INFLUENCE OF BLOOD GROUP, GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND HEMOGLOBIN GENOTYPES ON *FALCIPARUM* MALARIA OUTCOME IN CHILDREN UNDER 3 YEARS IN VIHIGA, KENYA

BY

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DEPARTMENT OF BIOMEDICAL SCIENCE AND TECHNOLOGY

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DECLARATION

I declare that this thesis is my original work and has not been presented to any other University or Institution for a degree or any other award.

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DEDICATION

To my family, husband Ahmed and children Swaleh, Hassan and Faridah for all the support and encouragement.

ABSTRACT

An estimated 198 million malaria cases were reported worldwide of which 128 million were in African region while 16 million were reported in Kenya by the end of 2013. The prevalence of malaria in Vihiga, Kenya was 52% and this is greatly driven by *Plasmodium falciparum* drug resistance, ecological and agricultural factors supporting malaria transmission and vector proliferation. Influence of host genetic factors like blood group, glucose-6-phosphate dehydrogenase (G6PD) and haemoglobin genotypes on *falciparum* malaria outcome varies geographically and ethnically. However, their influences on *Plasmodium falciparum* infection among children in Vihiga, Kenya, had not been determined. Studies on these genetic variants and their influences on malaria outcome have been done in isolation but none had looked at their concurrent inheritence. This hospital based cross-sectional study, therefore, determined the influence of blood group, G6PD, haemoglobin genotypes and the concurrent inheritence of G6PD and Hb genotypes on malaria infection among children under 3 years in Vihiga, Kenya. A total of 414 malaria uninfected healthy controls and 440 malaria infected cases (severe malaria, n=72, and uncomplicated malaria, n=368) were recruited and their socio-demographic, clinical and laboratory information collected. About 2 microliters of whole blood was collected from each study participant and used for malaria microscopy diagnosis as well as haemoglobin estimation, G6PD genotyping, blood group. Determination of blood groups was done by forward grouping using commercial antisera, G6PD done by flourescent spot test Hb genotypes by alkaline electrophoretic scan and malaria tests were performed microscopically after staining the films by giemsa. The influence of blood group, haemoglobin and G6PD genotypes on malaria was determined using multivariate logistic regression analysis with malaria uninfected healthy control as reference group controlling for age and gender. Blood group A (P=0.600), B (P=0.227), AB (P=0.279) and O (P = 0.787) had no influence on uncomplicated malaria and severe malaria infection (P > 0.05). G6PD normal (P=0.770), intermediate (P=0.327) and deficient (P=0.309) showed no influence on uncomplicated malaria. However, G6PD normal children were 4.81 times more likely to suffer from severe malaria (odds ratio [OR], 4.81; [95% CI], 2.10-8.01; *P* =0.001) relative to G6PD intermediate (odds ratio [OR], 1.61; [95% CI], 1.09-2.73; *P* = 0.015) and deficient (odds ratio [OR], 0.23; [95% CI], 0.14-0.85; *P* = 0.034) children. Haemoglobin AA (P=0.551), AS (P=0.509) and SS (P=0.753) had no influence on uncomplicated malaria. There was no influence of haemoglobin AA (P=0.654), AS (P=0.721) and SS (P=0.831) on severe malaria. Concurrent inheritance of hemoglobin genotype with either G6PD normal, intermediate and deficient had no influence on uncomplicated and severe malaria (P>0.005). The findings of this study provide better understanding of genetic variants and their influence on malaria *P. falciparum* malaria outcome. Determination of G6PD deficiency provides an insight into the choice of ant malarial medicine and better clinical management of patients suffering from malaria.

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ABBREVIATIONS

Bp	Base pair
CCC	Comprehensive care clinic
CI	Confidence interval
DNA	Deoxyribonucleic acid
G6PD	Glucose six phosphate dehydrogenase
Hb	Hemoglobin
Hb AA	Normal haemoglobin
Hb AS	Heterozygote sickle cell
Hb SS	Homozygote sickle cell
HBV	Hepatitis B virus
HC	Malaria uninfected healthy control
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
Kb	Kilobase
KNBS	Kenya National Bureau of statistics
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
OR	Odds ratio
SM	Severe malaria
UM	Uncomplicated malaria
WHO	World Health Organization

OPERATIONAL TERMS

Allele:	An alternative form of a gene (one member of a pair) that is located at a specific position on a specific chromosome
Gene:	Is a locus (or region) of DNA which is made up of nucleotides and is the molecular unit of heredity.
Genotype:	Is the set of genes in our DNA which is responsible for a particular trait.
Heterozygote:	An individual having two different alleles of a particular gene or genes, and so giving rise to varying offspring.
Homozygote:	An individual having two identical alleles of a particular gene or genes and so breeding true for the corresponding characteristic.
Rosetting:	The formation of clusters in which uninfected red blood cells (RBC) aggregate around a central *Plasmodium falciparum*-infected RBC (RBC) promoting RBC sequestration in the microvasculature.

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CHAPTER ONE: INTRODUCTION

1.1 Background information

Malaria is caused by a protozoan parasite of the genus *Plasmodium* and transmitted by the female anopheles mosquito through a bite. The four species that infect humans are *Plasmodium falciparum, P. vivax, P. ovale* and the recently discovered *P. Knowlesi. P. falciparum* is responsible for virtually all morbidities and mortalities (Snow *et al.,* 2005). An estimated 198 million malaria cases were reported worldwide of which 128 million were in the African region while 16 million were reported in Kenya by the end of 2013 (WHO, 2014). The prevalence of malaria at Kenya's highland of Vihiga was 52.0% (MOH, 2012). This high rates of malaria related morbidities is greatly driven by the malaria vaccine escape and drug resistant mutants resulting from host immune and drug pressure (Al-Harthi, 2011). Therefore, many investigations had been conducted to establish whether or not ABO blood group antigens, glucose-6-phosphate dehydrogenase (G6PD) and hemoglobin genotypes are associated with susceptibility, resistance, or severity of *P. falciparum* malaria (Driss *et al.*, 2011).

There are 33 blood group systems of which ABO blood group system remains to be the most important in transfusion and transplantation medicine (Logdberg *et al.,* 2011). *In-vitro* studies have shown that blood group O protects against severe *Plasmodium falciparum* malaria through the mechanism of reduced resetting among children residing at Kilifi, Kenya (Rowe *et al.*, 2007). Genetic diversity of ABO blood groups are specific to geographic area, race and ethnicity hence their ability to protect against malaria vary by regions and ethnic community (Dewan, 2015). However, the influence of ABO blood group on *P. falciparum* malaria among children living in Vihiga County Kenya, had not been determined.

Glucose-6-phosphate dehydrogenase (G6PD) catalyses the first step and controls the oxidative pentose phosphate pathway hence mutations cause instability of the enzyme or altered activity by decreasing affinity of G6PD for its substrates, Nicotinamide adenine dinucleotide phosphate ($NADP^{+)}$ or glucose-6-phosphate (G6P) (Luzzatto, 2006). While some studies reported that G6PD deficiency offer protection to malaria among male and female malarial nephropathy patients and children in Kenya, others have not (Otieno *et al.*, 1983; Suchdev *et al.*, 2014). These conflicting results are largely linked to genetic diversity within the G6PD locus, its effect on enzyme activity and its ability to protect against malaria which varies by geographic regions and ethnic groups (Maiga *et al.*, 2014; Manjurano *et al.*, 2015). However, the influence of G6PD deficiency on malaria among children in Vihiga county, Kenya, had not been determined.

Human haemoglobin is a tetrameric molecule composed of two pairs of α- and β-globins each encoded by α-globin and the β-globin genes, respectively (Schechter, 2008). Although some studies have reported that haemoglobin S genotype plays a significant role in modulating malaria in Kenyan children, others have not (McAuley *et al.*, 2010; Suchdev *et al.*, 2014). These conflicting findings are largely attributed to genetic diversity of haemoglobin gene ethnically, geographically and racially (Maiga *et al.*, 2014; Manjurano *et al.*, 2015). However, the influence of AS haemoglobin genotype on malaria among children living in Vihiga County, Kenya, had not been determined.

Protection by AS haemoglobin genotype and G6PD deficiency against *P. falciparum* malaria is usually thought to act independently (Lelliott *et al.*, 2015; Preuss *et al.*, 2012). Some studies in Africa reported that co-inheritance of both sickle cell trait and G6PD deficiency has been associated with increased protection against malaria (Awah *et al.*, 2012; Awah and Uzoengwe, 2006) while others have not (Guindo *et al.*, 2011) suggesting that the protective effect of concurrent G6PD deficiency and haemoglobin genotypes vary geographically within the African population. However, influence of concurrent inheritance of G6PD and haemoglobin genotypes on *p.falciparum* malaria in children under 3 years in Vihiga County Kenya, had not been determined. Young children have an incomplete immunity to malaria hence greatly burdened by *P. falciparum* parasitization (Jarra, 1983). Moreover, the acquisition of relative immunity with age greatly confounds the influence of ABO blood group, G6PD and haemoglobin genotype on malaria in older children and adults (Jarra, 1983). As such, the current study determined the influence of blood group, G6PD, and haemoglobin genotypes on *P. falciparum* malaria among children less than 3 years in Vihiga County Kenya.

1.2 Statement of the problem

Many investigations have been conducted to establish whether or not ABO blood group antigens, G6PD and Hb genotypes are associated with susceptibility, resistance, or severity of P. falciparum malaria (Driss et al.,2011). Findings have been conflicting and this is largely attributed to genetic diversity of ABO blood groups, G6PD and Hb genotypes and their ability to protect against malaria which vary geographically, ethnically and racially (Dewan, 2015., Maiga et al., 2014.,Manjurano et al.,2015) however, the same has never been done in Vihiga County. There is no literature review or published data on Vihiga, reports and communication from

clinicians reveal at least 2 children per week suffering from jaundice and malaria. It is thus important to determine the influence of ABO blood group, G6PD and Hb genotypes on *P. falciparum* malaria infection outcome in children below 3yeays of age living in Vihiga Kenya.

1.3 Objectives

1.3.1 General objective

To determine the influence of blood group, glucose-6-phosphate dehydrogenase and haemoglobin genotypes on *P. falciparum* infection among children under 3 years in Vihiga County Kenya.

1.3.2 Specific objectives

1. To determine the influence of A, B, AB, and O blood groups on *P. falciparum* malaria infection in children under 3 years in Vihiga County Kenya.
2. To determine the influence of G6PD normal, intermediate (heterozygous) and deficient (homozygous) genotypes on *P. falciparum* malaria infection in children under 3 years in Vihiga County Kenya.
3. To determine the influence of AA, AS, and SS haemoglobin genotypes on *P. falciparum* malaria in children under 3 years in Vihiga County Kenya.
4. To determine the influence of concurrent inheritance of G6PD and haemoglobin genotypes on *P. falciparum* malaria in children under 3 years in Vihiga County Kenya.

1.4 Hypotheses

1. H_0: There is no influence of A, B, AB, and O blood groups on *P. falciparum* malaria infection in children under 3 years in Vihiga County Kenya.
2. H_0: There is no influence of G6PD normal, intermediate (heterozygous) and deficient (homozygous) genotypes on *P. falciparum* malaria infection in children under 3 years in Vihiga County Kenya.
3. H_0: There is no influence of AA, AS, and SS haemoglobin genotypes on *P. falciparum* malaria infection in children under 3 years in Vihiga County Kenya.
5. H_0: There is no influence of concurrent inheritance of G6PD and haemoglobin genotypes on *P. falciparum* malaria infection in children under 3 years in Vihiga County Kenya.

1.5 Significance of the study

Findings of this study provide a better understanding of genetic variants and their influence on *P. falciparum* malaria infection outcome in the geographical location of Vihiga County, ethnic community of Luhyas in Kenya Determination of the association between ABO blood group antigens ,G6PD, and Hb genotype with *p. falciparum* malaria infection guides further investigations into the development of malaria vaccine, therapies, clinical management of patients suffering from malaria and also provide a clear consensus on the role of these genetic variants. Elucidation of ABO blood group distribution helps in effective establishment and management of blood bank inventory. Determination of G6PD deficiency provide an insight

into the choice of ant malarial medicine, guides further investigations into the development of vaccine and better clinical management of patients suffering from malaria.

CHAPTER TWO: LITERATURE REVIEW

2.1 Pathogenicity of *Plasmodium falciparum*

Infection by *Plasmodium falciparum* begins with the bite of an infected female *Anopheles* mosquito (Figure 1). After a silent infectious phase, primarily in the liver hepatocyte (Prudencio *et al.*, 2011), exoerythrocytic merozoite forms are passed into the blood stream as membrane-bound merosomes that rupture, allowing parasites access to circulating erythrocytes (Prudencio *et al.*, 2011; Sturm *et al.*, 2006). The merozoites rapidly invade erythrocytes, and as they grow and replicate, the intracellular parasite dramatically remodels the host red blood cell, giving rise to a rigid and poorly deformable cell with a propensity to adhere to a variety of cell types. These changes play a pivotal role in severe complications of *P. falciparum* malaria, with symptoms including fever, anaemia (Evans *et al.*, 2006), lactic acidosis, and in some cases coma and death (Miller *et al.*, 2002).

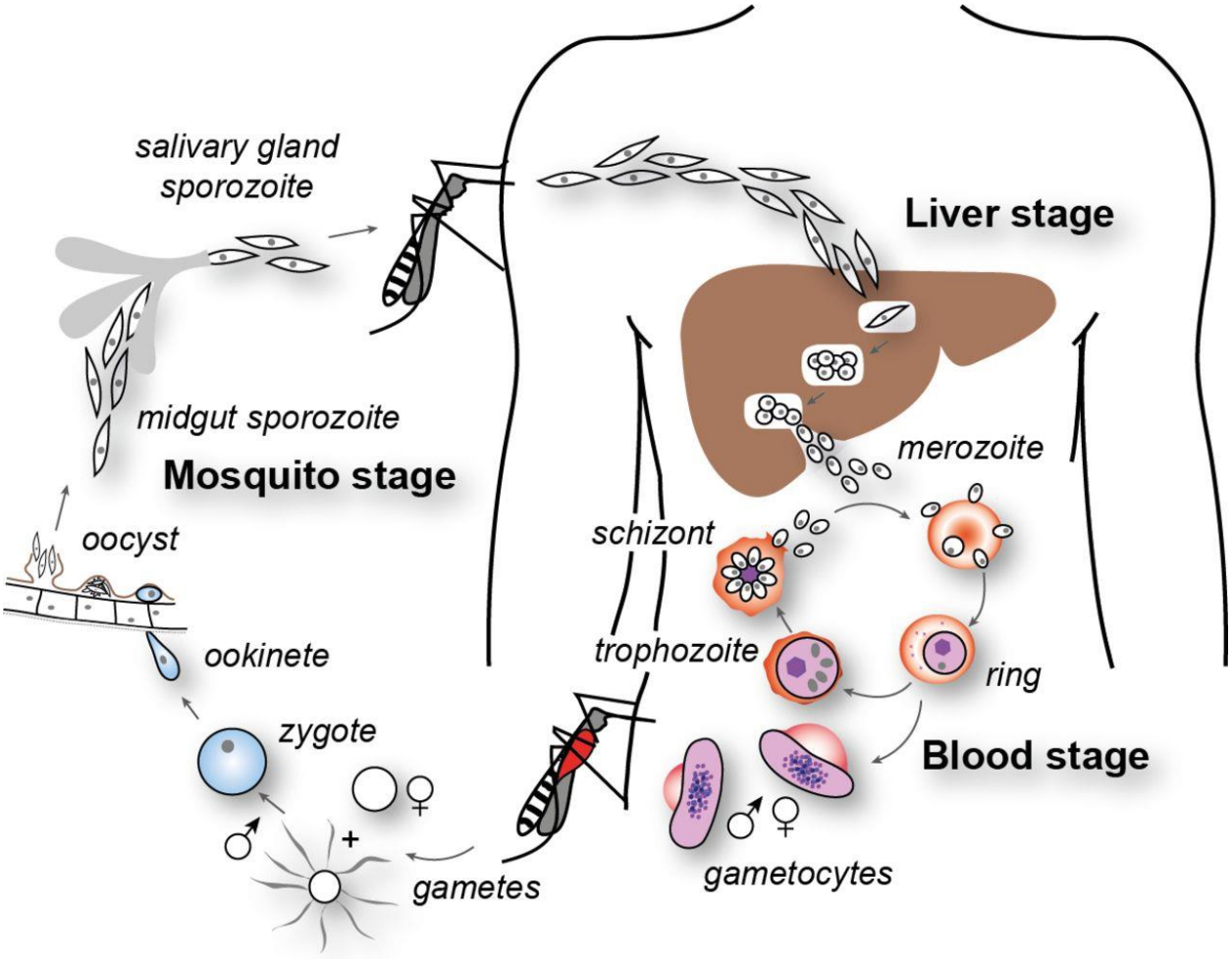

Figure 1: Lifecycle of *Plasmodium falciparum*. *Anopheles* mosquito bites a human and injects sporozoite forms. These move to the liver and invade hepatocytes, in which they develop to produce exoerythrocytic merozoite forms that are released into the blood stream. Merozoites invade erythrocytes and grow into trophozoites and mature schizonts. Merozoites are released that reinvade new erythrocytes. Gametocytes, formed from the asexual blood stage, are taken up by a feeding mosquito into the gut where they mature to form male and female gametes. The fertilized zygote develops to an ookinete and an oocyst and finally sporozoites that migrate to the salivary glands. Adopted from (Cowman *et al.*, 2012).

2.2 Epidemiology of malaria

Five species, *Plasmodium falciparum, P. vivax, P. ovale, P. Knowlesi* and *P. malariae, all* of these *Plasmodium* species cause malaria, and there is growing awareness of the importance of

each to global health (WHO, 2014). Despite malaria intervention policies and strategies, approximately 198 million cases of malaria (range: 124 million to 283 million) were reported globally in the year 2013 with Africa accounting for about 82% of the global malaria incidences (WHO, 2014). Moreover, 0.6 million (range: 0.4 million to 0.8 million) people mostly children succumbed to malaria (WHO, 2014). In Kenya, nearly 16.0 million malaria incidences and 40,000 deaths were reported in the year 2013 (WHO, 2014). Although about 55% of mortality and morbidity attributed to malaria is caused by *P. falciparum* (Snow *et al.*, 2005), *Plasmodium vivax* also causes a significant burden of disease (Guerra *et al.*, 2010).

2.3 Influence of ABO blood group antigens on *Plasmodium falciparum* malaria infection

To date, 33 blood group systems representing over 300 antigens have been listed by the International Society of Blood Transfusion (Logdberg *et al.*, 2011). Among the 33 systems, ABO remains the most important in transfusion and transplantation since any person possess clinically significant A, B and H carbohydrate antigens which can regulate protein activities during infection and antibodies against these antigens (Greenwell, 1997; Mitra *et al.*, 2014). Therefore, a number of studies have been conducted to investigate the association between ABO blood group systems with some diseases including malaria (Mitra *et al.*, 2014). Interestingly, the current geographic distribution of blood group O is consistent with a selection pressure by *P. falciparum* in favour of group O individuals in malaria-endemic regions (Cserti and Dzik, 2007).

Rosetting is characterized by the binding of *P. falciparum*-infected RBCs to uninfected RBCs to form clusters of cells that are thought to contribute to the pathology of *falciparum* malaria by obstructing blood flow in small blood vessels (Kaul *et al.*, 1991). The rosetting phenotype varies

between parasite isolates and correlates with severe *falciparum* malaria in sub-Saharan Africa (Rowe *et al.*, 2009). Rosetting parasites form larger, stronger rosettes in non-O blood groups (A, B or AB) than in group O red blood cells (Carlson and Wahlgren, 1992; Udomsangpetch *et al.*, 1993). Taken together, blood group O protects against severe malaria through reduced rossetting.

While no epidemiological study has determined the association between ABO blood group and susceptibility to severe *falciparum* infection *in-vivo* among Kenyan children, this has been demonstrated in African populations. A cross-sectional study in South-western Nigeria demonstrated the association between ABO blood group with malaria among children who were 3 years old (Amodu *et al.*, 2012). Similarly, ABO blood group has been linked to *P. falciparum* malaria in a cross-sectional study involving febrile patients attending a hospital in Southern Ethiopia (Zerihun *et al.*, 2011). In Zimbabwe, relationship between ABO blood and severe malaria has been shown among in- and out-patients (Fischer and Boone, 1998). However, conflicting results have also been reported in African populations. A cross-sectional study involving febrile patients attending a Health Center in Southern Ethiopia, reported equal vulnerability to malaria among the various ABO blood types (Degarege *et al.*, 2012). Also, a study targeting patients consulting at a health facility in Cameroon, showed no association between ABO blood group and *P. falciparum* malaria (Nkuo-Akenji *et al.*, 2004). Variations in these study findings are due to genetic diversity of ABO blood groups which are specific to geographic area, race and ethnicity (Dewan, 2015). Taken together, the ability of ABO blood group to modulate malaria infection varies by geographic area and ethnic community.

In Kenya, *in-vitro* studies have shown that blood group O protects against severe *Plasmodium falciparum* malaria through the mechanism of reduced rosetting (Rowe *et al.*, 2007). Interestingly, these rosettes are formed better depending on the blood cell types, with the blood cell type A and B having higher chances of forming rosettes (Carlson and Wahlgren, 1992; Udomsangpetch *et al.*, 1993). However, the influence of ABO blood group on *P. falciparum* malaria had not been determined in Vihiga Kenya. This study therefore, determined the influence of ABO blood groups on *P. falciparum* malaria among children under 3 years in Vihiga County Kenya.

2.4 Influence of G6PD on *P. Falciparum* malaria infection

The United States Food and Drug Administration (FDA) approved Primaquine, an 8-aminoquinoline, for treatment of malaria in 1952 despite the fact that it caused severe hemolytic anaemia upon ingestion (Hill *et al.*, 2006). Later in 1956, it was discovered that the hemolytic anaemia was caused by glucose-6-phosphate dehydrogenase (G6PD) deficiency (Alving *et al.*, 1956). The G6PD is 18 kb long and contains 13 exons and 12 introns, of which the exons' length varies between 12 bp and 236 bp and is located on the distal long arm of X chromosome (locus q28) (Martini *et al.*, 1986). Whereas G6PD deficiency is transmitted X-chromosomally, 140 mutations, mostly point mutations and small deletions which cause structural defects in the enzyme, have been established on the DNA sequence (Beutler and Vulliamy, 2002; Roos *et al.*, 1999). G6PD catalyses the first step and controls the oxidative pentose phosphate pathway hence mutations cause instability of the enzyme or altered activity by decreasing affinity of G6PD for its substrates, Nicotinamide adenine dinucleotide phosphate($NADP^{+)}$ or glucose-6-phosphate (G6P) (Luzzatto, 2006).

Potential protective mechanisms within G6PD deficient erythrocytes include increased oxidative stress in G6PD deficient cells accelerate ring stage erythrocyte senescence promoting phagocytic clearance of parasitized cells (Cappadoro *et al.*, 1998). G6PD deficient erythrocytes are also more susceptible to eryptosis, which is an additional factor mediating their early clearance when parasitized (Lang *et al.*, 2002). In addition, impaired *P. falciparum* growth within G6PD deficient cells is due to glutathione instability and oxidants such as alloxan, phenylhydrazine, and divicine (Preuss *et al.*, 2012).

Worldwide, between 300 to 400 million people carry at least one deficient G6PD gene making G6PD deficiency a significant enzymopathy (Nkhoma *et al.*, 2009). G6PD deficiency rate is prevalent in areas such as Africa where malaria is endemic or has been endemic (Tishkoff *et al.*, 2001). Therefore, numerous studies in Africa hypothesized that G6PD deficiency offers protection against malaria. By examples, a longitudinal study involving Ugandan children confirmed the protective nature of G6PD deficiency to malaria infection (Bwayo *et al.*, 2014). A cross-sectional study in Nigeria reported a positive malaria prognosis in G6PD deficient children (Orimadegun and Sodeinde, 2014). Meanwhile, conflicting findings have also been reported in Africa. A multinational study involving Kenya, Burkina Faso, Ghana, Nigeria, Tanzania and Mali showed no association between G6PD and malaria (Carter *et al.*, 2011). Similar results were reported in a cross-sectional descriptive study involving asymptomatic Nigerian children (Jeremiah *et al.*, 2010). G6PD deficiency was not associated with malaria in another study involving malaria infected and uninfected Nigerian children (Martin *et al.*, 1979). Therefore,

previous literature suggests that G6PD gene that protects against malaria varies by geographic area and ethnic community.

Inconclusive findings on the association between G6PD and malaria have also been reported in Kenya. Along the Lake Victoria region of Kenya, G6PD deficiency has been shown to offer protection to malaria among male and female malarial nephropathy patients (Otieno *et al.*, 1983). A cross-sectional study in Nyando reported that G6PD deficiency offer protection against malaria in males aged between 6-35 months (Suchdev *et al.*, 2014). However, the same study showed that G6PD deficiency does not offer protection against malaria in females of the same age group (Suchdev *et al.*, 2014). Meanwhile, genetic diversity within the G6PD locus, its effect on enzyme activity and its ability to protect against malaria varies between regions, race and ethnic groups (Maiga *et al.*, 2014; Manjurano *et al.*, 2015). However, association between G6PD deficiency and malaria in the ethnic community of Vihiga, Kenya, had not been determined. Therefore, this study will determine the influence of G6PD deficiency on *P. falciparum* malaria in children under 3 years in Vihiga, Kenya.

2.5 Influence of hemoglobin genotype on *P. falciparum* malaria infection

Human haemoglobin is a tetrameric molecule that consists of two pairs of identical polypeptide subunits, the α- and β-globins, each encoded by a different family of genes. The human α-like globin genes (*ζ, α_1*, and *α_2*) are located on chromosome 16, and the β-like globin genes (*ε, Gγ, Aγ, δ,* and *β*) are located on chromosome 11 (Frenette and Atweh, 2007). At birth, although the α genes remain fully active, the γ genes are effectively down-regulated and the β-like (δ and β) genes are up-regulated so that by the end of the first year of life, the "adult" haemoglobin (Hb)

phenotype, haemoglobins A ($\alpha_2\beta_2$) and A_2 ($\alpha_2\delta_2$), is predominant (Frenette and Atweh, 2007). In some cases, expression of the γ-globin persists in adult erythroid cells; this largely asymptomatic state is known as hereditary persistence of fetal hemoglobin (HPFH) (Forget, 1998). Upon completion of the switch from Hb F to Hb A, patients with disorders of the *β-globin* genes start manifesting the clinical features of sickle cell disease. Sickle cell disease etiology has been linked to single nucleotide change (A to T) in the *β-globin* gene resulting in valine for glutamic acid substitution in the β-globin protein (Ingram, 1956). This in turn allows the formation of stable intermolecular interactions in the concentrated intracellular solutions of deoxyhemoglobin S ($\alpha_2\beta_2^S$) (Serjeant, 2013).

Mechanical and immunological changes in infected Hb AS red blood cells have been shown to alter disease progression. Rosette formation, the binding of *P. falciparum*-infected red blood cells to uninfected red blood cells, leads to microcirculatory obstruction in cerebral malaria (Aikawa *et al.*, 1990). Rosette formation is impaired in *P. falciparum*-infected Hb AS red blood cells under deoxygenated conditions (Carlson *et al.*, 1990). Impaired rosette formation with Hb AS red blood cells is due to increased sickling of these cells in deoxygenated conditions or to reduced expression of erythrocyte surface adherence proteins contribute to protection against severe malaria (Gong *et al.*, 2013). Cytoadhearance enables parasites to sequester in the vasculature and avoid clearance by the spleen, leading to endothelial activation and associated inflammation in the brain and other organs, important in the progression to severe malaria (Dondorp *et al.*, 2004). Therefore, reduced cytoadherence of Hb AS and Hb SS erythrocytes likely leads to increased spleenic clearance, and may in part explain lower parasite densities and a lower incidence of severe malaria in Hb AS individuals (Cholera *et al.*, 2008). It has been

reported that phagocytosis by monocytes of Hb AS red blood cells infected with ring-stage *P. falciparum* is enhanced compared to that of infected Hb AA cells (Ayi *et al.*, 2004). This enhanced phagocytosis is due to increased presentation of opsonins, including membrane bound IgG, C3c, membrane-bound hemichromes, and aggregated band 3 (Ayi *et al.*, 2004). The lymphoproliferative and gamma globulin response to purified *P. falciparum* soluble antigens is higher in Hb AS children compared to Hb AA children suggesting a more robust cellular and humoral responses to *P. falciparum* in Hb AS children (Abu-Zeid *et al.*, 1991; Gong *et al.*, 2013).

In the year 2010, a geostatistical modelling proved a strong geographical link between the highest haemoglobin S allele frequencies and high malaria endemicity regions in Africa (Piel *et al.*, 2010). Indeed, malaria is a selective force for haemoglobin S alleles in Africa. A case-control study in Mali revealed the protective effect of haemoglobin S gene against malaria (Toure *et al.*, 2012). Similarly, a cross-sectional study in Nigeria showed that haemoglobin S gene modulates malaria severity in infected children (Amodu *et al.*, 2012). In Uganda, children harbouring haemoglobin S gene had lower episode of malaria and lower parasitemia (Parikh *et al.*, 2004). On the contrary, homozygous haemoglobin A was associated with protection against higher parasitemia among f*alciparum* infected Gabonese school children (Migot-Nabias *et al.*, 2000). In Cameroon, homozygous haemoglobin A and heterozygous haemoglobin S children are equally vulnerable to malaria (Bernstein *et al.*, 1980). Taken together, haemoglobin S variants regulating malaria vary by geographic area and ethnic community.

In Kenya, the association between haemoglobin and malaria has also been determined. In coastal Kenya, haemoglobin S genotype has been shown to offer protection against malaria in children (Williams *et al.*, 2005b). A prospective study in Western lowland of Kenya demonstrated that heterozygous haemoglobin S provides significant protection against severe malaria and high-density parasitaemia among children (Aidoo *et al.*, 2002). A retrospective study in Kilifi reported that children harbouring haemoglobin S gene have lower *Plasmodium falciparum* parasitemia compared to normal haemoglobin genotype children (Komba *et al.*, 2009). In Kilifi, a case control study demonstrated the protective effect of haemoglobin S genotype against uncomplicated malaria and severe malaria in children (McAuley *et al.*, 2010). On the other hand, a cross-sectional study in Nyando revealed that susceptibility to malaria in children is not influenced by haemoglobin genotype (Suchdev *et al.*, 2014). Since diversity of haemoglobin gene is specific to geographic area, race and ethnic group (Mohammed *et al.*, 2006; Sabahelzain and Hamamy, 2014), it is important to determine associated effect on haemoglobin genotypes on malaria among children from the Highlands of Kenya. However, relationship between haemoglobin genotypes and malaria among residents of the Vihiga County Kenya, has not been determined. As such, the present study determined the influence of haemoglobin genotypes on malaria in children under 3 years residents of Vihiga County Kenya.

2.6 Influence of concurrent G6PD and hemoglobin genotypes on *P. falciparum* malaria

Erythrocytes undergo several age-related physiological changes during their normal lifespan, which eventually trigger their phagocytosis and recycling by macrophages. This senescence exist in tow major models. In the first model, referred to as “band 3 senescence”, a build-up of haemichromes (products of haemoglobin degradation) results in crosslinking between

cytoplasmic domains of band 3 proteins and formation of aggregates (Pantaleo *et al.*, 2008). Aggregation is facilitated by phosphorylation of band 3, another hallmark of senescence, which reduces its affinity for the cytoskeletal protein, ankyrin, and increases its mobility in the membrane (Ferru *et al.*, 2011). Aggregated band 3 is recognized by naturally occurring antibodies, which in turn promotes complement protein C3 binding and activation (Pantaleo *et al.*, 2008). This finally results in complement-mediated phagocytosis of the senescent cell (Pantaleo *et al.*, 2008). The second model, referred to as "eryptosis", is characterized by increased intracellular calcium, activation of proteases and phosphatidylserine (PS) exposure on the external surface of the plasma membrane (Bratosin *et al.*, 2001). PS exposure leads to phagocytosis of the eryptotic cell (Foller *et al.*, 2009). Both eryptosis and band 3 senescence are elevated in parasitized erythrocytes (Eda and Sherman, 2002), and in direct correlation with increasing parasite maturation (Giribaldi *et al.*, 2001). An increased oxidative burden imparted on the cell by the parasite is believed to be a central cause of these phenomena (Giribaldi *et al.*, 2001). Naturally occurring antibodies to band 3 are associated with improved malaria outcome, indicating that accelerated band 3 senescence may be an important contributor to host defence (Hogh *et al.*, 1994).

In several of the haemoglobinopathies, aberrant haemoglobin is thought to contribute towards the build-up of haemichromes in erythrocytes, thereby accelerating band 3 aggregation (Arese *et al.*, 2005). In ring stage infected erythrocytes, senescence of HbAS occurs more rapidly than equivalently infected normal erythrocytes (Ayi *et al.*, 2004). This renders ring stage parasites within these cells more susceptible to phagocytosis, which could contribute toward the resistance phenotype conveyed by these disorders. Eryptosis is also enhanced in HbAS and contributes to

the early clearance of parasitized cells through PS exposure and phagocytosis (Lang *et al.*, 2002). Potential protective mechanisms within G6PD deficint erythrocytes include increased oxidative stress in G6PD deficient cells accelerate ring stage erythrocyte senescence promoting phagocytic clearance of parasitized cells (Cappadoro *et al.*, 1998). G6PD deficient erythrocytes are also more susceptible to eryptosis, which may be an additional factor mediating their early clearance when parasitized (Lang *et al.*, 2002). Finally, impaired *P. falciparum* growth within G6PD deficient cells (Preuss *et al.*, 2012) suggest that protection by G6PD deficiency and sickle cell trait are mutually exclusive.

In Africa, studies have investigated concurrent G6PD and haemoglobin genotypes. Two separate studies in Nigeria and Cameroon reported that co-inheritance of G6PD deficiency and AS haemoglobin is associated with increased protection against malaria (Awah and Uzoengwe, 2006; Awah. *et al.*, 2012). However, a study in Mali revealed that concurrent G6PD deficiency and sickle cell trait have no effect on severity of malaria (Guindo *et al.*, 2011). In Kenya, including Vihiga County, the influence of concurrent inheritance of G6PD and haemoglobin genotype on *falciparum* malaria has not been determined. Therefore, this study determined the influence of concurrent inheritance of G6PD and haemoglobin genotype on *P. falciparum* malaria in children under 3 years in Vihiga County Kenya.

CHAPTER THREE: METHODOLOGY

3.1 Study Site

This study was conducted at Vihiga Count Rereferal Hospital. There has been a marked increase in malaria in the Vihiga highland, nearly 1.3 times the overall rate in Kenya, largely due to the rise of drug-resistant strains of *Plasmodium falciparum* parasites (MOH, 2012; Zhong *et al.*, 2008). The ecology of the Vihiga highlands of Kenya supports stable transmission and increasing population pressure has led to agricultural changes creating ideal conditions for malaria vector proliferation (Munga *et al.*, 2006). Finally, *Anopheles* mosquitoes are generally highly zoophilic, rather than anthropophilic, that is, they greatly prefer animals to humans for blood meals thus becoming an efficient human malaria vectors in Vihiga highland (Ndenga *et al.*, 2016).

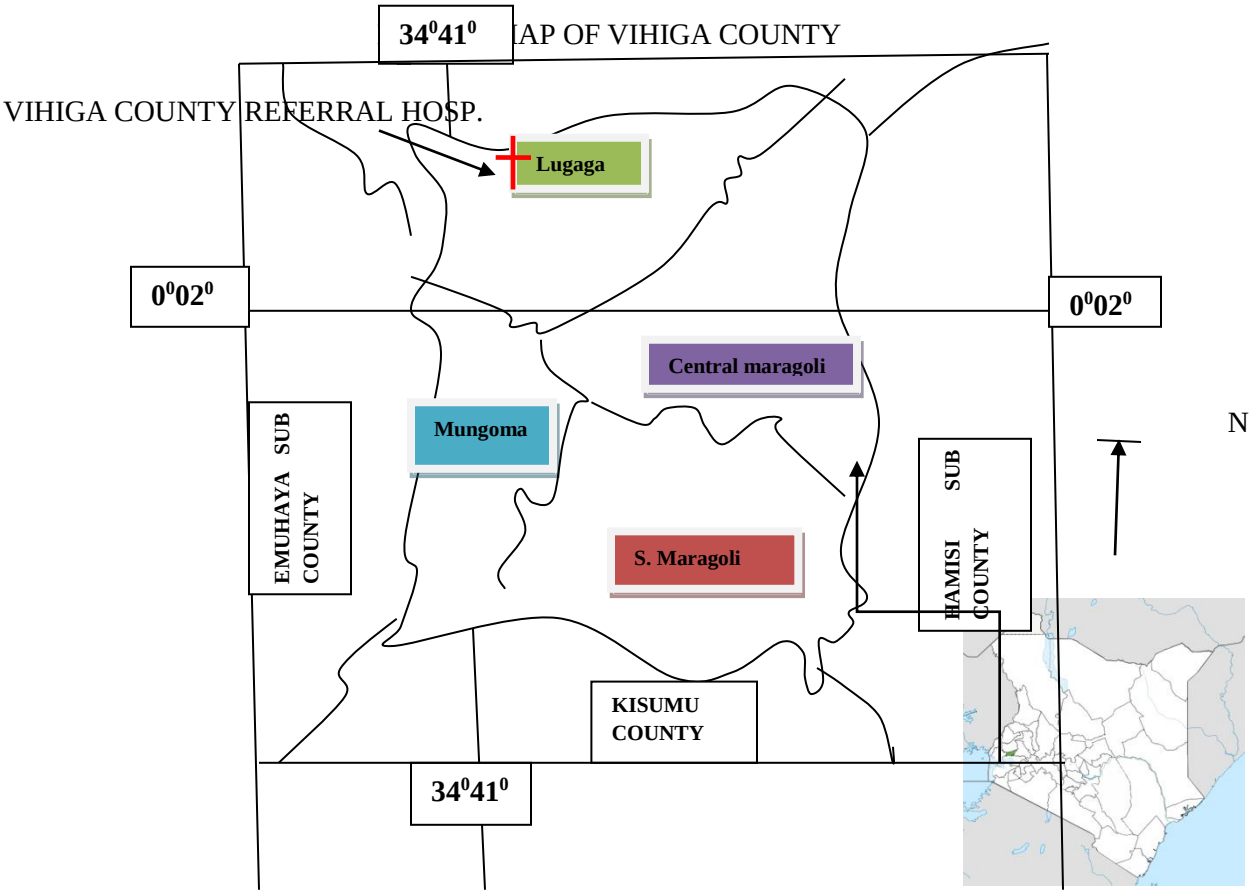

Figure 2: Map of Vihiga County, Kenya

3.2. Study design and study population

This was a hospital based cross-sectional study targeting children less than three years residents of Vihiga County, Kenya. The target population was under three years old because their immunity is not fully developed and this allowed clear observation of the genetic variants. Children were recruited via random sampling method and stratified into either malaria infected and uninfected healthy controls based on blood smear malaria microscopy diagnosis. Malaria infected were further classified into severe malaria (cerebral malaria, severe anaemia; haemoglobin concentration < 5 g/dl and circulatory collapse; systolic blood pressure < 50 mmHg) as outlined by World Health Organization (WHO) (WHO, 2012). Malaria infected children who lacked these features were categorized as uncomplicated malaria (WHO, 2012).

3.3 Eligibility criteria

3.3.1 Inclusion criteria

Children who were under 3 years, and their parents signed an informed consent form, in addition they disclosed any information that was required during the study period.

3.3.2 Exclusion criteria

1. *Plasmodium falciparum* malaria positive children who had received anti-malarial treatment within 48 hrs prior to the microscopical confirmation of their blood slides for malaria parasites.
2. Children co-infected with *P. falciparum* , other species of plasmodium, HIV-1, HBV and HCV.

3.4 Sample size determination

The sample size was determined using the formula $n = Z^2pq/d^2$ (Bryan, 1992.).

Where:

n = the sample size required,

z = 1.96: confidence level test statistic at the desired level of significance,

p = 52.0%: prevalence of malaria (MOH, 2012).

q=1-p: proportion of malaria uninfected and d = 0.05: acceptable error willing to be committed.

Therefore $n = (1.96 \times 1.96 \times 0.52 \times 0.48) \div 0.05^2$

The optimum sample size estimated was n = 384. Therefore, this study required at least 384 study participants.

3.5 Data collection

Socio-demographic characteristics such as age, gender and anti-malarial therapy information were collected using a questionnaire (Appendix 1). Questions were read exactly as they appeared on the interview questionnaire. Answers to the questions were either close-ended or open-ended.

3.6 Laboratory techniques

3.6.1 HIV-1 and 2

Approximately, 2.0 ml of venopuncture blood was collected in BD™ vacutainer anticoagulant tubes (Becton, Dickinson and Company, Franklin Lakes, USA) from each study participant. A drop of the blood was used for HIV-1, HBV and HCV serological testing. HIV counselling and

testing was conducted by trained counsellors. HIV-1 sero-testing was performed using KHB™ (Shanghai Kehua Bio-engineering Co. Ltd, China), First Response™ (Premier Medical Corporation Ltd., Kachigam, India) and Unigold™ (Trinity Biotech Plc, Bray, Ireland) rapid tests for human immunodeficiency virus type 1 and 2 (HIV 1-2) according to the Kenyan HIV testing algorithm (NASCOP, 2010). Hepatitis B and C infection status was determined using Determine™ HBV and HCV rapid test strips, respectively.

3.6.2 Determination of hemoglobin level

Hemoglobin level was measured using Hb Hemocue 301(kuvettgattan 1,SE-26271 Angelholm Sweden) in line with manufacturer's recommendations. The system was calibrated every morning before sample analysis. All haemoglobin measurements were performed within 10 minutes from the time of blood collection to minimize variability in the measurements.

3.6.3 ABO blood group determination

ABO blood groups typing was performed by forward grouping using commercial antisera (Biotech laboratories Ltd, Ipswich, Suffolk, UK) according to manufacturer's protocol. Briefly, one drop of whole blood was placed in three different places on a grease-free clean glass slide labelled A,B and D. A drop of antisera A was added on the area labelled A, anti B on B and anti D on D. The blood cells and the antiseras were mixed with a wooden applicator stick. The slide was then tilted to check for agglutination and the result recorded accordingly.

3.6.4 Malaria diagnosis

Thick and thin blood films were prepared from venous blood, stained with 10% Giemsa stain for 10 minutes and examined under a microscope. Parasite densities were calculated using the thick films by the WHO method (parasite count × 8,000 divided by the number of WBCs counted which was 200) (WHO, 2012), and the thin films were used to establish the species of the parasites present. Films were classified as negative when no parasites are seen after two hundred microscopic fields had been examined. For purposes of quality control, slides were cross-examined independently by a senior microscopist and the results compared.

3.6.5 Hemoglobin genotyping

Hemoglobin genotypes were determined by cellulose acetate electrophoresis with Titan III plates according to the manufacturer's protocols (Helena Bio-Sciences, Oxford, United Kingdom). Hemolysates prepared from blood samples and Hemo AFSC controls was dispensed onto the acetate paper, and hemoglobin variants separated by electrophoresis with an alkaline buffer at pH 8.6. The plates were then stained using Ponceau S stain, and hemoglobin genotypes scored using the Hemo AFSC control.

3.6.6 G6PD genotyping

Glucose-6-phosphate dehydrogenase (G6PD) deficiency was determined by a fluorescent spot test (Trinity Biotech Plc., Bray, Ireland) as per the manufacturer's protocol. Blood was hemolyzed and spotted onto a filter paper. Assay solution containing glucose-6-phosphate and oxidized NADP ($NADP^+$) was added, and samples excited with UV light at 340 nm. Based on

the presence or absence of fluorescence emissions, the samples were scored as normal (high emission), intermediate (moderate emission), or deficient (no emission).

3.7 Statistical analysis

Statistical analysis was performed using SPSS version19.0. Age and haemoglobin level were summarized as medium and interquartile ranges while categorical variable such as gender was summarized as proportion (%). Age and haemoglobin level were compared across group using Kruskal Wallis and Dunn's multiple comparison tests. Gender was compared across group using Chi-square test. The distribution of blood group, G6PD and hemoglobin genotypes on malaria was determined using Chi-square test. The influence of blood group, G6PD and hemoglobin genotypes on malaria was determined using multivariate logistic regression analysis. Significance was set at $P \leq 0.05$.

3.8 Ethical considerations

Ethical approval for this study was obtained from Maseno University Ethical Review Committee (Appendix 2) while research permit was acquired from County government of Vihiga department of health (Appendix 3). This study was conducted according to Helsinki's declaration. Written informed consent (Appendix 4) was obtained from all participants before enrolment. Confidentiality was ensured throughout the study by removing all personal identifiers (that is, no individual names were used) and codes were used for study participant records. All study forms and data records were archived in secure cabinets and office while biological samples were stored in lockable freezers with access to the data and biological samples limited only to the investigators. Blood smear positive study participants were treated for malaria according to the

national guidelines. HIV-1 infected children were referred to comprehensive care clinic at Vihiga County Hospital for special care and management.

CHAPTER FOUR: RESULTS

4.1 Demographic and laboratory measurements of children under 3 years in Vihiga County, Kenya

The demographic and laboratory measurements of the study participants are presented in Table 1. A total of 854 children were enrolled in to this study comprising of 414 (48.5%) malaria uninfected healthy controls and 440 (51.5%) malaria infected cases (severe malaria, n=72, and uncomplicated malaria cases, n=368). Gender distribution was similar across the study groups (P = 0.060). Age distribution was also similar across the study groups (*P* = 0.851). Most importantly, children with severe malaria presented with lower haemoglobin level (median, 4.3; IQR, 1.0) compared to uncomplicated malaria (median, 8.1; IQR, 2.5) and malaria uninfected healthy controls (median, 10.6; IQR, 1.6; P<0.0001).

Table 4.1. Demographic and clinical characteristics of study participants

Characteristics	**HC(n=414)**	**SM (n=72)**	**UM (n=368)**	***P* value**
Gender				
Females	180 (43.5)	33 (45.8)	191 (51.9)	0.060
Males	234 (56.5)	39 (54.2)	177 (48.1)	
Age in months	17.2 (11.3)	18.0 (11.8)	19.0 (11.5)	0.851
Laboratory information				
Hb g/dl	10.6 (1.6) [a]	4.3 (1.0) [b]	8.1 (2.5)	**<0.0001**

Data are presented as medians (IQR, interquartile range) or indicated as number (n) and proportion (%) of subjects. Statistical analysis was performed using Kruskal–Wallis test for continuous measures and Chi-square test for gender distribution.

4.2 Influence of ABO blood group on falciprum malaria infection in children under 3 years in Vihiga County, Kenya

The distribution of blood group on *p falciprum* infection in children under 3 years in Vihiga county, Kenya, is presented in table 4.2 (a). The prevalence of blood group A was 23.4%, 27.85%, and 26.1% and that of blood group AB was 2.4%, 2.8%, 4.1% in the malaria uninfected

healthy control (HC), severe malaria (SM) and uncomplicated malaria (UM) group, respectively. The proportion of children with blood type B was 30.4%, 33.3%, and 25.5%, while that of blood group O was 43.7%, 36.1%, and 44.3%, in the malaria uninfected health controls (HC), severe malaria (SM) and uncomplicated malaria (UM), respectively.

To determine the influence of ABO blood group on falciparum malaria, multivariate logistic regression was conducted controlling for age and gender (table 4.2 (b)). Malaria uninfected healthy controls had the highest frequency of study participants hence it was used as a reference category. There was no influence of blood group A (P=0.600), B (P=0.227), and O (P = 0.787) on uncomplicated malaria group. Likewise, there was no influence of blood group A (P=0.263), B (P=0.680), and O (P=0.891) on severe malaria.

Table 4.2(a). Distribution of ABO blood group on falciparum malaria in children under 3 years in Vihiga County, Kenya

Blood group	HC(n=414)	SM (n=72)	UM (n=368)	*P* value
A	97 (23.4)	20 (27.8)	96 (26.1)	0.585
AB	10 (2.4)	2 (2.8)	15 (4.1)	-
B	126 (30.4)	24 (33.3)	94 (25.5)	0.206
O	181 (43.7)	26 (36.1)	163 (44.3)	0.429

Data shown are number (n) and proportions (%) of study participants. HC, malaria uninfected healthy control. SM, severe malaria. UM, uncomplicated malaria. -, Statistical analysis not performed because more than 20% of the expected counts are less than 5. A, blood group A. B, blood group B. AB, blood group AB. O, blood group O. Statistical comparison was performed using Pearson's Chi-square test.

Table 4.2(b). Influence of ABO blood group on falciparum malaria in children under 3 years in Vihiga County, Kenya

STUDY GROUP	Blood Group	OR	95 % CI	P-Value
UM	A	1.09	0.77-1.56	0.600
	B	1.67	0.73-3.81	0.227
	AB	-	-	-
	O	1.06.	0.46-3.75	0.787.
SM	A	1.44	0.76-2.70	0.263
	B	1.39	0.29-6.71	0.680
	AB	-	-	-
	O	1.10	0.48-5.78	0.891

Odds ratios (OR) and 95% confidence intervals (95% CI) were determined using multivariate logistic regression controlling for age and gender. Health controls (HC) were used as reference category. SM, severe malaria. UM, uncomplicated malaria. A, blood group A. B, blood group B. AB, blood group AB. O, blood group O. -, multivariate logistic regression was not performed because there were less than five cases of AB blood type children in the severe malaria group.

4.3 Influence of glucose-6-phosphate dehydrogenase on falciparum infection in children under 3 years in Vihiga county, Kenya.

The distribution of glucose-6-phosphate dehydrogenase genotype on *falciparum malaria* infection in children under 3 years in Vihiga county, Kenya, is presented in table 4.3 (a). The prevalence of G6PD normal was significantly higher in the malaria uninfected healthy control (35.7%) compared to severe malaria (48.6%) and uncomplicated malaria group (48.9%; P=0.001). The proportion of G6PD intermediate was 34.3%, 43.1%, and 33.2% in the malaria uninfected health control, severe malaria and uncomplicated malaria group, respectively. Higher prevalence of G6PD deficient was observed in the malaria uninfected healthy control (30.0%) relative to severe malaria (8.3%)and uncomplicated malaria group (17.9%; P< 0.0001).

The influence of G6PD on falciparum malaria is presented in table 4.3 (b). Using multivariate logistic regression with malaria uninfected healthy control as reference group adjusting for age and gender, there was no association between G6PD normal (P=0.770), intermediate (P=0.327) and deficient (P=0.309) with uncomplicated malaria. G6PD normal children had higher odds of severe malaria (odds ratio [OR], 4.81; 95% confidence interval [95% CI], 2.10-8.01; P=0.001). Children with G6PD normal genotype were 4.81 times more likely to suffer from severe malaria (odds ratio [OR], 4.81; 95% confidence interval [95% CI], 2.10-8.01; P=0.001) relative to children with G6PD intermediate (odds ratio [OR], 1.61; 95% confidence interval [95% CI], 1.09-2.73; P= 0.015) and deficient (odds ratio [OR], 0.23; 95% confidence interval [95% CI], 0.14-0.85; P=0.034) genotypes.

Table 4.3 (a). Distribution of glucose-6-phosphate dehydrogenase on *falciparum malaria* infection in children under 3 years in Vihiga county, Kenya

G6PD type	HC (n=414)	SM (n=72)	UM (n=368)	*P* value
Normal	148 (35.7)	35 (48.6)	180 (48.9)	**0.001**
Intermediate	142 (34.3)	31 (43.1)	122 (33.2)	0.268
Deficient	124 (30.0)	6 (8.3)	66 (17.9)	**< 0.0001**

Data shown are number (n) and proportions (%) of subjects. HC, malaria uninfected healthy control. G6PD, glucose-6-phosphate dehydrogenase. Normal, normal G6PD. Intermediate, heterozygous G6PD. Deficient, homozygous G6PD. SM, severe malaria. UM, uncomplicated malaria. Statistical comparison was performed using Pearson's Chi-square test.

Table 4.3 (b). Influence of glucose-6-phosphate dehydrogenase on *falciparum malaria* infection in children under 3 years in Vihiga County, Kenya

Study Group	G6PD Status	OR	95 % CI	P-Value
UM	Normal	0.93	0.54-1.58	0.770
	Intermediate	1.37	0.76-2.23	0.327
	Deficient	0.56	0.31-1.59	0.309
SM	Normal	4.81	2.10-8.01	**0.001**
	Intermediate	1.61	1.09-2.73	**0.015**
	Deficient	0.23	0.14-0.85	**0.034**

Odds ratios (OR) and 95% confidence intervals (95% CI) were determined using multivariate logistic regression controlling for age and gender. Health controls (HC) were used as reference category. G6PD, glucose-6-phosphate dehydrogenase. Normal, normal G6PD. Intermediate, heterozygous G6PD. Deficient, homozygous G6PD. SM, severe malaria. UM, uncomplicated malaria.

4.4 Influence of hemoglobin genotype on falciparum infection in children under 3 years in Vihiga County, Kenya

The distribution of haemoglobin genotype in children under 3 years in Vihiga County Kenya, is presented in table 4.4 (a). The prevalence of haemoglobin AA was higher in the severe malaria group (93.1%) relative to malaria uninfected healthy controls (71.7%) and uncomplicated malaria group (77.7%; P<0.0001). The frequency of haemoglobin AS was lower in the severe malaria group (6.9%) compared to malaria uninfected healthy control (27.3%) and uncomplicated malaria (22.3%; P=0.001). Haemoglobin SS was only observed in the malaria uninfected healthy control group.

To investigate the influence of hemoglobin genotype on *P. falciparum* infection in children under 3 years in Vihiga County, Kenya, multivariate logistic regression was performed controlling for age and gender. Malaria uninfected healthy control was used as a reference control. Haemoglobin AA (P=0.551), and AS (P=0.509) had no influence on uncomplicated

malaria. Consistent with the observation in the uncomplicated malaria group, there was no influence of haemoglobin AA (P=0.654), and AS (P=0.721) on severe malaria.

Table 4.4 (a). Distribution of hemoglobin genotype on falciparum infection in children under 3 years in Vihiga county, Kenya

Haemoglobin	HC (n=414)	SM (n=72)	UM (n=368)	*P* value
AA	297 (71.7)	67 (93.1)	286 (77.7)	< **0.001**
AS	113 (27.3)	5 (6.9)	82 (22.3)	< **0.001**
SS	4 (1.0)	0 (0.0)	0 (0.0)	-

Data shown are number (n) and proportions (%) of subjects. HC, malaria uninfected healthy control. SM, severe malaria anemia. UM, uncomplicated malaria. AA, normal hemoglobin. AS, heterozygote sickle cell. SS, homozygote sickle cell. -, Statistical analysis was not performed because more than 20% of the expected counts are less than 5. Statistical comparison was performed using Pearson's Chi-square test.

Table 4.4 (b). Influence of hemoglobin genotype on falciparum infection in children under 3 years in Vihiga county, Kenya

Study Group	Haemoglobin Status	OR	95 % CI	P-value
UM	AA	0.46	0.21-3.44	0.551
	AS	1.30	0.75-4.01	0.509
	SS	-	-	-
SM	AA	0.31	0.21-1.80	0.654
	AS	1.55	0.59-5.17	0.721
	SS	-	-	-

Odds ratios (OR) and 95% confidence intervals (95% CI) were determined using multivariate logistic regression controlling for age and gender. Health controls (HC) was used as reference category. SM, severe malaria anemia. UM, uncomplicated malaria. AA, normal hemoglobin. AS, heterozygote sickle cell. SS, homozygote sickle cell. -, multivariate logistic regression was not performed because there were less than five cases of haemoglobin SS children in the severe malaria and uncomplicated malaria group.

4.5 Influence of co-inheritance of hemoglobin and G6PD genotype on falciparum infection in children under 3 years in Vihiga County, Kenya

The distribution of co-inheritance of haemoglobin and G6PD genotype on *p. falciparum* infection in children under 3 years in Vihiga county, Kenya, is presented in table 4.5(a). There was a higher proportion of children harbouring both Hb AA and G6PD intermediate genotypes in the severe malaria (38.9%) relative to healthy controls 24.4%) and uncomplicated malaria (25.8%; P=0.035). There was a higher prevalence of children harbouring both Hb AA and G6PD normal in the severe malaria group (45.8%) compared to uncomplicated malaria (37.8%) and malaria uninfected healthy control (29.0%; P = 0.003). The prevalence of children harbouring both Hb AA and G6PD deficiency genotype was non-significantly higher in the malaria uninfected health control group (18.4%) relative to uncomplicated malaria (14.1%) and severe malaria group (8.3%; P= 0.054). The proportion of children harbouring both Hb AS and G6PD intermediate (9.4%, 4.2% and 7.3%), Hb AS and G6PD normal (6.8%, 2.8% and 11.1%), Hb AS and G6PD deficiency (11.1%, 0.0%, and 3.8%), Hb SS and G6PD intermediate (0.5%, 0.0%, and 0.0%), Hb SS and G6PD normal (0.0%, 0.0%, and 0.0%) and Hb SS and G6PD deficiency (0.5%, 0.0%, and 0.0%) genotype in the healthy control, severe malaria and uncomplicated malaria group, respectively.

To investigate the influence of concurrent inheritance of haemoglobin and G6PD genotype on *P. falciparum* infection, multivariate logistic regression was performed (Table 4.5b). There was no influence of Hb AA and G6PD intermediate (P=0.061), Hb AA and G6PD normal (P=0.069) and Hb AA and G6PD deficiency (P=0.095) on uncomplicated malaria. Likewise, there was no infect

on Hb AA and G6PD intermediate (P=0.063), Hb AA and G6PD normal (P=0.072) and Hb AA and G6PD deficiency (P=0.071) on severe malaria.

Table 4.5(a). Distribution of co-inheritance of haemoglobin and G6PD genotype on falciparum infection in children under 3 years in Vihiga county, Kenya

Haemoglobin and G6PD type	HC (n=414)	SM (n=72)	UM (n=368)	*P* value
Hb AA and G6PD intermediate	101 (24.4)	28 (38.9)	95 (25.8)	**0.035**
Hb AA and G6PD normal	120 (29.0)	33 (45.8)	139 (37.8)	**0.003**
Hb AA and G6PD deficiency	76 (18.4)	6 (8.3)	52 (14.1)	0.054
Hb AS and G6PD intermediate	39 (9.4)	3 (4.2)	27 (7.3)	-
Hb AS and G6PD normal	28 (6.8)	2 (2.8)	41 (11.1)	-
Hb AS and G6PD deficiency	46 (11.1)	0 (0.0)	14 (3.8)	-
Hb SS and G6PD intermediate	2 (0.5)	0 (0.0)	0 (0.0)	-
Hb SS and G6PD normal	0 (0.0)	0 (0.0)	0 (0.0)	-
Hb SS and G6PD deficiency	2 (0.5)	0 (0.0)	0 (0.0)	-

Data shown are number (n) and proportions (%) of subjects. HC, malaria uninfected healthy control. SM, severe malaria anemia. UM, uncomplicated malaria. AA, normal hemoglobin. AS, heterozygote sickle cell. SS, homozygote sickle cell. -, Statistical analysis was not performed because more than 20% of the expected counts are less than 5. Statistical comparison was performed using Pearson's Chi-square test.

Table 4.5(b). Influence of co-inheritance of haemoglobin and G6PD genotype on falciparum infection in children under 3 years in Vihiga County, Kenya

Study Group	Haemoglobin and G6PD type	OR	95 % CI	P-value
UM	Hb AA and G6PD intermediate	0.44	0.21-2.44	0.061
	Hb AA and G6PD normal	1.20	0.75-2.01	0.069
	Hb AA and G6PD deficiency	0.454	0.944-2.376	0.095
	Hb AS and G6PD intermediate	-	-	-
	Hb AS and G6PD normal	-	-	-
	Hb AS and G6PD deficiency	-	-	-
	Hb SS and G6PD intermediate	-	-	-
	Hb SS and G6PD normal	-	-	-
	Hb SS and G6PD deficiency	-	-	-
SM	Hb AA and G6PD intermediate	1.20	0.77-1.57	0.063
	Hb AA and G6PD normal	1.65	0.73-1.81	0.072
	Hb AA and G6PD deficiency	1.09	0.77-1.56	0.071
	Hb AS and G6PD intermediate	-	-	-
	Hb AS and G6PD normal	-	-	-
	Hb AS and G6PD deficiency	-	-	-
	Hb SS and G6PD intermediate	-	-	-
	Hb SS and G6PD normal	-	-	-
	Hb SS and G6PD deficiency	-	-	-

Odds ratios (OR) and 95% confidence intervals (95% CI) were determined using multivariate logistic regression controlling for age and gender. Health controls (HC) was used as reference category. SM, severe malaria anemia. UM, uncomplicated malaria. Hb, haemoglobin. AA, normal hemoglobin. AS, heterozygote sickle cell. SS, homozygote sickle cell. -, multivariate logistic regression was not performed because there were less than five cases of per independent variable.

CHAPTER FIVE: DISCUSSION

5.1 Introduction

The present study was set to determine the influence of blood group, G6PD and haemoglobin genotypes on malaria infection outcome. Results revealed that blood groups A, B, AB, and O, had no influence on falciparum malaria. However, G6PD genotypes had influence on *p. falciparum* malaria. Consistent with the influence of ABO blood group on malaria, haemoglobin genotypes had no influence on falciparum malaria. Co-occurrence of hameglobin AA with either G6PD normal, intermediate or deficient does not influence malaria infection.

5.2 Influence of blood group genotype on falciparum infection in children under 3 years in Vihiga County, Kenya

Several studies have established the effect of blood classification system ABO on malaria, specifically between group AB and severe malaria and refer that people with blood type O are relatively resistant to develop severe malaria by *P. faciparum* (Amodu *et al.*, 2012; Tadesse and Tadesse, 2013; Zerihun *et al.*, 2011). In the present study, influence of ABO blood group on malaria was not found. These findings are consistent with previous studies in Cameroon (Nkuo-Akenji *et al.*, 2004), Colombia (Herrera *et al.*, 2009) and Brazil (Cavasini *et al.*, 2006). However, the findings of these studies are inconsistent to previous studies in Kilifi County, Coastal Kenya, reporting effect of ABO blood group on malaria and that blood type O protects against malaria through reduced rosetting (Rowe *et al.*, 2007). Other studies (Maina and Nyandieka, 2010; Timmann *et al.*, 2012) have shown that this is due to geographic and ethnic distribution of the different ABO genetic blood polymorphisms associated with malaria protection in the country. Most importantly, CDH13, encoding cadherin1, and HS3ST3B1, encoding heparan sulfate 3-O-

sulfotransferase 3B1, which play an important role in the resistance to the invasion of erythrocytes by *P. falciparum,* have been detected in Kilifi County, Coastal Kenya (Mackinnon *et al.*, 2016; Persson *et al.*, 2008). Taken together, the influence of ABO blood group on malaria depends on the characteristics of the studied population, as every region and ethnic community in the world seems to have different ABO genetic blood polymorphisms. So it varies geographically and ethnically.

5.3 Influence of glucose-6-phosphate dehydrogenase genotype on falciparum malaria infection in children under 3 years in Vihiga county, Kenya.

The present study revealed that G6PD genotype influenced *P. falciparum* malaria infection outcome with higher odds ratio of severe malaria infection associated with G6PD normal children compared to G6PD intermediate and deficient children. These observations are consistent with previous studies involving children in Kilifi and Nyando counties of Kenya (Suchdev *et al.*, 2014; Uyoga *et al.*, 2015). Likewise, these findings are similar to previous studies involving children in Uganda (Bwayo *et al.*, 2014) and Nigeria (Orimadegun and Sodeinde, 2014) children. These observations are attributed to the protective mechanisms of G6PD deficient erythrocytes to malaria parasite. For instance, increased oxidative stress in G6PD deficient cells as well as susceptibility to eryptosis accelerates phagocytic clearance of parasitized cells (Cappadoro *et al.*, 1998; Lang *et al.*, 2002). In addition, there is impaired *P. falciparum* growth within G6PD deficient cells is due to glutathione instability and oxidants such as alloxan, phenylhydrazine, and divicine (Preuss *et al.*, 2012). Taken together, there is a negative and positive prognosis, respectively, of malaria infection outcome, in G6PD normal and deficient children under three years in Vihiga County Kenya.

5.4 Influence of hemoglobin genotype on falciparum malaria infection in children under 3 years in Vihiga County, Kenya

The findings of this study showing no association between haemoglobin genotypes with falciparum malaria is similar to previous studies involving children under three years in Ghana (Danquah *et al.*, 2010) and Tanzania (Makani *et al.*, 2010). However, the findings of this study are inconsistent to previous studies in Kilifi, Kenya, showing the influence of haemoglobin genotype on malaria infection among children younger than 13 years (Komba *et al.*, 2009; McAuley *et al.*, 2010; Williams *et al.*, 2005b). A potential reason for these discrepancies is that haemoglobin AS protection against malaria increases with age from only 20% in the first 3 years of life to a maximum of 56% by the age of 10 years, returning to 30% in individuals greater than 10 years indicating that malaria protection by Hb AS involves the enhancement of not only innate but also of acquired immunity to the parasite (Williams *et al.*, 2005a). Taken together, the influence of haemoglobin genotype on falciparum malaria varies by age.

5.5 Influence of co-inheritance of hemoglobin and G6PD genotype on falciparum infection in children under 3 years in Vihiga County, Kenya

This study demonstrated that concurrent inheritance of haemoglobin AA with either G6PD normal, intermediate and deficient gene does not influence malaria infection in children under three years in Vihiga county, Kenya. These findings are partly similar to previous studies involving Malian (Guindo *et al.*, 2011), Nigerian (Awah *et al.*, 2012) and Cameroonian (Awah and Uzoengwe, 2006) children. In this study, more G6PD normal children were observed in the severe malaria and uncomplicated malaria groups relative to healthy. The present study also showed that G6PD intermediate genotype has no influence on malaria infection outcome. Taken

together, it is possible that Hb AA alter the influence of G6PD intermediate genotype but not G6PD normal genotype on malaria infection outcome in children under three years in Vihiga, kenya. Therefore, the higher proportion of children harbouring both Hb AA and G6PD intermediate genotypes in the severe malaria relative to the healthy control and uncomplicated malaria group suggest increased susceptibility to malaria infection of the two conditions. In addition, the higher proportion of children harbouring both Hb AA and G6PD normal genotype in the severe malaria and uncomplicated malaria compared to health controls suggest that there was no synergistic or additive effect of two conditions.

CHAPTER SIX: SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATIONS

6.1 Summary of findings

There was no influence of ABO blood group, on malaria infection outcome. However, G6PD genotypes had influence on *falciparum* malaria. Haemoglobin genotypes had no influence on *falciparum* malaria infection and likewise co-inharitance of both G6PD and Hb genotype had no influence on p.*falciparum* malaria infection outcome.

6.2 Conclusion

In summary these results indicate that:

i. There is no influence of blood groups A, B, AB, and O, on *P. falciparum* malaria infection outcome in children under three years in Vihiga County Kenya.

ii. G6PD normal, intermediate and deficient genotypes influence *p. falciparum* severe malaria infection outcome in children under three years in Vihiga County Kenya.

iii. Hemoglobin genotypes have no influence on *P. falciparum* malaria infection in children under three years in Vihiga County Kenya.

iv. Concurrent inheritance of hemoglobin genotype with either G6PD normal, intermediate and deficient have no influence on *P. falciparum* malaria infection outcome in children under three years old in Vihiga County Kenya.

6.3 Recommendations from the current study

i. Variants A, B, AB and O blood groups seem not to alter *P.falciparum* malaria infection outcome in this population and as such may not be targets for vaccine trials in Vihiga County Kenya.

ii. G6PD deficiency alter *P. falciparum* malaria infection outcome and as such can be targeted for vaccine development and choice of ant malaria medicines in Vihiga County Kenya.

iii. Genetic variant of Hb genotype has no influence on *P. falciparum* malaria infection outcome hence cannot be targeted for vaccine trial in Vihiga County.

iv. Combination of both Hb genotype and G6PD variants dont alter *p. falciparum* malaria infection outcome hence, not targets for vaccine development in Vihiga County Kenya.

6.4 Recommendations for future studies

i. Host ABO blood group genetic polymorphisms linked to *P. falciparum* susceptibility, among children under 3 years in Vihiga County, Kenya, should be genotyped.

ii. Genome wide association studies should be conducted to identify malaria resistance loci within the G6PG, genotypes in Vihiga County Kenya.

iii. Genome wide association studies should be conducted to identify malaria susceptible loci within the Hb genotypes.

iv. Future studies should be conducted using larger sample sizes in order to establish more co inheritance patterns.

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APPENDICES

Appendix 1:Questionnaire

Influence of ABO blood group, glucose -6-phosphate dehydrogenase and haemoglobin genotype on *p. falciparum malaria* infection outcome in children under 3 years in Vihiga Kenya

Study participant ID .....
Volunteer's Name .....
Mother /Guardian 's Name .....
Date of Birth .....Sex.....
Recruitment Date .....Age .....

Contacts

Telephone no. ..... Village .....
County ..... Nearest Market.....
Location ..... Nearest Church.....
Chief ..... Nearest School.....

Ethnic group .....

Which of the following Malaria Prevention methods are used in the home(check all that apply)
Mosquito Nets [ ] Mosquito Repellant Gel [ ] None [ ]
Mosquito Coils/Sprays [ ] Other(specify) .....

Presenting signs and symptoms/pysical examination

(yes /no)
Fever ..... Convulsions.....
Vomiting ..... Unresponsiveness.....
Poor Feeding ..... Difficult breathing.....
Headache ..... Temperature.....
Pallor ..... Others.....
Jaundice .....

Treatment history

Has the child received any of the following medications in the past 48 hours ?
(yes /no)
Quinine .....
Panadol .....
Amodiaquine .....
Artemether/Lumefandrine..... .....
Others (specify) .....
Was the child hospitalized.....
Has the child ever participated in this study before.....

Diagnosis

Severe Malaria..... Healthy control.....
Uncomplicated Malaria.....

Appendix 2: Ethical approval

MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
Fax: +254 057 351 221
Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 30th March, 2016

TO: Jafaralli Sande Ahmed
PG/MSc/PH/00104/2014
Department of Public Health
School of Public Health and Community Development
Maseno University
P. O. Box, Private Bag, Maseno, Kenya

REF: MSU/DRPI/MUERC/00275/16

RE: Influence of ABO Blood Group, Glucose-6-Phosphate Dehydrogenase Deficiency and AS Hemoglobin Genotype on *Falciparum* Malaria in Children under 3 years in Vihiga County, Kenya. Proposal Reference Number: MSU/DRPC/MUERC/00275/16

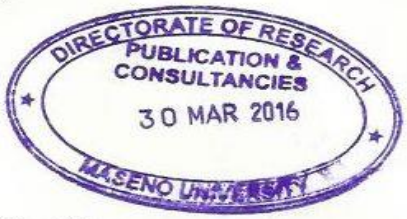
This is to inform you that the Maseno University Ethics Review Committee (MUERC) determined that the ethics issues raised at the initial review were adequately addressed in the revised proposal. Consequently, the study is granted approval for implementation effective this 30th day of March, 2016 for a period of one (1) year.

Please note that authorization to conduct this study will automatically expire on 29th March, 2017. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 28th February, 2017.

Approval for continuation of the study will be subject to successful submission of an annual progress report that is to reach the MUERC Secretariat by 28th February, 2017.

Please note that any unanticipated problems resulting from the conduct of this study must be reported to MUERC. You are required to submit any proposed changes to this study to MUERC for review and approval prior to initiation. Please advice MUERC when the study is completed or discontinued.

Thank you.



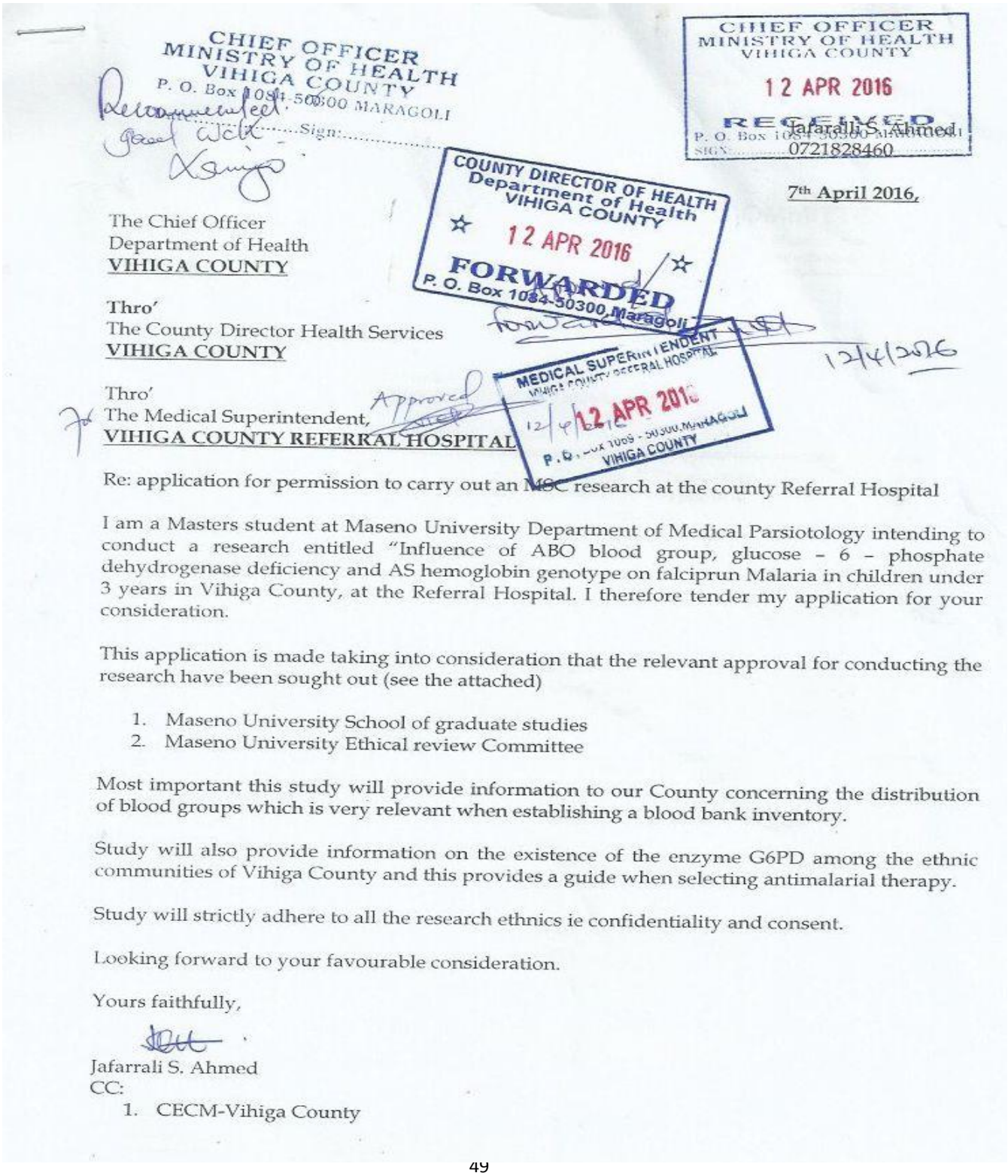
Yours faithfully,

Dr. Boniface Anyona,
Secretary,
Maseno University Ethics Review Committee.

Cc: Chairman,
Maseno University Ethics Review Committee.

MASENO UNIVERSITY IS ISO 9001:2008 CERTIFIED

Appendix 3: Research Permit



CHIEF OFFICER
MINISTRY OF HEALTH
VIHIGA COUNTY
P. O. Box 1084-50300 MARAGOLI
Recommended
Good Work
Sign:

CHIEF OFFICER
MINISTRY OF HEALTH
VIHIGA COUNTY
12 APR 2016
RECEIVED
P. O. Box 1084-50300 MARAGOLI
SIGN:

Jafarrali S. Ahmed
0721828460

COUNTY DIRECTOR OF HEALTH
Department of Health
VIHIGA COUNTY
12 APR 2016
FORWARDED
P. O. Box 1084-50300 Maragoli

7th April 2016,

The Chief Officer
Department of Health
VIHIGA COUNTY

Thro'
The County Director Health Services
VIHIGA COUNTY

Thro'
The Medical Superintendent,
VIHIGA COUNTY REFERRAL HOSPITAL

Approved

MEDICAL SUPERINTENDENT
VIHIGA COUNTY REFERRAL HOSPITAL
12/4/2016
12 APR 2016
P. O. Box 1084 - 50300 MARAGOLI
VIHIGA COUNTY

12/4/2016

Re: application for permission to carry out an MSC research at the county Referral Hospital

I am a Masters student at Maseno University Department of Medical Parsiotology intending to conduct a research entitled "Influence of ABO blood group, glucose – 6 – phosphate dehydrogenase deficiency and AS hemoglobin genotype on falciprun Malaria in children under 3 years in Vihiga County, at the Referral Hospital. I therefore tender my application for your consideration.

This application is made taking into consideration that the relevant approval for conducting the research have been sought out (see the attached)

1. Maseno University School of graduate studies
2. Maseno University Ethical review Committee

Most important this study will provide information to our County concerning the distribution of blood groups which is very relevant when establishing a blood bank inventory.

Study will also provide information on the existence of the enzyme G6PD among the ethnic communities of Vihiga County and this provides a guide when selecting antimalarial therapy.

Study will strictly adhere to all the research ethnics ie confidentiality and consent.

Looking forward to your favourable consideration.

Yours faithfully,

Jafarrali S. Ahmed
CC:
1. CECM-Vihiga County

Appendix 4: Consent Form

CONSENT TO PARTICIPATE IN RESEARCH

Study Title: Influence of ABO Blood Group, Glucose-6-Phosphate Dehydrogenase and Hemoglobin Genotype on Falciparum Malaria in Children Under 3 Years in Vihiga Kenya.

Dear participant,
You are invited to take part in this research study. This form tells you why this research study is being done, please read then can decide if you want to join this study or not. The Principal Investigator in this study is an MSc student at Maseno University. A study team will be working closely with principal investigator and the study will run for 3 months.

The purpose of this study is to determine the influence of ABO Blood Group, Glucose-6-Phosphate Dehydrogenase Deficiency and hemoglobin Genotype on Falciparum Malaria In Children Under 3 Years in Vihiga Kenya. If you choose to participate in this study, the team will require 3ml of blood for HIV/HBV/HCV voluntary testing, malaria diagnosis, ABO typing, G6PD testing and Complete Blood Count) from you. No drug or chemical will be introduced into your body

You can decide whether to take part in this study or not. You are free to say yes or no. If you say no, your regular medical care will not change. Even if you join this study, you do not have to stay in it, you may stop at any time. It is important to note that there is no financial benefit for participating in this study at the same time there will be no any cost implications to you.

Every effort will be made to keep your study records confidential but we cannot guarantee it. No funds have been set aside to pay any costs if you are harmed because of this study. If you think that you were harmed because of this study, contact the Principal Investigator.

By signing my name below, I confirm the following:
I have read (or been read to) this entire consent document. All of my questions have been answered to my satisfaction.

The study's purpose, procedures, risks and possible benefits have been explained to me.
I agree to let the study team use and share the health information and other information gathered for this study.

I voluntarily agree to participate in this research study. I agree to follow the study procedures as directed.

I have been told that I can stop at any time.
Subject's Name _____signature_____Date_____
Principal Investigator_____signature_____Date _____

Note: Below are some of the key contacts
Principle investigator: Jafarali Ahmed, (0721828460), Co-Investigator – Dr. Benard Guyah (0721206932), Prof. Sang (0722819165); MU-ERC-Dr Bonuke Anyona(0721543976).