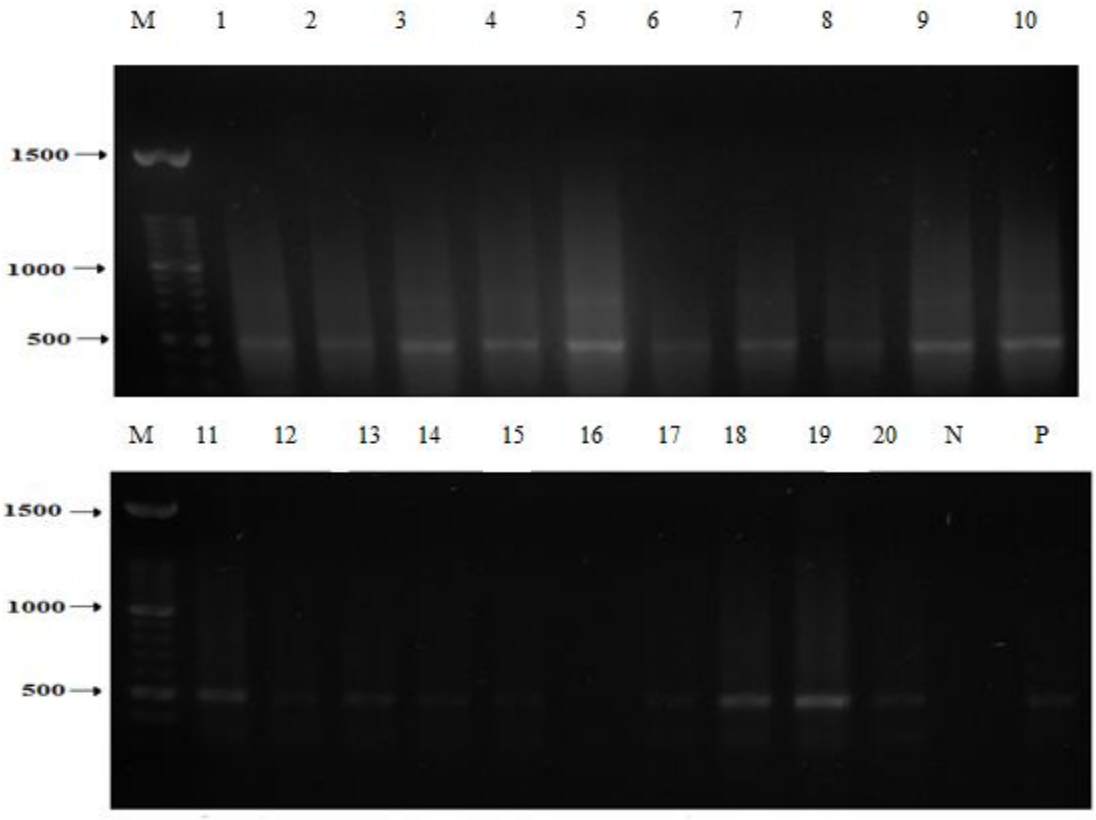

Figure 7: A 1% ethidium bromide stained agarose gel showing amplification of the Glycophorin B fragment on samples obtained from Kisumu. The 500bp band indicates amplified PCR products from the reaction with set two primers.

Nineteen out of 20 samples (samples 1-15, 17-20) and S+s+U positive control (P) were positive while one out of 20 (sample 16) was negative. A water sample was used as the negative control (N) in place of a DNA template.

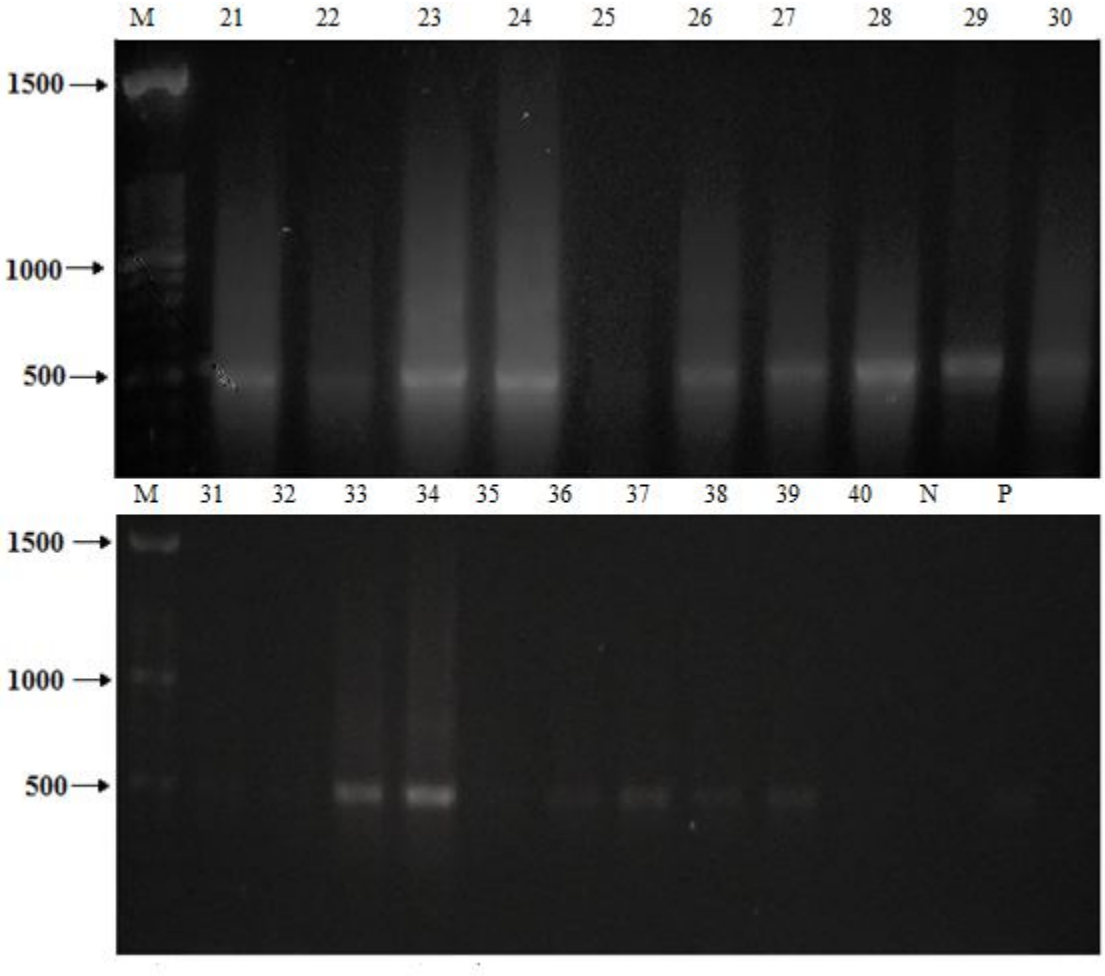

Figure 8: A 1% agarose gel showing position of the Glycophorin B amplified fragment on Mombasa samples. Sixteen of 20 samples (samples 33, 34, 36-39) and the positive control P (S+s+U+) were positive and gave the expected fragment of 500bp in position with that of the molecular marker (M). However four of twenty samples (samples 31, 32, 35 and 40) were negative as seen in the negative control (N) that contained water in place of a DNA template.

Samples from both Kisumu and Mombasa had a glycophorin B fragment detection rate of 95% and 80% respectively as shown in Figure 9. This indicates the Glycophorin B exon five still prevalent in both Districts.

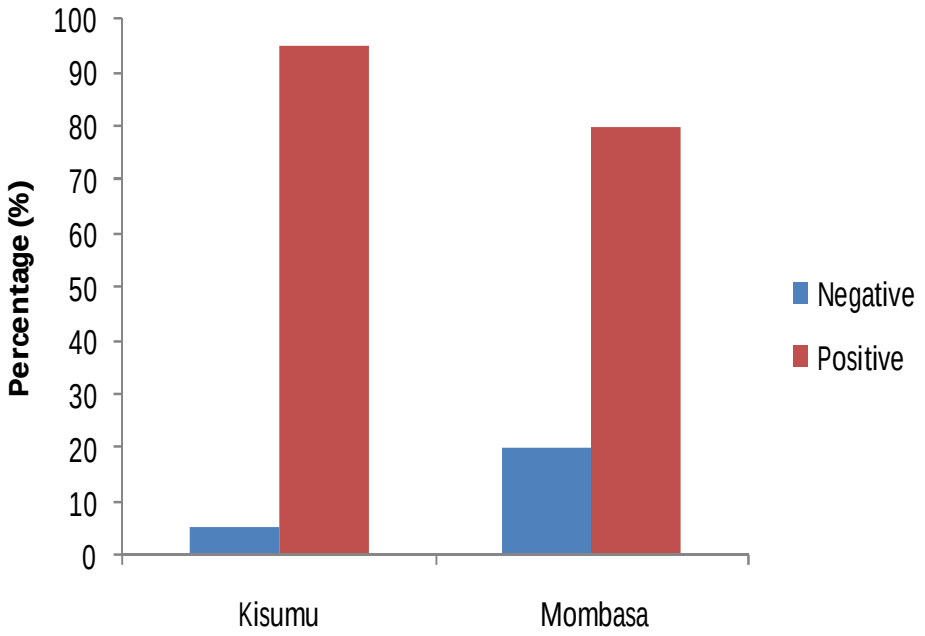

Figure 9: A chart showing the percentage of negative and positive results of glycophorin B in Kisumu and Mombasa samples. Nineteen (95%) and eighteen(80%) of twenty samples from Kisumu and Mombasa respectively were amplified by PCR using set two primers to detect the 500bp fragment of the glycophorin B gene.

4.1.2 Amplification of Erythrocyte binding ligand-1 gene

Eight samples each from Kisumu and Mombasa amplified with set one primers were positive for the EBL-1 gene fragment and gave the expected fragment of 650bp. This was determined by extrapolation from the 600bp band on the 1.5Kbp marker.

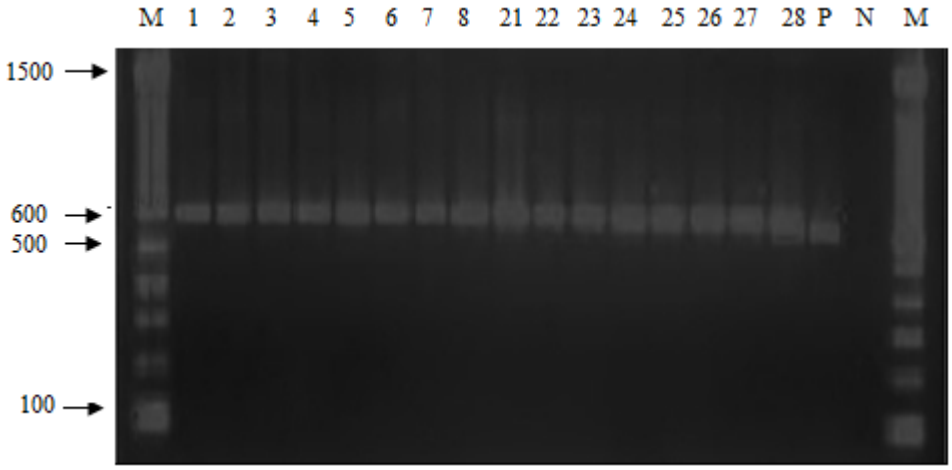

Figure 10: A 1% agarose gel showing amplification of the EBL-1 amplified fragment on Kisumu (1 through to 8) and Mombasa (21 to 28) samples. The band was also present on the positive control (P), a Dd2 *Plasmodium falciparum* laboratory strain sample (*ebl-1*gene present) while absent on the water sample used as a negative control (N).

4.1.3 Gene clean

Five PCR samples each from Kisumu and Mombasa with strong bands as shown in figures 6 and 7 that amplified the glycophorin B gene fragment using set two primers were purified in preparation for sequencing. These were chosen to give sufficient DNA for the sequencing reactions to produce results. The purified fragments are shown below (Figure 11). Three samples (lanes 5, 18 and 19 in Figure 11) gave multiple bands.

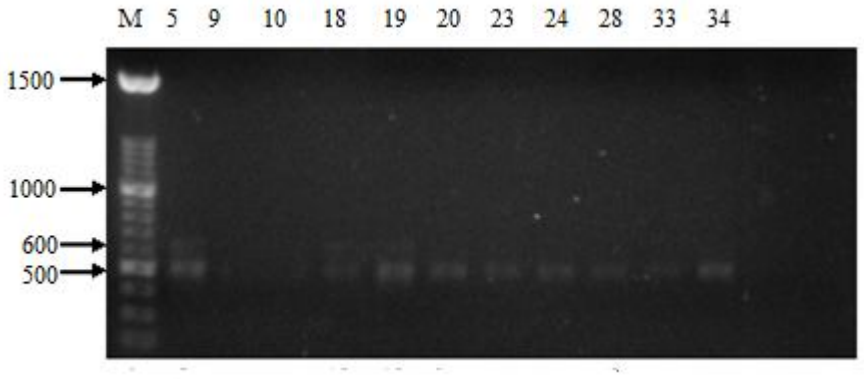

Figure 11: A 1% agarose gel presenting glycophorin B gene fragments that were purified. Samples 5, 18 and 19 gave two bands of 500bp and 600bp. Samples 9 and 10 did not give distinct bands and were therefore excluded from sequencing.

4.1.4 Sequencing

Purified Kisumu and Mombasa amplicons were sequenced following amplification using set two primers that were designed to amplify a region in exon 5 of GYP B. NCBI- BLASTn alignments confirmed the fragment was similar with a few mismatches to the Human MNS glycophorin B coding sequence, GenBank Accession number NG 007483.2. The identities, score and E-value were 91%, 490 and 5e- 121 respectively in Mombasa samples (Figure 12) and 83%, 244 and 2e-54 respectively in Kisumu samples (Figure 14). A representation of electrograms from the sequenced Mombasa and Kisumu samples is shown in Appendix 3 and 4 respectively. The electrograms indicated presence of impurities or more fragments from the one purified. There were clear peaks with additional peaks ("noise") below.

```
34    9          TCCGCCTCCTGAGTTCAGTTGAT-CTCGTGCTTCAGCCTCCACAGTAGTTGGGACTACAG  67
                 || ||||||   |||||  |||| ||||||||||||||||||||| ||||||||||||
GYP B  27640     TCTGCCTCCCTGGTTCAAGTGATTCTCGTGCTTCAGCCTCCACAGTAATTGGGACTACAG  27699

34     68        GTGCGGGCCACCATTCCTGACTAATTTTTGTATTTTTTTAATAGCACACATGGGGTTTCG  127
                 |||||||| ||||| |||||||||||||||||||||||||||||| | ||||||||||||
GYP B  27700     GTGCGGGCTACCATGCCTGACTAATTTTTGTATTTTTTTAATAGCAGAGATGGGGTTTCG  27759

34     128       CTGTGTTGGCCACGCTGGTTTCAAACTCCTGACCTCAAGTGATCCACCTGCCTTGGCCTC  187
                 |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
GYP B  27760     CTGTGTTGGCCAGGCTGGTTTCAAACTCCTGACCTCAAGTGATCCACCTGCCTTGGCCTC  27819

34     188       CCACCTGCTGGGATTACAGGAGTGAGCCACTGCGCCTGGCCCCAATTATGGTTTTTATAA  247
                 | |  ||||||||||||||| ||||||||||||||| ||||| ||||| ||||||||||||
GYP B  27820     CAAAGTGCTGGGATTACAGGTGTGAGCCACTGCGCCCGGCCCTAATTAGGGTTTTTATAA  27879

34     248       AACCAAAAAAAACTTGACAACACCCCTAGGTACTCTTTAGAAGACTCCAATTGGCTACATC  307
                 |||||||| |||||| |||||||||||||||| ||||||||| ||||||||||||||| |
GYP B  27880     AACCAAAAGAACTTGGCAACACCCCTAGGTACCCTTTAGAAGCCTCCAATTGGCTACAGC  27939

34    308        CTATGAACGATTGGC  322
                 || |||| |||||||
GYP B  27940     CTGTGAAGGATTGGC  27954
```

Figure 12: NCBI- BLASTn alignment results of sequence data from purified PCR products. Samples amplified from blood collected in Mombasa aligned to glycophorin B sequence in the database following introduction of a gap (1/ 315) indicated in red and an arrow below the base.

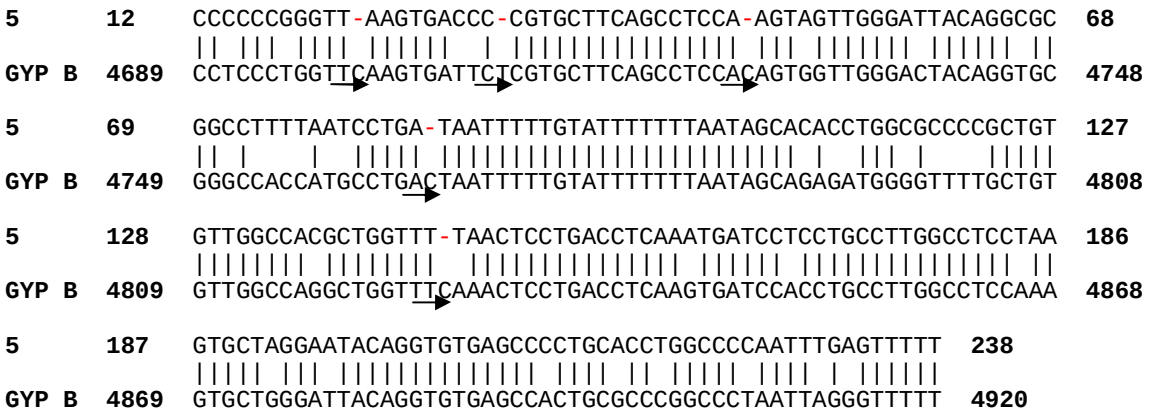

Figure 13: NCBI- BLASTn alignment results showing the fragment sequence in Kisumu samples aligned to glycophorin B sequence in the database following insertion of gaps (5/ 232) indicated in red and an arrow below the bases.

4.1.5 Sequence Analysis

Sequencing results from Kisumu and Mombasa samples were aligned with the Homo Sapiens Glycophorin B sequence in the NCBI database (gi|170763509|ref|NG_007483.1). There were various matches (indicated by dots below) and mismatches as shown in Figure 16 below.

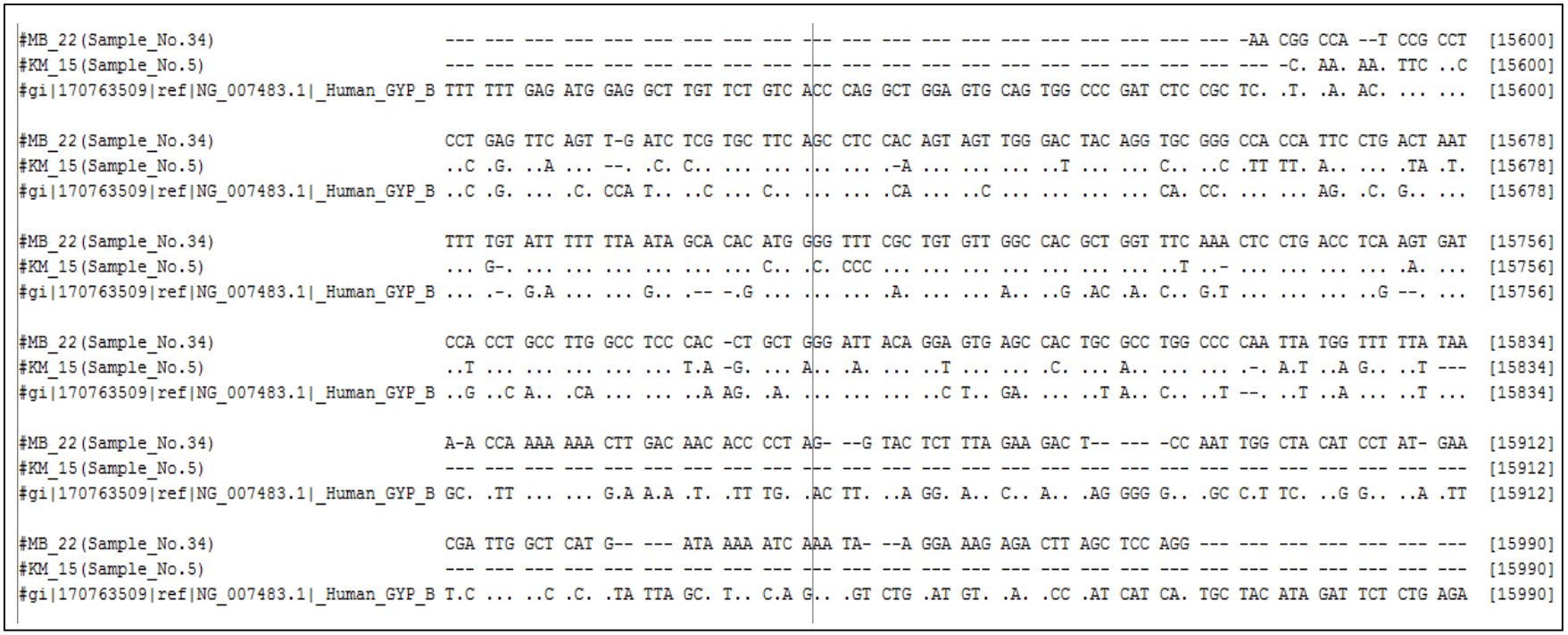

Figure 14: A schematic representation of Clustal W (Mega 5 Software) alignment of Kisumu and Mombasa sequenced samples with the human GYP B sequence in the database (gi|170763509|ref|NG_007483.1). There are various matches shown by dots (.) below the corresponding base. Dashes (-) show regions of misalignments.

4.1.6 Glycophorin B detection rate in Kisumu

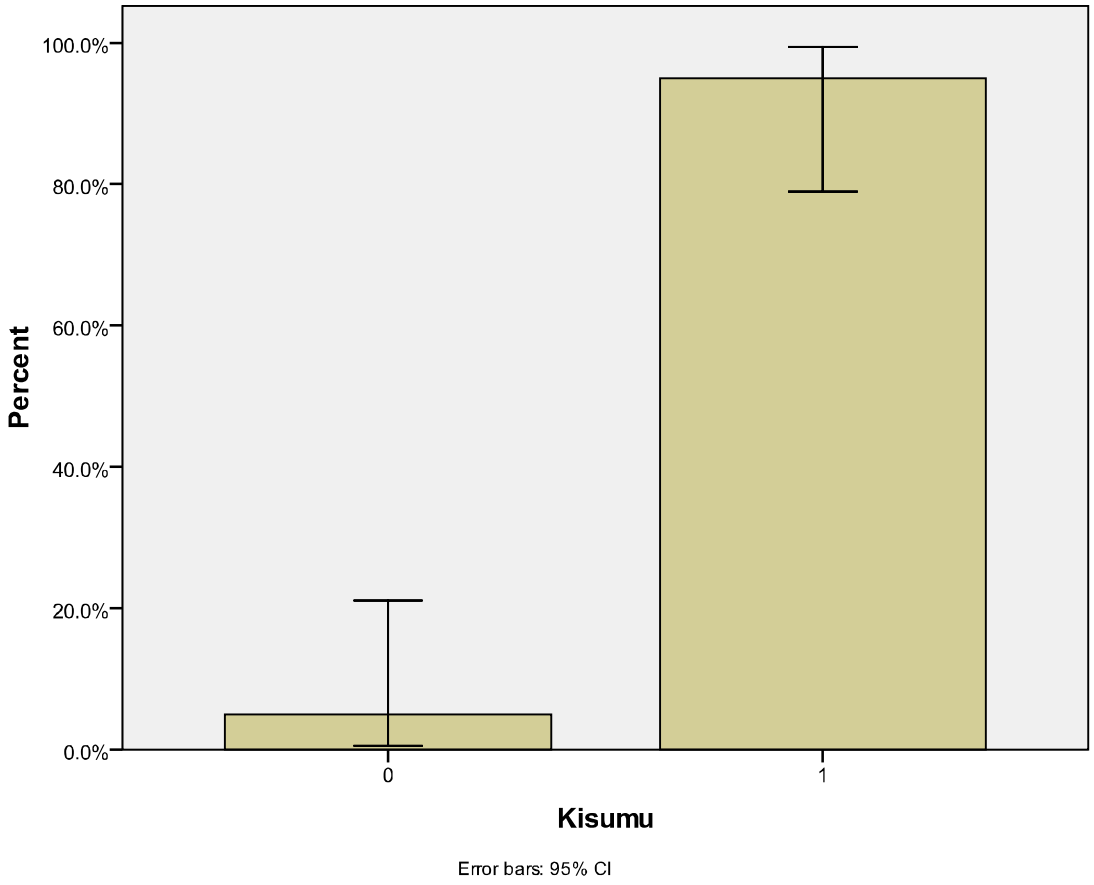

Figure 15: A 95% confidence interval in Kisumu samples

4.1.7 Glycophorin B detection rate in Mombasa

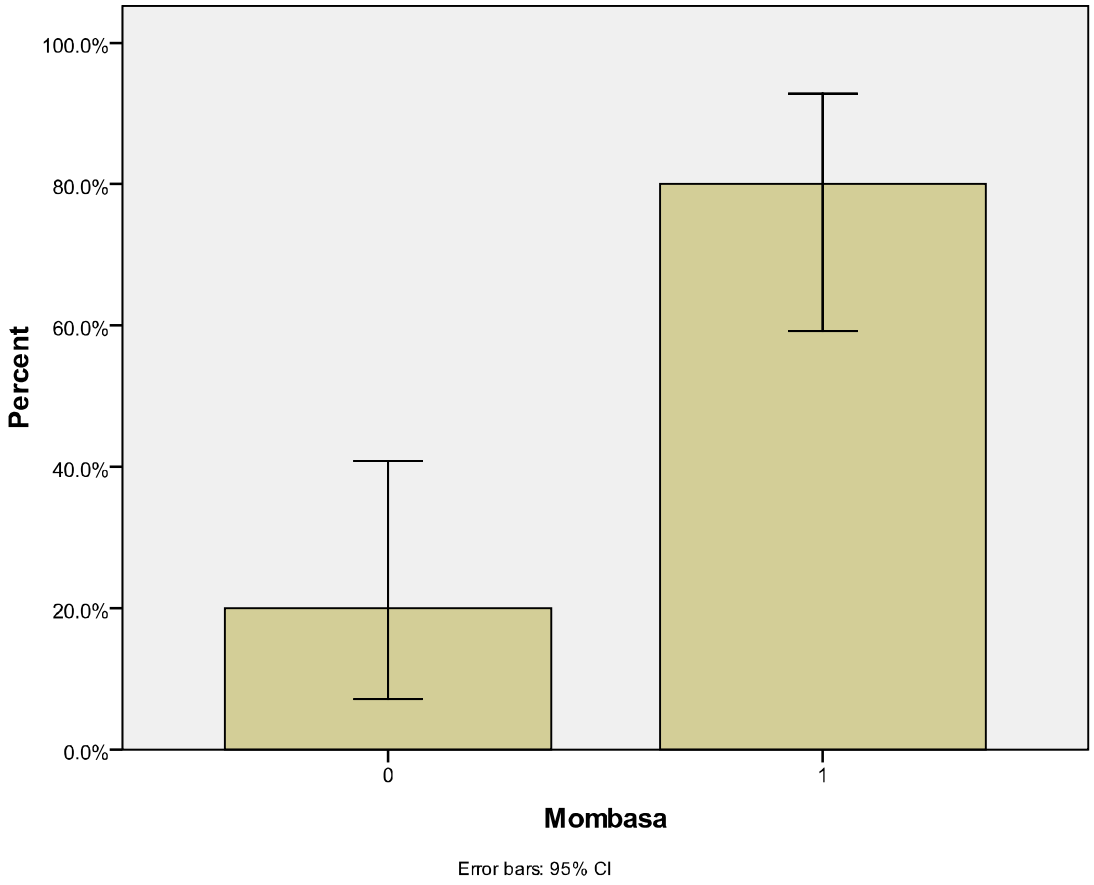

Figure 16: A 95% confidence interval in Mombasa samples

4.2 Discussion

Survival for hosts and parasites in malaria endemic regions results in changes that promote natural selection in both genomes. The evolution process is slow but with significant impacts on adaptability of the parasite and as a result, the disease burden has reduced (Ko *et al.*, 2011; Mackinnon and Marsh, 2010). Recently there has been some decrease in malaria in sub- Saharan Africa, Kenya included, in part, due to improved prevention strategies. These strategies include the distribution and use of insecticide treated bed nets as well as use of new drug interventions (Okiro *et al.*, 2007; Mmbando *et al.*, 2010).

Prolonged plasmodium species presence in Africa has led to development of various resistance adaptations. These include presence of truncated genes as seen in deletions that occur in glycophorin C gene at exon 3. This deletion causes poor binding to its corresponding ligand (EBA-140) in the *Plasmodium falciparum* malaria parasite (Jiang *et al.*, 2009). In addition *Plasmodium vivax* in West Africa has induced emergence of the duffy negative phenotype through a single nucleotide polymorphism that introduces a premature stop codon in the *FYab* gene that codes for the duffy antigen on RBCs (Heathcote *et al.*, 2011). This polymorphism is observed in glycophorin B, thereby protecting hosts homologous to the deletion against malaria.

Glycophorins A, B and E are located on chromosome four and share over 90% sequence homology. The coding regions are more diverse than the non coding regions (Storry *et al.*, 2003). The primers used in this study targeted a region in the exon five of GYP B. The 5´ end flanking region of the forward primer attached to intron five of the gene. In Figures 7 and 8, the amplification gave the targeted band of 500 bp in addition to other bands in the background which is a possibility that even glycophorins A and E were partially amplified. This is likely to have occurred since the forward primer amplified part of intron 5 in the glycophorin B gene. Glycophorins B and E originated from gene duplications in GYP A which gives them sequence homology particularly at the N terminus (Kudo and Fukuda, 1994). Glycophorins B and E resulted from gene duplications in GYP A which gives them sequence homology particularly at the N terminus (Kudo and Fukuda, 1994).

Glycophorin B gene fragments that were sequenced showed similarity with those deposited in the National Centre for Biotechnology Information database. However with a 91% similarity which is still considerably low for identical proteins, the results suggested the PCR products might have had a low quality thereby reducing the identity. At the same time the same query gave out some glycophorin E results which were indicative of the high sequence homology among glycophorins. This was evident in the 95% confidence interval, with error bars (Figures 15 and 16) showing need for a larger sample size.

Glycophorin B in the *Ife Pygmies* in the Democratic Republic of Congo has been eliminated from the local gene pool and this is likely to provide them with partial immunity against *Plasmodium falciparum* malaria (Fraser *et al.,* 1966; Mayer *et al.,* 2009). The partial protection is due to the ability of the parasite to used multiple receptors on the erythrocytes (Culleton and Kaneko, 2009). These receptors include glycophorin A and C that bind to *Plasmodium falciparum* ligands EBA-175 and EBA-140 respectively (Curtidor *et al.,* 2004). Additionally *Plasmodium falciparum* utilizes proteins in the reticulocyte biding like family (PfRh ligands) that are also expressed on the merozoites during invasion. A study conducted by Lopaticki *et al.,* (2010), showed that loss of function in erythrocyte binding ligands can be compensated by upregulation of *PfRh* genes.

Glycophorin B is composed of five exons and a pseudoexon that generates a 3´ untranslated region. Exon B1 is responsible for a leading peptide of the glycophorin B protein, while exons B2, B3 and B4 encode for the extracellular domain of the protein. Exon B5 on the other hand encodes for the transmembrane domain and a short cytosolic segment (Mullen *et al.,* 2009; Ko *et al.,* 2011).

Polymorphisms resulting in the glycophorin B gene silencing occur either as a gene deletion of exons 2 to 5 or presence of a deletion break point in a region between exon 1 and 2 of the gene (Rahuel *et al.,* 1991). Results shown in Figures 6 and 7 above suggest that a majority of people living in Kisumu and Mombasa have maintained the integrity of glycophorin B genes. Only 5% in Kisumu and 20% in Mombasa based on the results from the samples analyzed in this study indicated possibility of a gene deletion. The Mega 5 alignment (Figure 14) show

areas of alignment with the GYP B sequence in the NCBI database but were not conclusive of whether there were mutations in the Kisumu and Mombasa samples since the mismatches were on the higher side. Nevertheless, the matched base pairs indicate that this study has established a basis for further evaluation using the methods established in this thesis. Thus further studies would provide a definitive conclusion. A study conducted by Ko *et al.* (2011), in four ethnic groups in Kenya (Boni, Borana, Luo and Sengwer) showed presence of a haplotype in the extracellular domain of *GYP B* that might have adaptively evolved due to prolonged malaria exposure suggesting positive selection on glycophorin B.

Plasmodium falciparum success in manifestation of malaria is highly dependent on the invasion process that involves its ligands interaction with the host's receptors (Blumenfeld and Huang, 1995). EBL-1 is one of the multiple ligands used by the parasite and in the samples used in this study the parasites appear to maintain the gene as shown in Figure 10. Red blood cells without glycophorin B on their surface will fail to be invaded by the parasite through the GYP B – EBL-1 receptor – ligand interaction leading to the parasite utilizing its other available multiple receptors – ligand interactions. These include ligands such as EBA-175 responsible for attaching to GYP A the most abundant sialoglycoproiteins on the RBC surface (Sim *et al.*, 1994; Heddini *et al.*, 2001; Tham *et al.*, 2010).

The malaria parasite's *ebl-1* gene is composed of four cysteine conserved regions shown in Figure 3. Region 2 is thought to be responsible to binding to sialoglycoproiteins on the RBC surface (Mayer et al., 2004). Primers used in this study aimed at amplifying this region in the samples collected from Kisumu and Mombasa. Amplification of the ebl-1 gene shown in Figure 10 suggested that the parasite DNA used in this study may still contain the conserved region two. This was thought to be the case particularly due to amplification in the Dd2 laboratory strain (used as the positive control in ebl-1 gene amplification) that has been identified to carry an *ebl-1* gene with this region (Githui *et al.*, 2010). Samples used in this study were positive for malaria and amplification of EBL-1 further emphasized this diagnosis. However this needs further study for conclusive determination since the *ebl-1* gene fragments were not sequenced. Prevalence of *GYP B* and *EBL-1* genes was seen to be similar as indicated in Figures 7, 8 and 10 suggesting interdependence in both particularly in

the receptor- ligand interaction during *P. falciparum* invasion. It is only recently that EBL-1 *Plasmodium falciparum* ligand was shown to bind to GYP B sialoglycoprotein on the RBCs (Mayer *et al.*, 2009).

CHAPTER FIVE

5.0 Conclusion and Recommendation

5.1 Conclusion

People living in Kisumu and Mombasa have deletions in their GYP B genes. However the integrity of the gene in largely maintained. The parasites present in these regions also posses the erythrocyte binding ligand-1 gene that codes for the respective ligand for glycophorin B. This study serves as pilot study to give direction in evaluating these genes. People living in Kisumu and Mombasa are therefore likely to be susceptible to Malaria. These findings emphasis that better policies and strategies (e.g. insecticide treated nets and responsive treatment) should be employed to curb Malaria. Therefore more resources should be channeled towards these areas.

5.2 Recommendations

1. Exons 1 and 2 of GYPB have been shown to have a distinct break point of the gene that would ultimately lead to deletion and should be highly studied.
2. Nested PCR should be used to target specific fragments in *GYP B* due to its sequence homology with *GYP A* and *E*.
3. A study comparing polymorphism of *GYP B* gene prevalence during pre and post malaria reduction phase in Kisumu and Mombasa should be done.

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APPENDICES

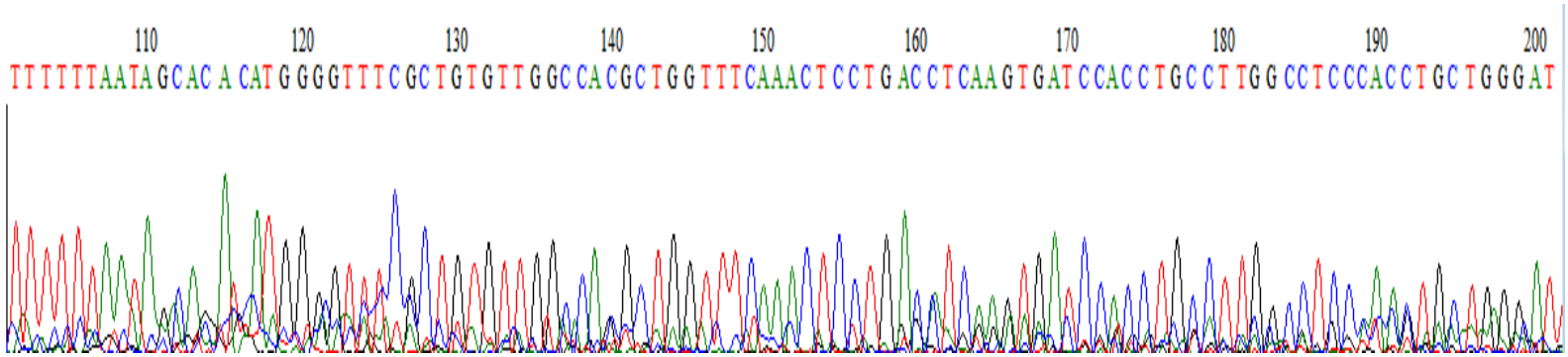
Appendix 1: Glass milk Preparation Protocol

1. In a 1litre glass beaker, resuspend 400g glass powder in 800mL ddH_20
2. Stir for 1 hour.
3. Turn off the stir plate and allow the slurry to settle for 90 minutes
4. Remove the supernatant, which contains the fine particles used for glass milk, and put into a 250ml centrifuge bottle.
5. Pellet the glass particles by spinning at 6000rpm for 10 minutes
6. Resuspend the pellet in 250mlddH_20
7. In fume hood, add concentrated nitric acid to 50%.
8. Stir the solution gently and turn on the heat to "high"
9. Bring the temperature of the slurry almost to a boil, then turn off the heat
10. Continue stirring and allow the slurry to return to room temperature
11. Pellet the glass as in step 5
12. Resuspend glass in 250ml ddH_20
13. Spin as in step 5
14. Continue washing glass particles and spinning as above until the pH of the slurry is neutral.
15. After slurry is neutralized, spin as in step 5
16. Resuspend glass pellet to make a50% slurry based on volume
17. Make 1ml aliquots in microcentrifuge tubes
18. Store indefinitely at room temperature

Appendix 2: Sample Numbers, Identification, Place and Year of Isolation.

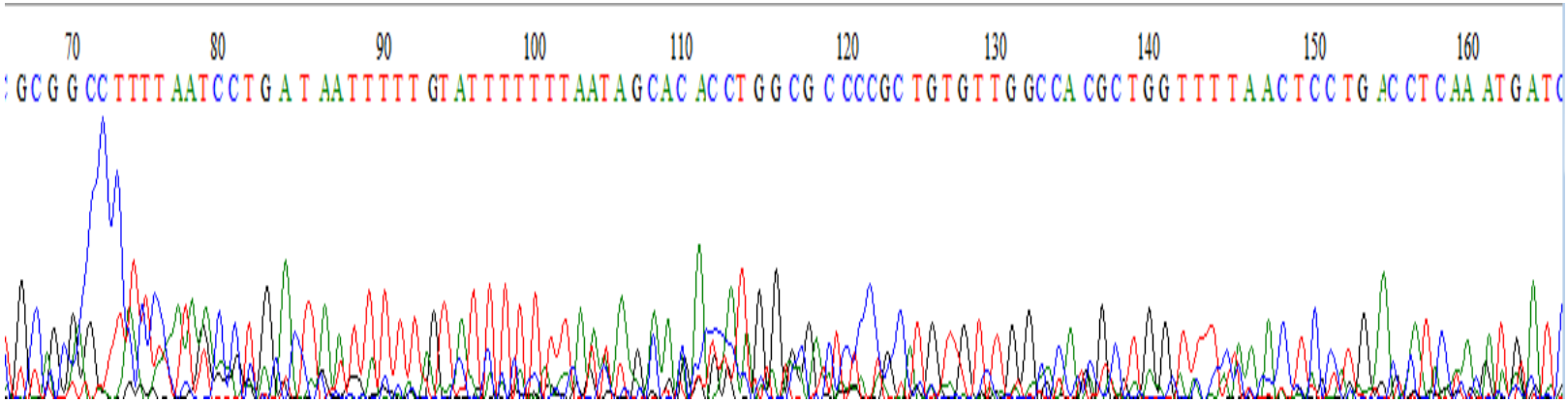
Sample Number	Sample ID	District	Year of isolation
1	KM 10	Kisumu	2009
2	KM 30	Kisumu	2009
3	KM 5	Kisumu	2009
4	KM 21	Kisumu	2009
5	KM 15	Kisumu	2009
6	KM 7	Kisumu	2009
7	KM 8	Kisumu	2009
8	KM 16	Kisumu	2009
9	KM 23	Kisumu	2009
10	KM 40	Kisumu	2009
11	KM 16	Kisumu	2009
12	KM 25	Kisumu	2009
13	KM 35	Kisumu	2009
14	KM 23	Kisumu	2009
15	KM 15	Kisumu	2009
16	KM 6	Kisumu	2009
17	KM 22	Kisumu	2009
18	KM 5	Kisumu	2009
19	KM 10	Kisumu	2009
20	KM 27	Kisumu	2009
21	MB 9	Mombasa	2009
22	MB 19	Mombasa	2009
23	MB 3	Mombasa	2009
24	MB 2	Mombasa	2009
25	MB 27	Mombasa	2009
26	MB 5	Mombasa	2009
27	MB 29	Mombasa	2009
28	MB 16	Mombasa	2009
29	MB 9	Mombasa	2009
30	MB 27	Mombasa	2009
31	MB 25	Mombasa	2009
32	MB 10	Mombasa	2009
33	MB 15	Mombasa	2009
34	MB 22	Mombasa	2009
35	MB 12	Mombasa	2009
36	MB 31	Mombasa	2009
37	MB 29	Mombasa	2009
38	MB 34	Mombasa	2009
39	MB 36	Mombasa	2009
40	MB 4	Mombasa	2009

Appendix 3



A representative electrogram showing the sequencing output in Mombasa sample number 34 (Figure 8) that shows clear peaks of the four bases (A, T, G and C). Additional small peaks below suggest that the PCR samples could have contained small amounts of impurities.

Appendix 4



A representative electrogram showing the sequencing output in Kisumu sample number 5 (Figure 7) that shows clear peaks of the four bases (A, T, G and C). Additional small peaks below suggest that the PCR samples could have contained small amounts of impurities.