ANTIBODY PREVALENCE AND LEVELS TO *PLASMODIUM FALCIPARUM* CIRCUMSPOROZOITE PROTEIN AND SCHIZONT EXTRACT IN INDIVIDUALS LIVING IN KIPSAMOITE, NANDI COUNTY, KENYA

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DECLARATION

I hereby declare that this thesis is my original work and has not been presented for a degree or other award in any other university.

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DEDICATION

This work is dedicated to my beloved wife Jeniffer Nanzakho. May God bless her for her patience, resource support and her relentless encouragement.

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ACRONYMS AND ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AMA	Apical Membrane Antigen
AMANET	African Malaria Network
AU	Arbitrary Units
CD	Cluster of Differentiation
CDC	Centre of Disease Control
CSA	Chondroitin Sulfate A
CSP	Circumsporozoite Protein
DOMC	Division of Malaria control
DNA	Deoxyribonucleic Acid
EEFs	Exo-Erythrocytic Forms
ELISA	Enzyme Linked immunosorbent Assay
EPR	Epidemic Preparedness and Response
GLURP	Glutamic Rich Protein
GPI	Glycosylphosphatidylinositol
GSK	GlaxoSmithKline
Hb	Haemoglobin
HIV	Human Immunodefficiency Virus
ICAM	Intercellar Adhesive Molecule
IgG	Immunoglobulin G
IL	interleukin
IPTp	Intermittent Prevention and Treatment in pregnancy

IRS	Indoor Residue Spraying
ITNs	Insecticide Treated Nets
KEMRI	Kenya Medical Research Institute
LLINs	Long Lasting Insecticidal Nets
LSA	Liver Stage Antigen
MIM	Multilateral Initiative on Malaria
MIP	Malaria In Pregnancy
MMV	Medicines for Malaria Venture
MOH	Ministry Of Health
MSP	Merozoite Surface Protein
MVI	Malaria Vaccine Initiative
NANP	Asparagine-Alanine-Asparagine-Proline peptide
NaOH	Sodium Hydroxide
NK cells	Natural Killer cells
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polimerase Chain Reaction
Pf	*Plasmodium falciparum*
PfEMP	*Plasmodium falciparum* Erythrocyte Membrane Protein
RBC	Red Blood Cell
RBM	Roll Back Malaria
RDTs	Rapid Diagnostic Tests

RTS,S	CSP repeat region (R), T-cell epitopes (T), hepatitis B surface antigen (S), ratio of RTS to additional S antigen 1:4 (RTS,S)
SE	Schizont Extract
TBVs	Transmission Blocking Vaccines
TNF	Tumor Necrotic Factor
TRAP	Thrombospondin Related Anonymous Protein
UMN	University of Minnesota
UNICEF	United Nations Children Education Fund
WBC	White Blood Cell
WHO	World Health Organization
µL	Micro litre

ABSTRACT

Malaria continues to be a major public health concern despite the concerted efforts to eliminate it. The quest for a vaccine remains a top priority. Antibodies to *Plasmodium falciparum* antigens are involved in prevention of infection and disease in animal models. The role of these antibodies has also been demonstrated in human with some of the antigens being targeted as lead candidates in vaccine development. However, the association of age and gender with antibody responses to these antigens is not clearly understood. Moreover, most of the studies have been done in endemic areas with little emphasis in areas of low and unstable malaria transmission. This study sought to determine whether age and gender affect antibody responses to *P. falciparum* antigens by measuring antibody levels and prevalences to recombinant circumsporozoite protein (CSP) and crude schizont extract (SE) in individuals of all ages living in an area where malaria transmission is low and unstable in western highlands of Kenya. Both male and female of all ages were recruited, blood samples collected and plasma obtained. Sixty samples were randomly selected and categorized into three age groups; <8years (n=25), 8-18years (n=21) and >18years (n=14). The participants were also categorized into males (n=30) and females (n=30) to determine the effect of gender on antibody responses. Seven samples from malaria naïve individuals from North America were used as negative controls while 30 pooled plasma samples from individuals in areas of stable malaria transmission were used as positive controls. Measurement of parasitaemia in all samples was done by light microscopy using both thin and thick blood smear. Haemoglobin levels were measured by photometry while IgG antibodies levels in plasma were measured by Enzyme Linked Immunosorbent Assay (ELISA). Data analysis was done by Graphpad Prism 6 using non-parametric Wilcoxon rank sum, Mann Whitney U, Kruskall-Wallis and Spearman rank correlation tests. The prevalence of antibodies was generally low across all age groups ranging from 0% to 14.29% at arbitrary units (AU)>2 for the two antigens. The antibody prevalence however increased with age. Males had significantly higher antibody prevalence than females with males having 10% while females 3.33% at AU>2 for both antigens $P<0.05$. The levels of IgG antibodies were generally low and there were no significant differences among the age groups and between male and females ($P>0.05$). There was a correlation between antibody levels to CSP and SE ($r=0.5977$; $P<0.05$). The study provides preliminary findings associating antibody responses with the exposure to malaria infection. It therefore recommends a longitudinal study on more antigens to inform exploration of multi-antigen vaccines and also adopt several control measures including Epidemic Preparedness and Response (EPR). It further recommends profiling immune responses of individuals living in epidemic prone areas.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Malaria is a major global health problem more so in sub-Saharan Africa. It is one of the most important infectious diseases worldwide, causing an estimated 214 million clinical cases and 438,000 deaths annually (WHO, 2015). Whereas better use of available control measures and the search for new, cheaper, and effective drugs are the main aims of current efforts in malaria research, sustainable control of malaria would be enhanced if effective vaccines were available for use in combination with the present control measures (Schwartz *et al.,* 2012). Malaria vaccine development is hindered by the fact that the parasite progress through a succession of stages in the human host, with stage specific expression of proteins, a high level of antigen polymorphism, redundancy of essential invasion pathways in host cells, and utilization of a number of immune evasion mechanisms (Pierce and Miller, 2009).

Identification of reliable and reproducible immune correlates of protection against *Plasmodium falciparum* infection will be important in the development and testing of malaria vaccines. Immunoglobulin-G (IgG) antibodies are produced against a number of malaria vaccine candidate antigens including the pre-erythrocytic antigen CSP and schizont extract (SE) (John *et al.,* 2008). It has been associated with some degree of protection from clinical malaria in areas of stable transmission. However, the mechanisms by which this antigen induces protection against malaria in humans have been the subject of multiple investigations.

Most of information to date is based primarily on observations of naturally infected individuals living in areas where there is stable malaria transmission. Residents of such

protection has been achieved in experimental settings using high doses of irradiated sporozoites, making the pre-erythrocytic stages of *Plasmodium* a focus of the malaria vaccine effort (WHO, 2012). Subunit vaccines based on circumsporozoite protein (CSP), such as RTS,S, are at the center of this endeavor. To date, the development of a malaria vaccine has been empirically driven and has yielded promising though modest results (Ballou, 2009).

Antibodies to the CSP occur frequently in individuals living in areas of hyperendemic malaria (Fontaine *et al.,* 2010). The antibody levels and prevalence are also proposed to be indicators of malaria transmission (Jun *et al.,* 2008). Most of the studies have been done in areas of high and stable malaria transmission and little done in areas of low and unstable malaria transmission.

In the present study, the responses of IgG antibodies against CSP antigen contained in recombinant five repeat peptide of Asparagine-Alanine-Asparagine-Proline $(NANP)_5$ and crude schizont extract which might serve as correlate of protective immunity in human vaccine trials were evaluated in an area with low unstable malaria transmission.

1.2 The statement of the problem

Malaria has for long remained a major global cause of morbidity and mortality. WHO statistics show that children under 5 years and pregnant women are at a greater risk of malaria infection with an estimated 24 million pregnant women and 75,000 to 200,000 infants affected each year (WHO, 2012). Although there are concerted efforts to eliminate malaria, the quest for a vaccine remains a top priority. Vaccines based on the circumsporozoite protein (CSP) are among the lead candidates, with the RTS,S vaccine

having undergone phase 3 efficacy trials at 11 sites in 7 African malaria endemic countries, namely; Gabon, Ghana, Kenya, Malawi, Burkina Faso, Tanzania and Mozambique (WHO, 2011). The phase 3 trial was Launched in 2009 and so far, first results showed that RTS,S reduced the risk of children experiencing clinical malaria and severe malaria by 56% and 47% respectively. These results confirm findings from phase 2 studies and support the ongoing effort of advancing RTS,S as malaria vaccine candidate. RTS,S is currently recommended by WHO for large scale trial (WHO, 2015). A major impediment to malaria vaccine development has been a lack of understanding of which immunologic responses are important in protection. Findings from many studies have reported that naturally acquired antibodies to *P. falciparum* play a key role in immunity against malaria (Sabchareon *et al.,* 1991). However, it is still unclear which antibody responses are important in protection from disease and at what age despite a number of immunological and epidemiological studies that have attempted to answer this question. It is also not known if gender affects development of immunity. Furthermore, most studies have been done in endemic areas with stable malaria transmission with little done in areas with low unstable malaria transmission. The present study therefore aimed at establishing whether there are differences in age and gender in frequencies and levels of antibodies to *P. falciparum* antigens in individuals living in areas with low unstable malaria transmission.

1.3 Study justification

Many studies have measured antibody responses to *P. falciparum* in individuals living in areas where malaria is endemic and prospectively assessed associations between antibody responses and the subsequent risk of malaria with age (Kreuels *et al.*, 2008). Children

under the age 5 years are at greater risk of infection with *P. falciparum* in malaria holoendemic areas with older children and adults developing partial immunity to malaria. The protective immunity that develops with age has been greatly associated with acquisition of protective antibodies more so to pe-erythrocytic antigens of which CSP and schizont extract are among them. However, in areas of low and unstable malaria transmission, older children and adults are also at risk of malaria infection. There are few studies that have been done in areas of low and unstable malaria transmission and it is not clearly understood why individuals of all ages are at risk of malaria. Moreover, there is sparse information on association of antibody responses and gender. Kipsamoite, located in western highlands Kenya, was chosen as a study site because it is prone to malaria epidemics and is documented as an area of low and unstable malaria transmission (Some, 1994).

1.4 Research questions

i. What is the prevalence of IgG antibodies to *P. falciparum* CSP and crude SE in the study area?

ii. What are the antibody levels to *Plasmodium falciparum* CSP and crude schizont extract in individuals living in an area with low unstable malaria transmission?

iii. What are the effects of age and gender on antibody responses to *P. falciparum* CSP and crude schizont extract in individuals living in an area with low unstable malaria transmission?

1.5 Hypotheses

i. There are no differences in antibody levels between *Plasmodium falciparum* CSP and schizont extract in individuals living in areas with low unstable malaria transmission.

ii. Age and gender have no effect on antibody responses to *P. falciparum* antigens in an area of low and unstable malaria transmission.

1.6 Objectives

1.6.1 General Objective

To determine prevalence and levels of antibodies to *Plasmodium falciparum* cicumsporozoite protein and crude schizont extract in individuals living in an area of low and unstable malaria transmission.

1.6.2 Specific Objectives

i. To determine IgG prevalence to *Plasmodium falciparum* CSP and crude schizont extract in individuals living in an area where malaria transmission is low and unstable in Western Kenya.

ii. To determine the relationship between specific antibody levels to CSP antigen and schizont extract in individuals living in an area of low and unstable malaria transmission.

iii. To evaluate the effects of age and gender on antibody responses to *P. falciparum* in an area of low and unstable malaria transmission.

1.7 Significance of the study

The study findings will provide knowledge on the antibody responses to crucial vaccine antigen (CSP) in relation to age and gender which will be of great relevance to vaccine developers and policy implementers such as Kenya's Ministry of Health (MOH) to understand the timing and frequency of administering the vaccines once developed and also understanding where and when booster vaccines are required. The information is also crucial in the development of a broad spectrum malaria vaccine.

CHAPTER TWO: LITERATURE REVIEW

2.1 Malaria disease

Malaria disease is as ancient as man himself. In the fourth century B.C, the Greeks associated the disease with the swamps and drained the swamps to control the disease (Nester *et al.,* 2004). The Italians later named the disease "malaria" meaning 'bad air'. Donald Ross in 1902 was awarded a Nobel Prize for demonstrating the life cycle of a malaria causing protozoa (Nester *et al.,* 2004).

Malaria is the most common parasitic infectious disease worldwide. It affects red blood cells (RBC) and hepatocytes. It is caused by protozoan parasites of the genus *Plasmodium* (phylum Apicomplexa). In humans, malaria is caused by *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium vivax*. *Plasmodium knowlesi* which is a parasite of monkeys also infect humans (Ashley, 2006; White, 2008). *Plasmodium falciparum* is the most common cause of infection and is responsible for about 80% of all malaria cases, and is also responsible for about 90% of the deaths from malaria (UNICEF, 2007). In 1955, the World Health Organization (WHO) commenced worldwide elimination of malaria with some success at the beginning. Out of fifty two nations which undertook the control programs, ten of them had eradicated malaria by 1960 (Brown, 2002). The appearance of insecticide resistant strains of *Anopheles* mosquitoes and logistical problems thwarted the efforts of WHO of eradicating the disease leading to its resurgence. The eradication program by WHO failed by 1976 (Farid, 1981).

Today malaria is not only the greatest killer disease but also the most widely spread infectious disease. It is a threat to approximately 40% of human population with currently

3.2 billion people at risk globally (WHO, 2015). Efforts are so far made to control malaria especially through treatment of infected people and mosquito vector control strategies either physical or chemical. It should however, be appreciated that with rapid increase in multi-drug resistant parasites and minimal investment in control measures, there will be increased morbidity and mortality due to malaria in the coming years. Development of new, alternative and cost-effective strategies of malaria control is the solution. Due to complex life cycle of malaria parasites and their interactions with insect and human hosts, most strategies have not been effective. Therefore, this calls for a more rational approach that involves the parasite biology and the immune responses the parasite elicit in the human host.

2.2 Life cycle of malaria parasite

Infection with malaria parasites is transmitted by female mosquitoes of genus *Anopheles* with *Anopheles gambiae* and *A. funestus* being the main vectors in Africa. The life cycle of malaria parasite is very complex and may differ slightly among the various species (Figure 2.1).

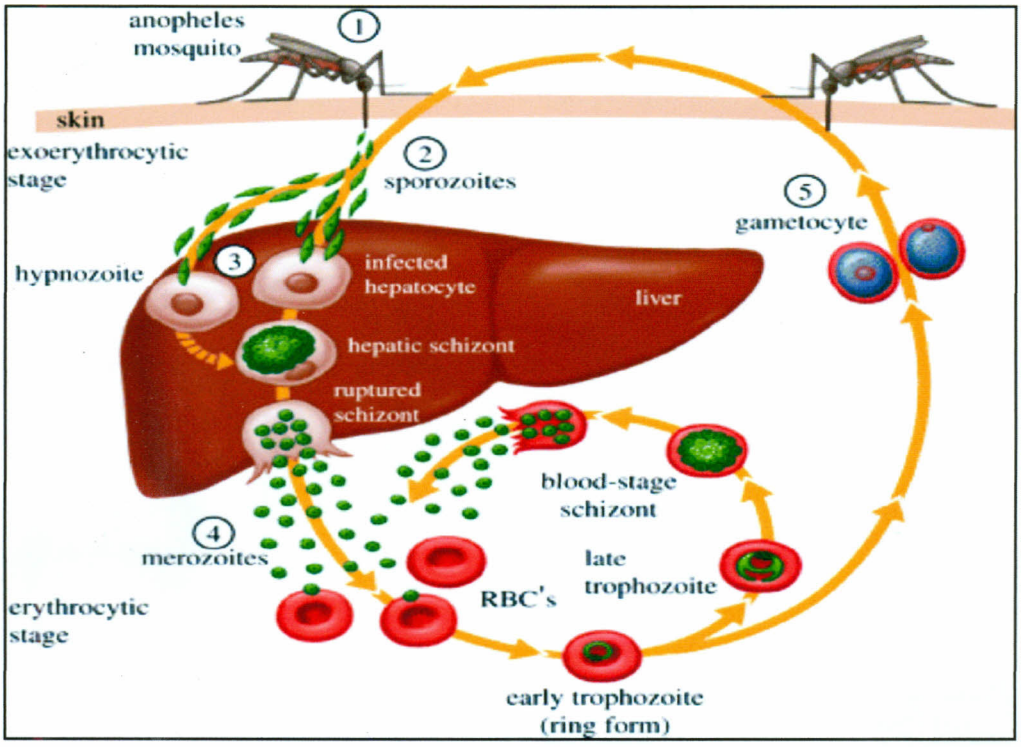

Figure 2.1: Life cycle of the malaria parasite illustrating the various stages that are relevant to vaccine design (Adopted from Hill, 2011). These are (1) the anopheline mosquito vector, used in experimental protocols to immunize with irradiated sporozoites administered by mosquito bite; (2) the sporozoite, the target of several vaccines, including RTS,S; (3) the liver-stage, usually targeted by vectored vaccines; (4) the blood-stage, usually targeted by protein in adjuvant vaccine candidates. Merozoite antigens have been most often included in blood-stage vaccines; (5) the gametocyte which along with the ookinete, formed after fertilization in the mosquito midgut, is the source of parasite antigens used in sexual-stage transmission-blocking vaccines. Pre-erythrocytic vaccines, which target the sporozoite and the liver-stage parasite, are intended to prevent infection as well as disease while blood-stage vaccines are intended to prevent clinical illness and death.

Following infection by sporozoites during mosquito's blood meal, the sporozoites travel to the liver where they grow and multiply within the hepatocytes to form exoerythrocytic schizonts (ES) within 5-15 days. *Plasmodium ovale* and *Plasmodium vivax* have a dormant stage called hypnozoite which may persist in the liver for weeks or years resulting in relapses of infection (Nester *et al.*, 2004). *Plasmodium falciparum* and *Plasmodium malariae* have no such stage. The ES contains thousands of merozoites

(Figure 2.2) (about 10000 to 30000) which rupture the liver cells and escape back to blood stream where they invade red blood cells and proliferate in them to form ring forms then trophozoites then schizonts (erythrocytic schizogony) constituting asexual life cycle.

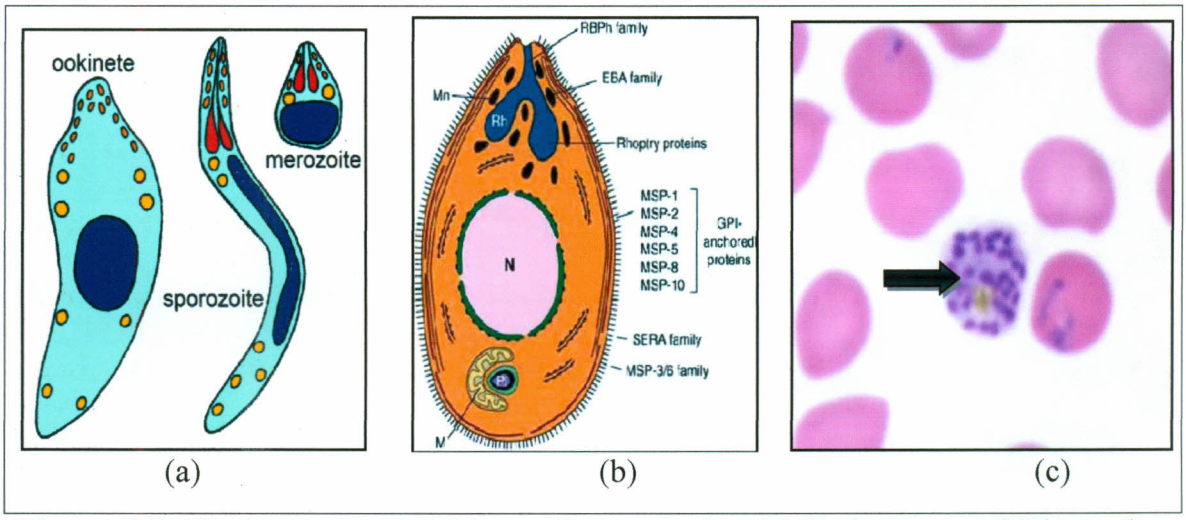

Figure 2.2: Various stages of *Plasmodium* parasite. (a) Early stages of *Plasmodium* adopted from Wikimedia commons (b) Schematic diagram of a merozoite. Adapted from the Walter and Eliza institute website(www.wehi.edu.au/facweb/ id=53) (c) *P. falciparum* schizont stage parasite (center pointed with arrow) showing merogony, recognizable by approximately 24 new merozoites identified by the Giemsa-stained nuclei (purple), and showing hemozoin pigment (yellow) (www.cdc.gov/malaria/diagnosis/microscopy) (May 20, 2014)

This causes red blood cells to burst to release merozoites which invade more erythrocytes. Some marozoites differentiate into sexual forms (gametocytes), which start to form after a number of asexual life cycles. Female and male gametocytes fuse on ingestion by female *Anopheles* mosquito to form the zygote; this develops into motile ookinete which transform into oocyst. Each oocyst forms more than 10000 new motile sporozoites which migrate to the salivary glands thus completing the life cycle. The Symptoms of malaria include fever, shivering, arthralgia (joint pain), vomiting, anemia (caused by haemolysis), haemoglobinuria, and convulsions. Pregnant women and their infants in endemic regions are particularly vulnerable to the effects of *P. falciparum*

infection accounting for an estimated 700,000 to 2.7 deaths annually (UNICEF, 2007). Knowledge of the life cycle of malaria parasite is crucial in development of new and alternative strategies for malaria control more importantly vaccine development and timing of vaccine administration and boosting.

2.3 Distribution of Malaria

2.3.1 Global Distribution of Malaria

It is estimated that approximately half of the world's population is at risk of malaria. Most malaria cases and deaths occur in sub-Saharan Africa. However, Asia, Latin America, and to a lesser extent the Middle East and parts of Europe are also affected (Figure 2.2). In 2011, 99 countries and territories had ongoing malaria transmission (WHO, 2013). According to World Health Organization (WHO) 2015 updates, 3.2 billion people, about half of world population are at risk of malaria. In 2015, there were 214 million cases of malaria and an estimated 438,000 deaths worldwide with most cases and deaths occurring among children living in sub-Saharan Africa. Between 2000 and 2015, malaria incidence rate fell by 37% and mortality fall by 60% globally (WHO, 2015).

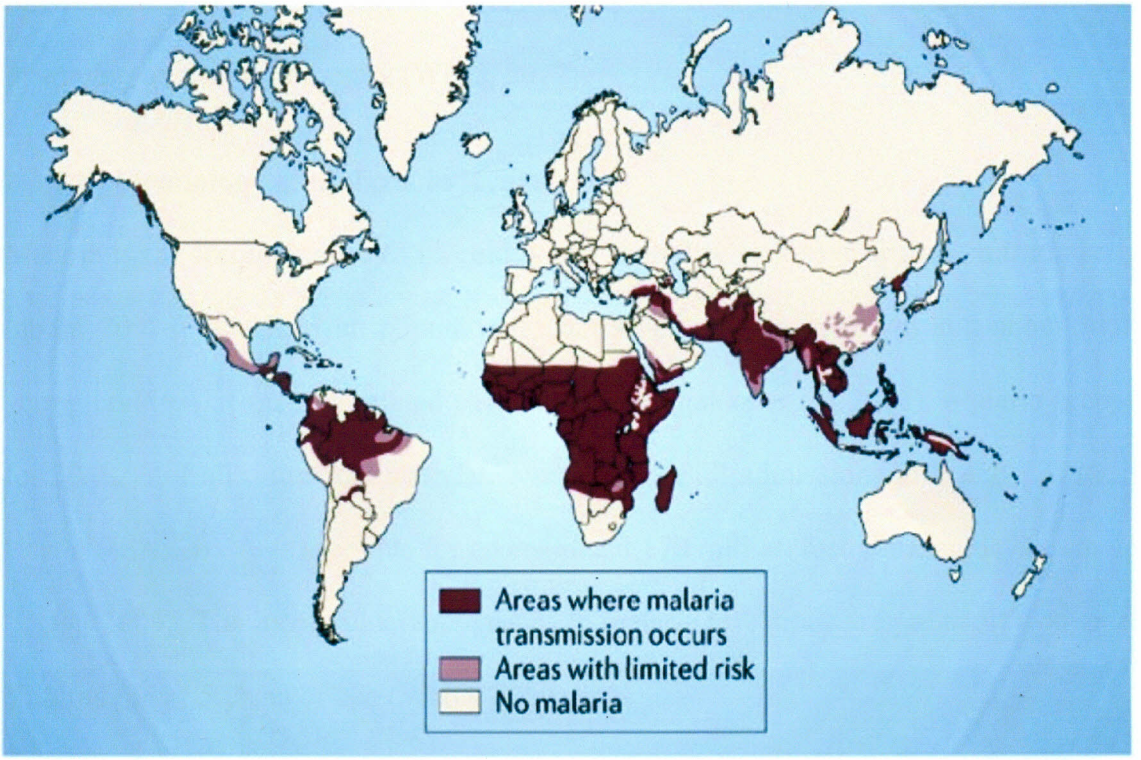

Figure 2.3: The world map showing distribution of malaria. Adopted from Nature website, (May,20, 2013)
http://www.nature.com/nrmicro/journal/v4/n9_supp/ nrmicro1525_F2.html

The specific population risk group include; young children in stable transmission areas who have not yet developed protective immunity against the most severe forms of the disease; non-immune pregnant women as malaria causes high rates of miscarriage and can lead to maternal death; semi-immune pregnant women in areas of high transmission where malaria can result in miscarriage and low birth weight, especially during first and second pregnancies and semi-immune HIV-infected pregnant women in stable transmission areas, during all pregnancies. Women with malaria infection of the placenta also have a higher risk of passing HIV infection to their newborns; people with HIV/AIDS; international travelers from non-endemic areas because they lack immunity; immigrants from endemic areas and their children living in non-endemic areas and

returning to their home countries to visit friends and relatives are similarly at risk because of waning or absent immunity (WHO, 2012)

2.3.2 Epidemiology of malaria in Kenya

Malaria is the leading cause of morbidity and mortality in Kenya with 27 million people (about 70% of the Kenyan population) at risk of malaria infection and about 34,000 young children of malaria-related cases annually (Ojakaa *et al.*, 2011). Malaria accounts for 30-50% of all outpatient attendance and 20% of all admissions to health facilities in Kenya (IEA, 2011). It accounts for an estimated 170 million lost working days each year (GOK, 2009). The most vulnerable groups to malaria infections are pregnant women and children under 5 years of age (WHO, 2009).

Kenya has four malaria epidemiological zones namely, endemic areas (areas of stable malaria transmission in altitudes of 0-1300m), areas of seasonal malaria transmission (arid and semi arid areas), Malaria epidemic prone areas of western highlands of Kenya (high altitudes) and low risk malaria areas (central highlands of Kenya including Nairobi) (DOMC, 2011). In western highlands of Kenya, malaria transmission is seasonal with considerable variations year after year and experience epidemics which are attributed to increase in minimum temperature during long rains period which favours and sustains vector breeding. The whole population is at risk during the epidemic and the fatality can be up to ten times greater than in areas with stable malaria transmission (Brooker *et al.*, 1999).

2.4 Manifestations of Malaria

2.4.1 Pathogenesis of Malaria

All the manifestations of malarial illness are caused by the infection of the red blood cells (RBC) by the asexual forms of the malaria parasite and the involvement of the RBC makes malaria a potentially multisystem disease since every organ of the body is served by the blood (Greenwood *et al.,* 2008). All types of malaria manifest with common symptoms such as fever and some patients may progress into severe malaria. Although severe malaria is more often seen in cases of *P. falciparum* infection, complications and even deaths have also been reported in non-*falciparum* malaria (Greenwood *et al.,* 2008).

At the completion of the schizogony within the red cells, newly developed merozoites are released by the lysis of infected erythrocytes and along with them, numerous known and unknown waste substances, such as red cell membrane products, hemozoin pigment, and other toxic factors such as glycosylphosphatidylinositol (GPI) are also released into the blood. These products, particularly the GPI, activate macrophages and endothelial cells to secrete cytokines and inflammatory mediators such as tumor necrosis factor (TNF), interferon-γ, interleukin-1, IL-6, IL-8, macrophage colony-stimulating factor, and lymphotoxin, as well as superoxide and nitric oxide (NO). Many studies implicate GPI as the key parasite toxin and is present in merozoite surface proteins (MSP) such as MSP-1, MSP-2, and MSP-4. The various cytokines released in response to these products are implicated to cause the systemic manifestations of malaria such as headache, fever and rigors, nausea and vomiting, diarrhea, anorexia, tiredness, aching joints and muscles, thrombocytopenia, immunosuppression, coagulopathy, and central nervous system manifestations (Clark *et al.,* 2006). Furthermore, the plasmodial DNA presented as

hemozoin is highly proinflammatory and can induce cytokinemia and fever (Parroche *et al.,* 2007; Schumann *et al.,* 2007). Hemozoin has also been linked to the induction of apoptosis in developing erythroid cells in the bone marrow, thereby causing anemia (Lamikanra *et al.,* 2009).

The cytokines of the proinflammatory cascade also act as double-edged swords in the pathogenesis of malaria. An early proinflammatory cytokine response helps in limiting the infection, with the cytokines inhibiting the growth of malarial parasites in lower concentrations. Failure to down-regulate this inflammatory response results in progressive immune pathology, leading to complications in malaria disease (Omer *et al.,* 2003; Clark *et al.,* 2006). It is therefore important to get rid of the parasite before reaching the blood stage since the pathogenesis of the disease occurs at this stage. This calls for understanding of the immune responses to the parasite at early stages.

2.4.2 Survival mechanisms of malaria parasite in the host

Plasmodium falciparum being the most severe species has a wide array of receptor families and highly redundant, alternate invasion pathways (Weatheral *et al.,* 2002) which gives the parasite the ability to invade RBCs of all ages, and with repeated cycles of development within the red cells, the parasite numbers exponentially grow into very high parasite burdens if the infection is uninhibited by treatment or host immunity (Weatheral *et al.,* 2002). Structural changes in the infected red cells and the resulting increase in their rigidity and adhesiveness are major contributors to the virulence for *P. falciparum* malaria (Ho and White, 1999). The red cells infected with late stages of *P. falciparum* have increased cytoadherence and also undergo rosetting. This results in

sequestration of the parasites in various important organs and deeper tissues escaping spleenic clearance. (Ho and White, 1999)

Certain proteins expressed on the surface of the infected red cells such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) mediate the adhesion of parasitized RBCs to the endothelium and to uninfected red cells enhancing cytoadherence (Miller *et al.,* 2002). PfEMP1 can bind to several adhesion receptors expressed on the endothelial cells with CD36, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) being the common ones (Srabasti *et al.,* 2008). Other receptors include; neural cell adhesion molecule, P-selectin and E-selectin, chondroitin sulfate A (CSA) and hemagglutinin. Chondroitin sulfate A acts as the receptor for binding in the placenta enhancing sequestration of the parasites in the placenta leading to placental malaria (Ho *et al.,* 1999). To limit this extend of malaria parasite and the associated damage, the immune mechanism of the body should clear the parasites during pre-erythrocytic stages.

2.4.3 Diagnosis of malaria

Diagnosis of malaria can be done in various ways which include; clinical observation, microscopic, antigen detection, Molecular diagnosis and serology. Clinical diagnosis is based on the patient's symptoms and physical findings at examination which are often not specific and are also found in other diseases. Microscopic diagnosis done by examining a smear of patient's peripheral blood under a microscope remains the gold standard technigue for laboratory confirmation of malaria (Figure 2.4). It however, depends on the quality of the reagents, the microscope, and the experience of the microscopist.

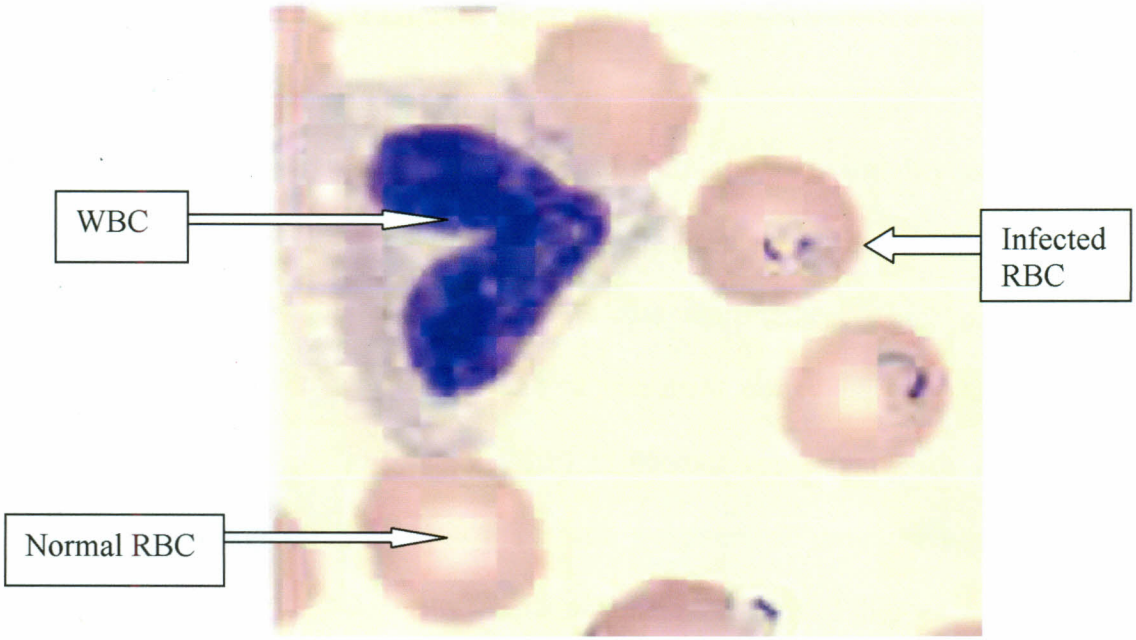

Figure 2.4: Blood smear stained with Giemsa, showing a white blood cell (on left side) and several red blood cells, two of which are infected with ***Plasmodium falciparum*** (on right side).adopted from CDC website (http://www.cdc.gov/malaria/diagnosis_treatmet/microscopy) (May 20, 2014)

Antigen detection involves various test kits which detects antigens from malaria parasites by immunochromatography. The tests are quick and give results within 2-15 minutes and are referred to as "Rapid Diagnostic Tests" (RDTs). They offer a useful alternative to microscopy in situations where reliable microscopic diagnosis is not available. However, several issues need to be addressed before being widely adopted. The accuracy of RDTs needs to be improved, their cost be lowered and their adequate performance under adverse field conditions be ensured (WHO, 2012). Molecular diagnosis involves detection of nucleic acids of the parasites using polymerase chain reaction (PCR). Despite being slightly more sensitive than smear microscopy, PCR results are often not available quickly enough to be of value in establishing the diagnosis of malaria infection. The technique is most useful for confirming the species of malaria parasite after the diagnosis has been established by either smear microscopy or RDT (WHO, 2012).

2.5 Malaria control and treatment strategies

2.5.1 Malaria control strategies

Global Malaria control strategy aims to reduce morbidity and mortality associated with malaria. In Kenya and other malaria endemic countries, the control strategies employ four main approaches. One of the approaches is case management which deals with the formulation and implementation of malaria treatment policy issues. Management of malaria and Anaemia in Pregnancy (MIP) is another approach which addresses the provision of malaria prevention measures and treatment of pregnant women such as Intermittent prevention and treatment in pregnancy (IPTp) (Filler *et al.*, 2006). In addition, Vector control approach is employed whose intention is to prevent transmission through use of (ITNs/ LLINs) and other methods through Integrated Vector Management (IVM) and Epidemic Preparedness and Response (EPR) which aims at establishment of malaria early warning systems and carrying out preventive measures such as the Indoor Residue Spraying (IRS) campaigns (Kenya Malaria Fact sheet, 2012). The strategies have been able to reduce malaria to a larger extend in the 21st century but they proof to be costly.

The use of personal protective methods such as long sleeved clothing, mosquito repellents including lotions and burning compounds containing insect repellents (mosquito coils) reduces the number of bites thus parasite transmission. This is, however, subject to personal discretion, often deterred by warm humid weather conditions and in some cases lotions and incense repellents cause irritation. The use of Insecticide treated nets has been found to reduce mortality by 44% in African children (Fegan *et al.*, 2007). Insecticide treated nets are a feasible, affordable and a sustainable method. Unfortunately

they do not prevent transmission during other periods because nets only protect from insect bites while in bed yet mosquitoes may bite at any given time especially evening and night.

2.5.2 Treatment of Malaria

Combination therapies are the current recommendations by the world health organization (WHO, 2012). This is due to the malaria parasite's ability to develop resistance to monotherapy treatments. Until the 1990s, Chloroquine was successfully used in the treatment of malaria before resistance was observed which was associated with an increase in malaria morbidity and mortality (Trape *et al.,* 2002). Artemisinin combination therapy studies in Africa found them more efficacious in treatment than previous first line treatments such as chloroquine and sulphodoxine-pyrimethamine (Okell *et al.,* 2008). The current combination therapy in use is Lumefantrine-artemether (for example, Co-artem) which is more efficacious than Sulphodoxine- Pyrimethamine (sp) (Mulenga *et al.,* 2006) although more expensive. Other therapeutic drugs for malaria include; Malarone which is a combination of two antimalarials, (atovaquone and proguanil) taken to treat malaria caused by chloroquine-resistant *P. falciparum*; Doxycycline for infections caused by *P. falciparum* and *P. vivax* in Thailand and Kenya; Quinine plus an antibiotic such as doxycycline, tetracycline, or clindamycin for most *P. falciparum* infections and is not to be used in Southeast Asia, where quinine effectiveness is declining.

Chemoprophylaxis is also an important tool to prevent malaria infection. It is important to travelers from malaria non-endemic regions to endemic areas. Some of the drug regimens available for malaria prophylaxis include; Proguanil, Chloroquine, Mefloquine, Atovaquone/proguanil and Doxycycline. They, however, have their disadvantages

including resistance, bulkiness in dosage, cost and even contraindication (Greenwood, 2012). Antimalarial medication is particularly prone to counterfeiting and is a contributing factor in malaria treatment failures and drug resistance (Newton *et al.*, 2006).

2.5.3 Initiatives to fight malaria

Over the past twenty years, there has been a proliferation of international, multi-lateral initiatives to co-ordinate and consolidate the efforts to control malaria. Roll Back Malaria (RBM) was launched by the WHO, World Bank, the United Nations Children's Fund and other partners and aimed to reduce the malaria burden by half by 2010 mainly through treatment and prevention strategies. The Global Fund aims to fight AIDS, Tuberculosis and Malaria and to reduce poverty. The Multilateral Initiative on Malaria (MIM) was created to promote greater research and leadership in Africa through capacity building and the facilitation of global collaboration. Medicines for Malaria Venture (MMV) are a private-public partnership whose goal is to develop at least one new affordable anti-malarial drug or drug combination. The Malaria Vaccine Initiative (MVI) speeds up the development of promising malaria vaccine candidates. The African Malaria Network (AMANET) focuses on regional training and organization of clinical and vaccine trials within the continent. These, and other partnerships against malaria not mentioned here reflect the growing global commitment to significantly reduce the burden of malaria (Alilio and Bygbjerg, 2004).

2.6 Immunity in malaria

Immunoglobulin from malaria exposed adults has been shown to provide immunity through passive transfer to non-immune individuals (Sabchareon *et al.*, 1991). Immunity

to malaria is naturally developed with continuous exposure to malaria antigen (Baird *et al.,* 1998). Natural immunity however wanes if the intensity, length and duration of exposure to malaria antigens decrease (Struik *et al.,* 2004). Genetic factors such as Haemoglobin (Hb) variants and thalassaemias have also been implicated in the acquisition of immunity to *P. falciparum* malaria (Modiano *et al.,* 2001). These traits are widely distributed in malaria endemic regions (Allison, 1954). Their protective mechanism is not known though HbS and β-thalassemia have been shown to enhance phagocytosis of parasitized erythrocytes (Ayi *et al.,* 2004).

2.7 Malaria vaccine

Vaccines are often the most cost-effective tools for public health yet no effective vaccine for malaria has so far been developed. Despite this, researchers remain hopeful. This is justified by the fact that individuals who are exposed to the parasite in endemic areas develop acquired immunity against disease and death (Marsh and Kinyanjui, 2006). In addition, research shows that immunity can be transferred from immune adults to individuals that have no protective immunity by use of purified immunoglobulin (Graves and Gelband, 2006). Furthermore, clinical and animal studies have shown that experimental vaccination has some degree of success when using attenuated sporozoites or the RTS,S/AS01 malaria vaccine candidate (Agnandji *et al.*, 2011). Several vaccines are under development with Surface *P. falciparum* 66 (SPf66) tested extensively in endemic areas in the 1990s and other vaccine candidates, targeting the blood-stage of the parasite's life cycle but were found to be insufficiently effective (Tanner and Alonso, 1996). Several potential vaccines targeting the pre-erythrocytic stage are being developed, with RTS,S showing the most promising results so far (Graves and Gelband,

2006). The most advanced subunit malaria vaccine to date, RTS,S/AS01 has undergone a phase III clinical trial and is based on a fusion protein of part of the CSP of *P. falciparum* with the hepatitis B surface antigen plus a chemical adjuvant to boost the immune system response (Alonso *et al.*, 2004). Studies with RTS,S in an endemic area of Mozambique showed that vaccination of children aged 1–4 years induced partial protection against infection and clinical malaria including severe disease (Alonso *et al.*, 2004). In October 2011, the group reported first findings from the Phase III trial of RTS,S indicating that 46% of 15,460 inoculated infants and children were protected for 15 months (Agnandji *et al.*, 2011). The vaccine is recommended for large scale trial (WHO, 2015). Therefore, there is need to understand in detail the host immune response to CSP to improve on the efficacy of the vaccine candidate and even develop a broad spectrum vaccine that could be used in other age groups.

2.8 Vaccine Candidates

The complexity of *Plasmodium* life cycle presents problems in vaccine development but also increases the number of potential targets for a vaccine (Figure 2.5). Malaria vaccine approach target the parasite at three distinct stages of the life cycle which provide numerous opportunities for antigen targets (Graves and Gelband, 2006).

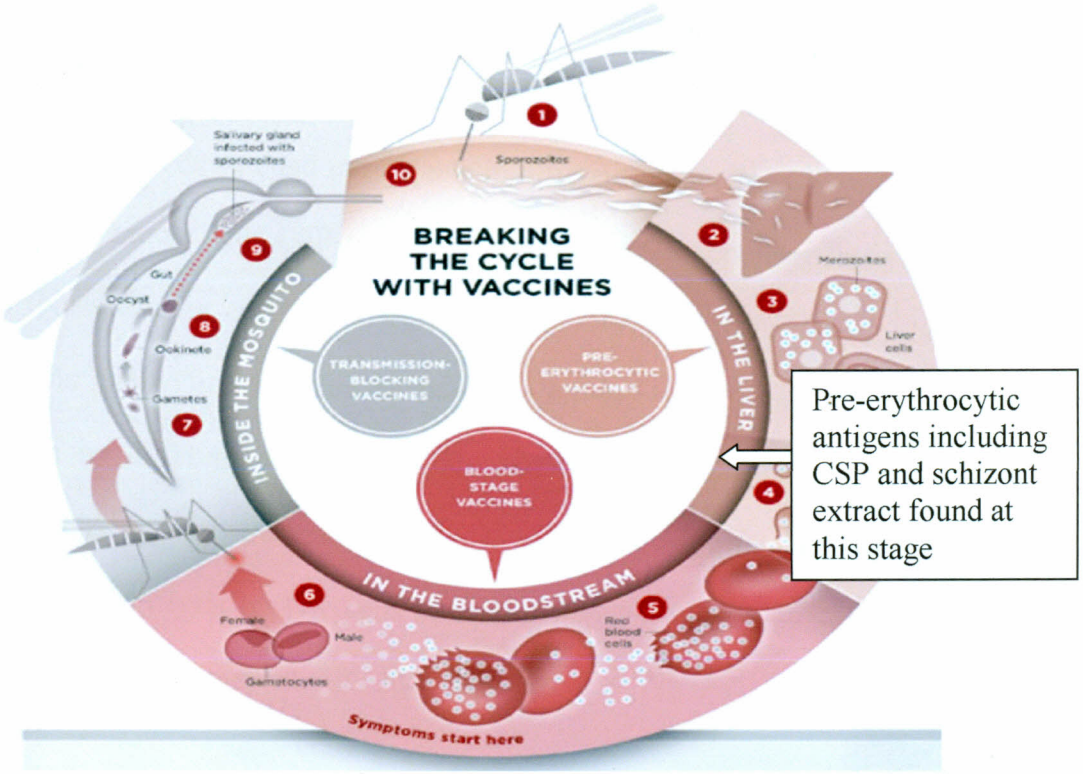

Figure 2.5: Malaria parasite life cycle showing stages for vaccine approach. (Modified from Baer *et al.*, 2007; Breman, 2009)

2.8.1 Pre-erythrocytic vaccine candidates

The vaccine candidates at this stage aim to protect against the early stage of malaria infection. The understanding of the interaction between the host immune system and the pre-erythrocytic stage would help arrest the disease before clinical manifestation. Indeed, the pre-erythrocytic stage of *Plasmodium* is the focus of the malaria vaccine effort (Hoffman *et al.*, 2002). The CSP which forms a dense coat on the parasite surface has been hypothesized to mediate many of the initial interactions between the sporozoite and its two hosts (Ménard *et al.*, 2000; Sinnis and Nardin, 2002). It is found to elicit initial immune response in the human host. Evidences against *P. falciparum* accumulating over the years indicate strongly that, high antibody titres against the CSP correlate with

protection (Greenwood, 2011). Circumsporozoite protein consists of a tetra peptide region (Asn-Ala-Asn-Pro) that is conserved between parasite variants making it a suitable marker for vaccine design and measurable in different populations. Various studies have found immunization with CSP to induce immune protection in monkeys, rodent and humans (Nussenzweig *et al.,* 1967; Gwadz *et al.,* 1979; Hoffman *et al.*, 2002). These CSP-specific antibodies may contribute to elimination of sporozoites and infected hepatocytes by different mechanisms like neutralization of sporozoites by inhibiting gliding motility and cell traversal, Fc receptor-mediated engulfment of sporozoites, Fc-receptor dependent lysis by NK cells or by activation of the complement system after antibody binding (Plassmeyer *et al.*, 2009).

2.8.2 Erythrocytic stage vaccine

These are blood-stage vaccine candidates that target the malaria parasite at its most destructive stage and the rapid replication of the organism in human red blood cells. Erythrocytic stage vaccines do not aim to block all infection but are expected to decrease the number of parasites in the blood, and in so doing, reduce the severity of disease (Graves and Gelband, 2006). Evidence suggests that people who have survived regular exposure to malaria develop natural immunity over time. The goal of a vaccine that contains antigens or proteins from the surface of the blood-stage parasite (the merozoite) would be to allow the body to develop that natural immunity with much less risk of getting ill.

In malaria endemic areas, effective immunity against malaria develops after repeated exposure that limits blood-stage parasitaemia and prevents symptomatic illness and severe complications (Marsh and Kinyanjui, 2006). Antibodies that inhibit blood stage

replication of *P. falciparum* are believed to be important in mediating both acquired immunity and immunity generated by candidate blood-stage vaccines (Good *et al.*, 2004). Serum antibodies that inhibit parasite growth *in vitro* have been isolated from clinically immune individuals, but are rarely detected in malaria-naive individuals (Brown *et al.*, 1982).

2.8.3 Transmission-blocking vaccine candidates

Transmission-blocking vaccine (TBVs) candidates seek to interrupt the life cycle of the parasite by inducing antibodies that prevent the parasite from maturing in the mosquito after it takes a blood meal from a vaccinated person (Wu *et al.*, 2008). These vaccines would not prevent a person from getting malaria, nor would they lessen the symptoms of disease. They would, however, limit the spread of infection by preventing mosquitoes that fed on an infected person's blood, from spreading malaria to new hosts by targeting the sexual stages of malaria parasites and blocking sexual mating so as to prevent the development of sporozoites in Anopheles mosquitoes (Kang *et al.*, 2013). This approach has the advantage of having robust *in vitro* assays that could be used to demonstrate proof-of-concept, as well as a relatively clear effector immune response (Carter, 2001). Several candidates are in clinical development, including vaccines that target the surface proteins, *Pfs25* and *Pfs28* or *Pvs25* and *Pvs28* of mosquito stages such as zygotes, ookinetes and young oocytes of *P. falciparum* and *P. vivax* respectively (Wu *et al.*, 2008; Kang *et al.*, 2013). A successful transmission-blocking vaccine would be expected to reduce deaths and illness related to malaria in at-risk communities. However, a major challenge for this vaccine approach is proving true field efficacy.

2.9 The future of malaria vaccine

Theoretically, each developmental stage could have a vaccine developed specifically to target the parasite. Moreover, any vaccine produced would ideally have the ability to be of therapeutic value as well as preventing further transmission and is likely to consist of a combination of antigens from different phases of the parasite's development (Yazdani *et al.*, 2006).

The testing of most vaccines is usually done in malaria endemic areas (WHO, 2011) and therefore little information is available on immune responses to *P. falciparum* vaccine candidate antigens in epidemic prone areas of low malaria transmission.

The aim of the present study was to establish a better understanding of the immune response to pre-erythrocytic antigens which are crucial for invasion of malaria parasite in humans by determining IgG antibodies to CSP and heterogeneous crude schizont extract of *P. falciparum* in areas of low and unstable malaria transmission. The findings of this study would provide prerequisite information for developing and testing of a wide spectrum vaccine to malaria and also understand the timing, and frequency of vaccine administration.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study site

This study was conducted in highland areas of Kipsamoite, Nandi County, Kenya (Appendix IV c), which is primarily comprised of the Nandi ethnic group, one of the Kalenjin speaking tribes. The study site lies between 0°21'52"N and 0°16'56"N in latitude and 35°5'20"E and 34°59'7"E in longitude and is located at an altitude of between 1829m and 2132m above sea level with a total population of ~7,800 as of 2009. There are usually two rainy seasons, short rains in October and long rains beginning in March. Malaria transmission at the site is low, unstable and seasonal. It is usually episodic and local outbreaks of malaria with high rates of morbidity and mortality have occurred in the past (Khan *et al.,* 1992). Both *P. falciparum* infection and *Plasmodium malariae* infection have been documented in the area (Some, 1994).

3.2 Study population

The study participants were males and females of all ages who had lived in the study area for at least six months by the start of the study and who were residents of the area during the study. This was to minimize the confounding effects of travel and acquisition of infection in nearby lowland areas where malaria is endemic. Signs and symptoms of malaria were recorded during enrolment.

3.3 Inclusion criteria

Subjects enrolled were both male and female. Age was not a limiting factor in this study. The subjects had lived in the study site for six months continuously by the start of the study. They were also willing to participate in the study and informed consent given by self or the guardian in the case of minors.

3.4 Exclusion criteria

Individuals were excluded from the study if they had moved out of the study area within the six months prior to commencement of the study. Furthermore, individuals who had haemoglobin values below 8gHb/dl and were evidently ill according to the assessment of the clinician were excluded. In addition, residents who were unwilling to participate in the study or their parents/guardians declined to give consent were excluded from the study. Lastly, non-residents of the study area were not allowed to be participants in the study.

3.5 Sample size determination

Sample size was determined using StatsToDo statistical software for estimating the correlation coefficient (www.statstodo.com) with the following parameters:

Probability of type 1 error (α) = 0.05

Expected Correlation coefficient rho (ρ) = 0.32

At power ($1-\beta$) = 0.8 (80%)

This gave n as 59

The assumption was that approximately 5% would not consent. Since the study involved one time point collection of samples and only those who had lived in the study site for minimum of six months were eligible, migration after sample collection would have no effect on the results. The number was adjusted to a minimum total of 60 subjects in the study.

3.6 Ethical consideration

The review and approval for this study was obtained from Kenyatta University Ethical Review committee and Ethical Review Committee of Kenya Medical Research Institute (Appendix VI). Permission to carry out the study was given by NACOSTI.

3.7 Informed consent

Since this study involved both adults and minors, written informed consent was obtained from the study participants or, in the case of minors, from their parents or guardians. The consent forms were signed by participants or their parents/guardians before witnesses. The forms were translated into the local language, Kalenjin (Nandi) for participants and parents/guardians who do not understand English (Appendix II).

3.8 Study design

The study was cross-sectional and was part of a larger study. The large study involved recruitment of one group of individuals both male and female, age not limiting. In this study, blood samples were collected at one time-point and plasma samples obtained. Diagnosis for malaria was done and Haemoglobin levels also measured to determine the extent of illness. Those found positive were treated for free at the Health centre using the recommended line of malaria treatment. Sixty plasma samples (n=60) were randomly selected from the samples of the larger study and used in the present study. The samples were categorized into three (3) groups based on the age; <8years (n=25), 8-18years (n=21) and >18years (n=14) to assess the effect of age on IgG responses. The samples were further categorized into male (n=30) and female (n=30) to determine if gender has an effect on IgG responses. Seven plasma samples (n=7) from North American individuals who were never exposed to malaria were used as negative controls. Thirty

(30) pooled plasma samples from adults living at Asembo Bay in Nyanza, an area of stable malaria transmission were used as positive control. Measurement of IgG responses to crude schizont extract (SE) and circumsporozoite protein (CSP) was done by Enzyme Linked Immunosorbent Assay (ELISA).

3.9 Laboratory procedures

Initial procedures like sample collection, diagnosis and plasma separation were carried out in the field. Prepared slides and plasma samples were ferried to UMN malaria laboratory at KEMRI Kisumu for further procedures.

3.9.1 Sample collection and handling

Blood was collected by venipuncture from adults (10 to 20 ml) and children (5 ml). Samples were collected in EDTA coated tubes from which plasma was separated, aliquoted and stored at -80°C until used for analyses. Ten microlitres (μL) of blood were obtained by finger prick from all individuals to measure the hemoglobin concentration and thick and thin blood films were prepared from finger prick blood samples for diagnosis of malaria by microscopy.

3.9.2 Microscopic diagnosis of malaria

Thick and thin blood films were air-dried and stained with 6% Giemsa. Each slide was read by two independent microscopists from KEMRI, Centre for Global Health Research, Kisumu. Asexual and sexual parasites were counted separately and species differentiated. Malaria parasites were counted against 200 white blood cells (WBC). A slide was declared negative only after reading against 2,000 WBC without observation of a malaria parasite. In the event of a discrepancy between the two readers in terms of the presence or absence of malaria parasites, or if parasite densities differed by more than 50%, the slide

was re-examined by a third microscopists. The arithmetic mean of the two final closest readings was used as the final parasite density. If there was no agreement after the third reading, the arithmetic mean of the three parasite densities was used.

3.9.3 Haemoglobin concentration

Hemoglobin level was determined by photometry (HemoControl, EKF Diagnostics, and Barleben, Germany). Hemoglobin values were adjusted by -0.8 g/dL for altitude. Cut-offs for anemia was defined as follows: children 0.5 – 4.9 yrs = 11.0 g/dL; children 5 – 11.9 yrs = 11.5 g/dL; children 12 – 14.9 yrs = 12 g/dL; non-pregnant females $\geq$ 15 yrs = 12.0 g/dL; males $\geq$ 15 yrs = 13.0 g/dL (medicinenet.com).

3.9.4 Antigen peptides and source

The presence of antibodies to CSP was tested by using recombinant central repeat sequence peptides of CSP consisting of five Asparagine-Alanine-Asparagine-Proline $(NANP)_5$ repeat tetrapeptide to which individuals from areas where malaria is endemic demonstrate IgG responses. Antigens for schizont extract (SE) were obtained from malaria culture using *P. falciparum* from 3D7 parasite isolates (Sarr *et al.,* 2007). Both antigens were donated by Dr. Chandy John of UMN.

3.9.5 Antibody measurement by ELISA

Immunoglobulin G (IgG) antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (John *et al.,* 2005). Circumsporozoite peptide and crude heterogeneous schizont extract was dissolved in 1x phosphate-buffered saline (PBS) to a concentration of 10µg/ml separately. Fifty microlitres of antigen solution was added to Thermo Immulon- 4HBX plates, (Milford, MA). Following overnight incubation at 4°C, washing

with PBS 0.05% Tween 20 was done 4×, using an automated plate washer and blocking in 5% (wt./vol) nonfat powdered milk in PBS for non specific binding followed. Fifty microlitres (µl) samples of plasma diluted to 1:100 in 5% non-fat powdered milk in PBS was added to wells in duplicates and incubated for 2 hours at room temperature. After washing with washing buffer, 50 µl of alkaline phosphatase-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:1,000 in 5% non-fat powdered milk in PBS was added and incubated for 1 hour. After extensive washing (6×) with washing buffer, *p*-nitrophenylphosphate substrate was added in accordance with the manufacturer's instructions (Sigma, S0942 St. Louis, MO). Reactions were stopped by addition of 50 µl of 3M NaOH. The optical density (OD) was measured at 405 nm on a 96-well ELISA plate reader (Molecular Devices, Sunnyvale, CA). All samples were tested in duplicates and samples were retested if there was a discrepancy of greater than 25% between the duplicates. Standardization of the plates was achieved using positive-control plasma pools on each plate from 30 individuals from malaria endemic region. Background (determined from the wells with no plasma) were deducted from the mean OD of each sample and a cut-off threshold of positivity was determined as the mean plus 3 standard deviations from the seven negative control plasma samples (North America residents) included in each assay. Immunoglobulin G antibody levels were expressed in arbitrary units (AU). The numbers of AU were calculated by dividing the optical density of a sample by the mean optical density plus 3 standard deviations (SD) for plasma from seven North Americans who had never been exposed to malaria.

3.9.6 Data analysis

Microsoft Office Excel 2007 was used in the initial processing and management of data and further analysis was done using GraphPad Prism 6 statistical software (GraphPad Inclusions). Wilcoxon rank sum test was used to determine summary statistics for the age groups and gender. Mann-Whitney U-test and Kruskall-Wallis test were used to assess differences in the antibody responses in the various age groups and between male and female. The non-parametric tests were used since the IgG response data was not normally distributed. The spearman rank correlation test was used to analyze the relationship between IgG responses to SE and CSP. Results were considered significant at $P<0.05$.

CHAPTER FOUR: RESULTS

4.1 Characteristics of the study population

The study involved participants of all ages both male and female who were categorized on the basis of age and gender. Microscopy results from peripheral blood smears of participants at the time of sampling demonstrated that the individuals were nonparasitemic. There were no blood stage malaria infection with *P. falciparum, P. malariae, P. ovale* or *P. vivax* (0%) in the study sample (n=60). At the time of sampling, all the participants were asymptomatic for malaria and did not show any evident sign for malaria illness. Measurement of haemoglobin (HB) levels revealed that 88.3% (53 out of 60) of the participants had HB levels above the cut-off of anaemia at their various age groups (children 0.5 – 4.9 yrs = 11.0 g/dL; children 5 – 11.9 yrs = 11.5 g/dL; children 12 – 14.9 yrs = 12 g/dL; non-pregnant females ≥ 15 yrs = 12.0 g/dL; males ≥ 15 yrs = 13.0 g/dL) while 11.7% (7 out of 60) had levels slightly below the threshold. Six of these were <8 years and 1 was in the age group of 8>18 years. No participant above 18 years had haemoglobin levels below the anaemia threshold. Analyses of their characteristics were as summarized (Table 4.1).

Table 4.1: Demographic characteristics of the participants

Sample population (N)	Age group (Yrs)	No (n)	sex		Parastaemia*				Mean Hb levels in g/dL
					P. falciparum	*P. malariae*	*P. vivax*	*P. ovale*	
60	<8	25	M	10	0	0	0	0	12.5
			F	15	0	0	0	0	11.4
	8-18	21	M	10	0	0	0	0	13.5
			F	11	0	0	0	0	13.2
	>18	14	M	10	0	0	0	0	15.9
			F	4	0	0	0	0	13.9

*** 0 indicate that the participants were not parastaemic.**

4.2 Overall antibody frequencies to schizont extract and circumsporozoite protein

The proportion of antibody-positive individuals at a cut-off of AU>1 (low level antibodies) was relatively low for both Schizont extract (SE) and circumsporozoite protein (CSP). The results showed that 18.3% (11 out of 60) were IgG positive for SE while 20% (12 out of 60) were IgG-positive for CSP. The proportion of IgG-positive individuals to CSP was slightly higher than to SE though not significantly different (P=0.05). However, when the cut-off was set at AU>2 (high level antibodies), the proportion of positive individuals was extremely low at 6.7% (4 out of 60) for both SE and CSP (Table 4.2).

Table 4.2: Proportion of antibody-positive individuals to *Plasmodium falciparum* Schizont extract and Cicumsporozoite protein at a cut-off of AU>1 and AU>2

Antigen	Number (N)	IgG Positive at AU>1		IgG Positive at AU>2	
		Number (n)	Percentage (%)*	Number (n)	Percentage (%)*
SE	60	11	18.3	4	6.7
CSP	60	12	20	4	6.7

***Percentage is calculated by the formula n/N × 100;** AU>1 is low level antibodies; AU>2 is high level antibodies; AU= optical density of sample/mean optical density + 3 standard deviations of –ve control.

4.3 Antibody levels to schizont extract and cicumsporozoite protein

Very low IgG levels to both schizont extract and CSP were registered among study participants. The total IgG levels to schizont extract (SE) ranged from 0.140-6.260 AU with median level of 0.575 AU and mean of 0.8602 AU in the 60 individuals of the study population. On the other hand the total IgG to circumsporozoite protein ranged from 0.020-4.22 AU and the median level was 0.530 AU and mean of 0.8075 AU in the 60 individuals (Table 4.3).

Table 4.3: IgG levels (AU) to *Plasmodium falciparum* antigens in the study population

Parameter	Antibody response to SE (AU)	Antibody response to CSP (AU)
Number of participants (N)	60[#]	60[#]
Minimum	0.1400	0.0200
25% percentile	0.4300	0.3475
Median	0.5750	0.5300
75% percentile	0.7775	0.9075
Maximum	6.2600	4.2200
mean	0.8602	0.8075
Std. deviation	1.0492	0.8110
Std. error of mean	0.1354	0.1047

[#] is the sample size

When the antibody levels were compared between the two *P. falciparum* antigens, the median level of antibodies to SE was slightly higher than median level of antibodies to CSP though not significantly different ($P>0.05$).

4.4 Correlation between IgG levels to CSP and SE antigens of *P. falciparum*

The correlation between IgG levels to SE and CSP was significant though the correlation was moderate ($r=0.5977$; $P<0.001$) (Figure 4.1).

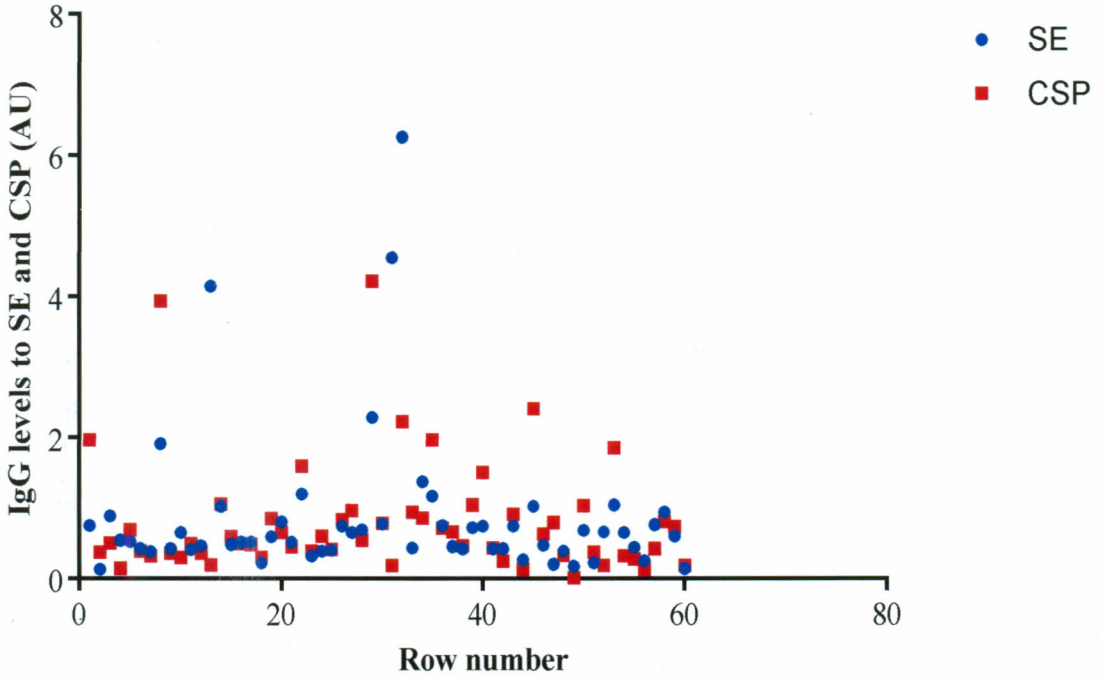

Figure 4.1: Correlation of antibody levels between schizont extracts and circumsporozoite protein for all participants (n=60) in an area of unstable malaria transmission. IgG levels were expressed as optical density (OD) in arbitrary units (AU). Correlation coefficient (r=0.5977), 95% confidence interval (0.3988 to 0.7428) and two-tailed P value of <0.001 (P<0.05) was considered significant.

4.5 Frequency of IgG to *P. falciparum* in participants of different age groups

Analysis was done for all the sixty individuals categorized in three age groups. When antibody-positive cut off was set at AU>1 (low level antibodies), 8% (2 out of 25) of participants aged <8 years, 23.8% (5 out of 21) of participants aged 8-18 years and 28.6% (4 out of 14) of participants above 18 years were IgG-positive to SE. On the other hand, the proportion of IgG-positive participants to CSP at cut-off of AU>1 was 16% (4 out of 25), 19.04% (4 out of 21) and 28.6% (4 out of 14) for participants of age groups <8 years, 8-18 years and > 18 years respectively. Generally, the frequency of IgG antibodies to *P. falciparum* antigens in the study area which is an unstable malaria transmission area increased with age for both SE and CSP when the IgG-positive threshold was considered

at AU>1 though not significantly different (P>0.05). It was observed that the antibody frequency was higher for CSP than SE in individuals aged <8 years but not significantly different (P>0.05). The antibody frequency for individuals aged >18 years was the same for both SE and CSP (Figure 4.2).

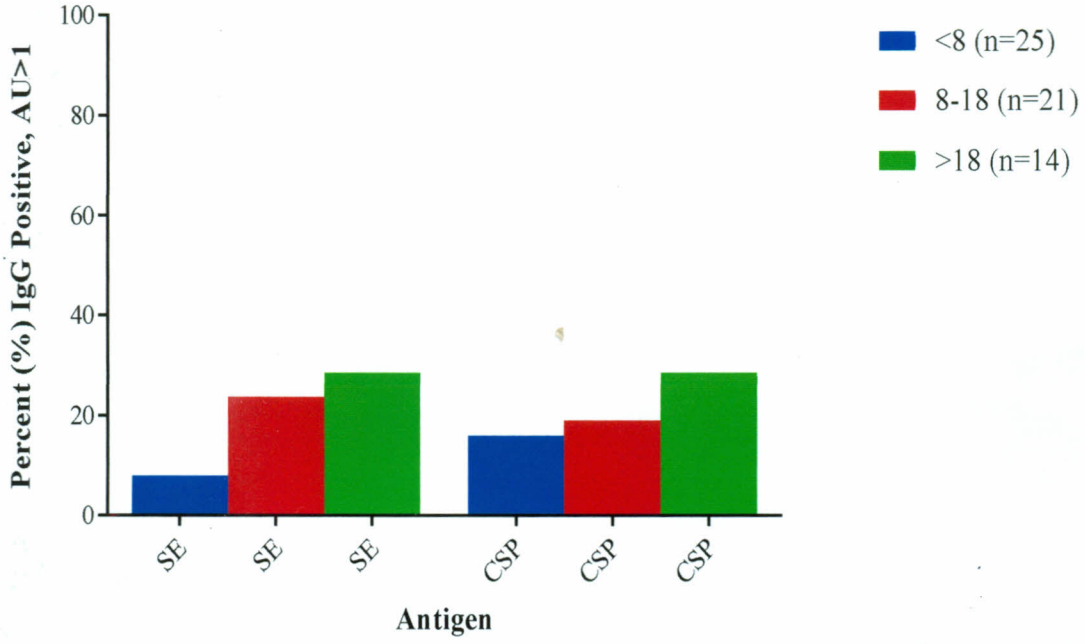

Figure 4.2: Prevalence of total IgG antibodies, determined by ELISA for *P. falciparum* Schizont extract and Cicumsporozoite Protein in participants of different age groups in an area of unstable malaria transmission. The frequencies were expressed in percentage (%) when the cut-off of AU>1was considered antibody-positive (low-level antibodies).

The trend was different in the study area when the threshold for antibody positivity was considered at AU>2 (High-level antibodies) where very low frequencies were recorded. There was no participant (0%) that was IgG-positive for both SE and CSP among individuals <8years. Interestingly, at cut-off of AU>2 the frequency of IgG to CSP was much lower in the individuals aged above 18 years (7.14%) than in individuals aged 8-18 years (14.29%) though not significantly different (P>0.05). Similarly the IgG frequency

in age group >18 years at cut-off of AU>2 was lower to CSP (7.14%) than to SE (14.29%). However, the difference was not significant (P>0.05) (Figure 4.3).

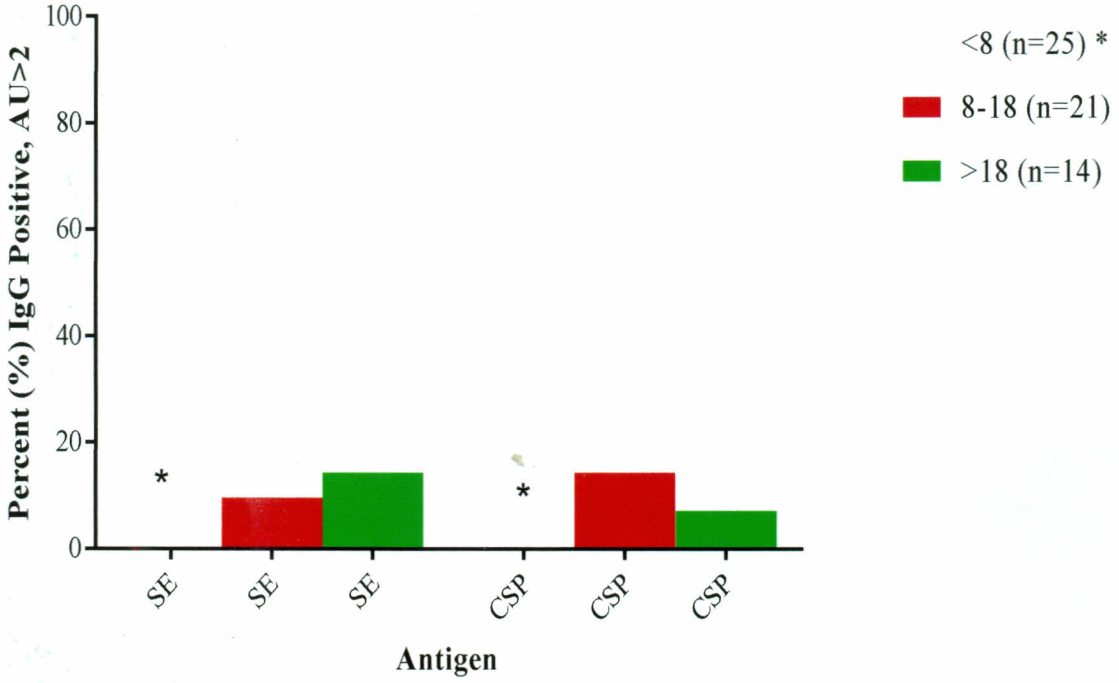

Figure 4.3: Prevalence of total IgG antibodies, determined by ELISA for *P. falciparum* Schizont extract and Cicumsporozoite Protein in participants of different age groups in an area of unstable malaria transmission. The frequencies were expressed in percentage (%) when the cut-off of AU>2 was considered antibody-positive (high-level antibodies). *= 0% prevalence of IgG antibodies.

4.6 Relationship between IgG levels to the two antigens and age

Generally the levels of IgG to SE and CSP were relatively low among the individuals of the study population. The median levels of IgG antibodies increased with age for both antigens though not significantly different (P>0.05). The median levels of IgG antibodies were slightly lower in individuals >18 years than those aged between 8-18 years (Table 4.4).

Table 4.4: Levels of IgG antibodies to *Plasmodium falciparum* Schizont extract and Cicumsporozoite protein for different age groups AU>1

antigen	Age (years)	Number of participants (N)	Median level (range)[a]	95% CI[b] of median
SE	<8	25	0.5500(0.1400-1.050)	(0.3721-0.6140)
	8-18	21	0.6000(0.2300-4.150)	(0.4700-0.7800)
	>18	14	0.6050(0.2700-6.260)	(0.4300-1.3800)
CSP	<8	25	0.3800(0.0200-1.970)	(0.3300-0.7000)
	8-18	21	0.6000(0.2000-4.220)	(0.4600-0.9200)
	>18	14	0.6950(0.1400-2.230)	(0.2500-1.5100)

[a] Median level and range in AU
[b] Confidence interval of the median

When the median levels of IgG antibodies to SE were compared to CSP among the three age groups, the levels to CSP were slightly higher than IgG levels to SE. However, the difference was not significant (P>0.05) (Figure 4.4).

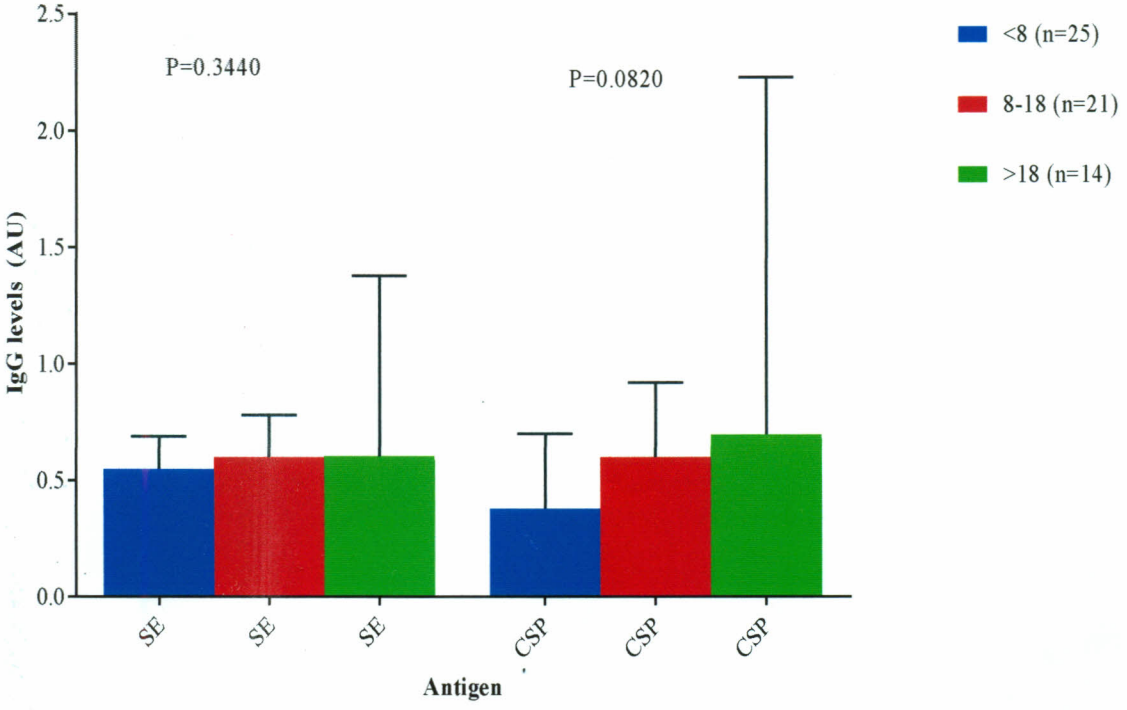

Figure 4.4: Relationship between IgG levels to schizont extract and circumsporozoite protein among participants of different age groups in an area of unstable malaria transmission. IgG levels were expressed as optical density (OD) in arbitrary units (AU), error bars represent standard error of the mean (SEM). There was no significant difference when median were compared by Kruskal-Wallis Test (P>0.05).

4.7 Prevalence of IgG antibodies to *P. falciparum* antigens in relation to gender

At cut-off of AU>1, the IgG levels to both SE and CSP were relatively low in both male and female participants. The prevalence of IgG antibodies to SE was higher in males at 26.67% (8 out of 30) than in females at 10% (3 out of 30) though not significant (P>0.05). Similarly, higher proportion of males were IgG antibodies positive to CSP at 23.33% (7 out of 30) than females at 16.67% (3 out of 30) though not significant (P>0.05). However, When the cut-off for antibody positivity was set at AU>2, the antibody prevalence was extremely low in both male and female participants. The expression of IgG antibodies to both SE and CSP was lower in females (3.33% for both antigens) as compared to males (10% for both antigens). When the prevalence of IgG was

compared between CSP and SE in both males and females, the prevalence IgG antibodies to SE were higher than IgG antibodies to CSP in males while in females IgG antibodies to CSP were higher than IgG antibodies to SE (Table 4.5).

Table 4.5: Proportion of antibody-positive individuals to *Plasmodium falciparum* Schizont extract and Cicumsporozoite protein at a cut-off of AU>1 and AU>2 in males and females

Antigen	sex	Number (N)	IgG Positive at AU>1		IgG Positive at AU>2	
			Number (n)	Percentage (%)*	Number (n)	Percentage (%)*
SE	Male	30	8	26.67	3	10
	Female	30	3	10.	1	3.33
CSP	Male	30	7	23.33	3	10
	Female	30	5	16.67	1	3.33

***Percentage is calculated by the formula n/N × 100**

Comparing the prevalence of IgG antibodies to the two antigens in both males and females at threshold of AU>2, the prevalence of IgG antibodies to both CSP and SE were extremely low. However, the proportion of antibodies was the same in males (10% for both CSP and SE) as well as in females (3.33% for both CSP and SE) (Figure 4.5).

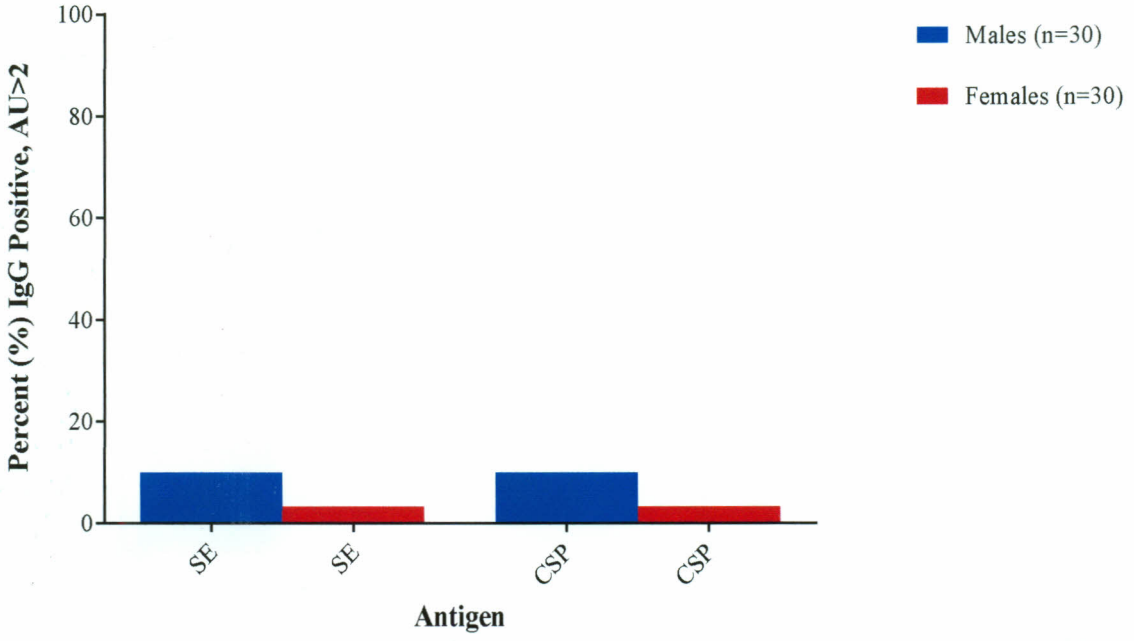

Figure 4.5: Prevalence of total IgG antibodies, determined by ELISA for *P. falciparum* Schizont extract and Cicumsporozoite Protein in male and female participants in an area of unstable malaria transmission. The frequencies were expressed in percentage (%) when the cut-off of AU>2 was considered antibody-positive (high-level antibodies).

4.8 Relationship between IgG levels to the two antigens and gender

The IgG antibody levels to SE and CSP in the study area were relatively low in both males and females. The median levels of IgG antibodies to SE in males (0.6050 AU) were slightly higher than in females (0.5600). In contrast, the median IgG levels to CSP were slightly higher in females (0.5750) than in males (0.5000). The differences that were recorded between antibody levels to the two antigens and between males and females were not significant (P>0.05) (Figure 4.6).

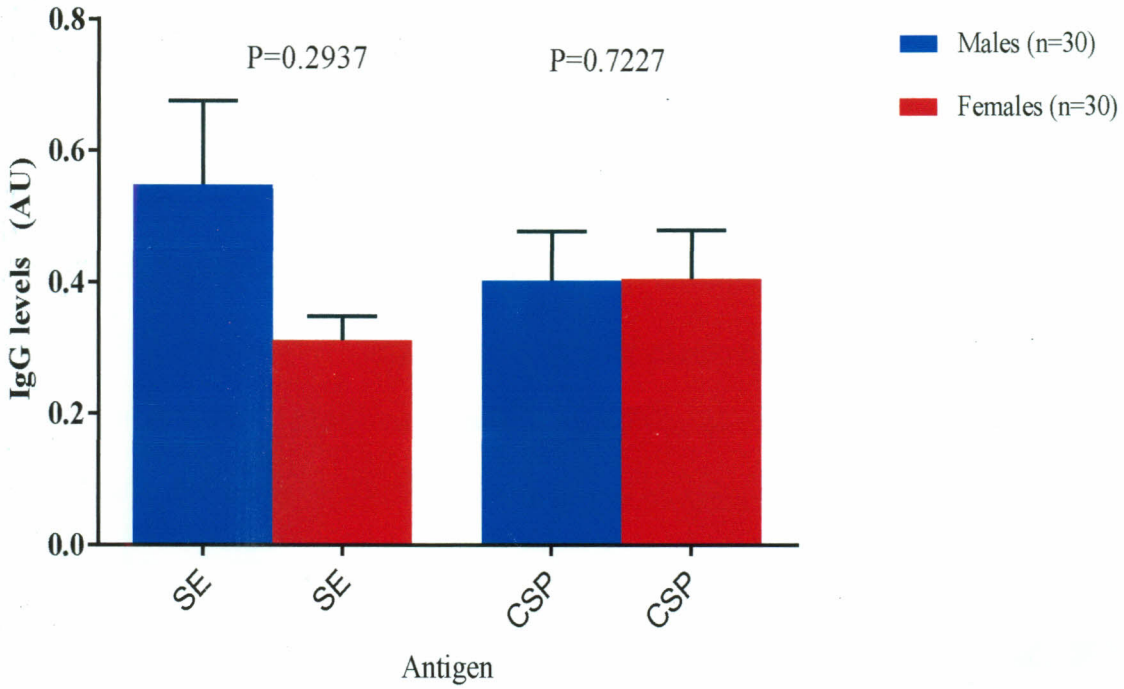

Figure 4.6: Relationship between IgG levels to schizont extract and circumsporozoite protein between males and females in an area of unstable malaria transmission. IgG levels were expressed as optical density (OD) in arbitrary units (AU), error bars represent standard error of the mean (SEM). There was no significant difference when median were compared by Mann-Whitney Test (P>0.05).

An effort was made to compare frequencies and levels of IgG antibodies to SE and CSP between parastaemic and non-parastaemic individuals. However, there were no parastaemic individuals in the study group (0 out of 60).

CHAPTER FIVE: DISCUSSIONS

5.1 Characteristics of the study population

Highland areas of western Kenya are prone to unpredictable epidemics of malaria due to low and unstable malaria transmission. Studies have documented that the inter-epidemic periods are characterized by low frequencies of asymptomatically infected individuals and increase in gametocyte prevalence is an indication of possible outbreak (John *et al.*, 2005). The absence of blood stage malaria parasite in the present study sample population and that all were asymptomatic to malaria conforms to the earlier findings (Noland *et al.*, 2008). Low parastaemia prevalence and low asymptomatic individuals have been reported in highland areas both during dry and rainy seasons (John *et al.*, 2005). Moreover, in the present study, sample collection was done in the month of May during transmission interruption period which indeed explains further the absence of parastaemic individuals and asymptomatically infected individuals. Haemoglobin level may also be an indicator for malaria illness such that levels below the set threshold are associated with anaemia which is a characteristic of malaria due to haemolysis of RBC (Federica *et al.*, 2007). The study population had normal haemoglobin levels as per the current set cut-off point for anaemia explaining lack of haemolysis caused by malaria parasites thereby supporting absence of parastaemic individuals.

5.2 Antibody prevalence to schizont extract and circumsporozoite protein

In vitro and *in vivo* studies of rodent malaria models demonstrate that antibodies to CSP or TRAP impair or prevent malaria infection (Gysin *et al.*, 1984), providing biological possibility for the idea that, antibodies to these antigens may play a role in protection from infection and disease. Studies have documented that the presence of high-level IgG

antibodies to pre-erythrocytic antigens especially to CSP correlates with protection from *P. falciparum* infection in adults and clinical malaria in children (John *et al.*, 2008). Data from the present study show that generally a very small proportion of individuals living in the study area had high-level IgG antibodies to the two pre-erythrocytic *P. falciparum* antigens, CSP and schizont extract. This percentage is relatively higher than 3.3% documented in an earlier study in the same area (Noland *et al.*, 2008). This difference may be partly due to timing in sample collection and sample size of the studies. All the same, the results of the present study demonstrate that the prevalence of high-level IgG antibodies in an area with unstable malaria transmission is extremely low as compared to documented results (44.3%) for an area of stable malaria transmission (Noland *et al.*, 2008). These findings suggests in part that the intermittent risk of clinical malaria in individuals living in an area of unstable malaria transmission may relate to the low or absence of high-level IgG antibodies to pre-erythrocytic antigens particularly CSP.

As much as presence of high-level IgG antibodies to pre-erythrocytic antigens may not completely prevent blood-stage infection, it may control the parasite density and the clonality of parasitemia by reducing the invasion of *P. falciparum* sporozoites into the liver or by impairing progressive development of parasites in the liver. This notion is supported by documented results in earlier studies that, increased levels of anti-CSP antibodies is associated with decreasing parasite densities in asymptomatic adults in malaria-holoendemic areas (Offula *et al.*, 2005). The control of parasite density may be a way through which partial protection against pre-erythrocytic stages of *P. falciparum* reduces clinical illness associated with malaria. Reduced parastaemia density may also reduce haemolysis of erythrocytes thereby resulting in high haemoglobin levels. Findings

in the present study document reduced prevalence of anti-CSP and anti-SE antibodies. This may in part explain the absence of protective immunity among individuals in the study area resulting in frequent epidemics.

5.3 Relationship between IgG antibodies levels to CSP and schizont extract

The present study generally reports very low IgG antibodies levels to both schizont extract and circumspozoite protein in highland area with low unstable malaria transmission. The levels compared to documented data from areas of high transmission are extremely low. Documented information associate high level of antibodies to CSP and other pre-erythrocytic antigens such as MSP, LSA and TRAP with protection against clinical malaria (John *et al.*, 2005). The low levels IgG antibodies to CSP and SE reported in the present study suggest in part why the highland areas are prone to epidemics.

The findings in the present study that there is a correlation between IgG antibodies levels to the two antigens conform to documented data of strong correlation in expression of antibodies to pre-erythtrocytic antigens especially CSP, MSP, LSA and TRAP in malaria holoendemic areas (John *et al.*, 2005). The results in the present study suggest that protection against malaria may be as a result of antibodies to multiple pre-erythrocytic antigens and that repeated and frequent infection appears to be the major determinant of high levels of IgG antibodies to these antigens.

The mechanism of association of antibodies to different pre-erythrocytic antigens in protection is not clearly understood. However, one potential mechanism for antibody related protection is the combined effects of antibodies that target different steps during

the pre-erythrocytic phase of infection life cycle. Circumsporozoite protein is important in adhesion of the sporozoite to the basolateral membrane of the hepatocyte (Frevert *et al.,* 1993) and monoclonal antibodies to CSP inhibit parasite invasion of hepatoma cells (Chatterjee *et al.,* 1995). Thrombospondin related adhesive protein (TRAP) is essential for sporozoite gliding motility (Sultan, 1999) and hepatocyte invasion (Muller *et al.*, 1993) and antibodies to TRAP have also been shown to prevent sporozoite invasion of hepatocytes (Muller *et al.*, 1993).

The low levels of IgG antibodies to pre-erythrocytic antigens particularly CSP in highland areas of Western Kenya therefore is attributed to infrequent exposure to malaria parasites hence lack of protective immunity. The degree of natural exposure to malaria required to attain high levels of IgG antibodies to pre-erythrocytic antigens is not known. These high levels of IgG are usually attained by some children in malaria holoendemic areas thereby developing immunity but not others. However, findings of the present study present very low levels of anti-CSP and anti-SE IgG antibodies which may in part be attributed to very low degree of natural exposure to malaria.

5.4 Relationship between age and antibody prevalence and levels to schizont extract and circumsporozoite protein

In highland areas of Africa, older children and adult continue to be at risk for clinical malaria, although the risk is somewhat lower than that for young children (Hay *et al.,* 2002). This is in contrast with areas with stable, high level malaria transmission, where clinical immunity to malaria develops by the age of 3 to 5, with intermittent episodes of mild malaria and very few episodes of malaria occurring after this age (Snow *et al.,* 1997). Results from the present study demonstrate very low prevalence of high-level antibodies to both CSP and schizont extract in individuals of all ages. These frequencies

are generally low in comparison to results documented for studies done in areas of stable malaria transmission where over 50% individuals show high level IgG antibodies to pre-erythrocytic antigens (John *et al.*, 2005).

Several previous studies have demonstrated that high levels of IgG antibodies to multiple pre-erythrocytic antigens are associated with protection against *P. falciparum* infection in adults living in malaria-holoendemic area (John *et al.*, 2008). Majority of this protection is associated with high-level IgG antibodies to CSP and LSA not assessed in the present study (John *et al.*, 2005). The findings of the present study suggest that the persistent risk of clinical malaria in older children and adults in areas of unstable transmission may be as a result of low prevalence of high-level IgG antibodies to pre-erythrocytic antibodies especially CSP.

The immunity to malaria that develops with age in areas of stable malaria transmission is not completely characterized. However, several studies have documented a correlation with protection from clinical malaria for anti-CSP IgG antibodies assessed in this study and IgG antibodies to other antigens not tested in the present study such as LSA-1, TRAP, AMA-1, MSP-1, MSP-2, MSP-3 and GLURP (Riley *et al.*, 1992; Scarselli *et al.*, 1993; Roussilhon *et al.*, 2007; John *et al.*, 2008; Osier *et al.*, 2008). These previous studies confirm the association of age-dependent acquisition of anti-malaria antibodies with cumulative exposure to malaria in areas with high and stable malaria transmission. However, little comparable information is available for individuals residing in areas of unstable, epidemic-prone malaria transmission. A study conducted in Tanzania dwelt majorly on blood-stage antigens including MSP-1_{19}, MSP-2, AMA-1 and glycosylphosphatidylinositol (GPI) across a wide range of ages and transmission

intensities. The Tanzanian study reported that antibody isotypes for the tested antigens relate to age and transmission intensity (Tongaren *et al.*, 2006).

The present study dwelt on pre-erythrocytic antigens which the Tanzanian study did not test. The key finding of the present study is that highland areas of western Kenya with unstable malaria transmission present extremely low levels of IgG antibodies as compared to documented data of areas of stable malaria transmission. Furthermore the results of this study indicate that there is no significant difference in IgG antibody levels to CSP and schizont extract in the study area. Although results of the present study indicate that the levels of IgG antibodies to CSP and schizont extract increase with age, adults have very low levels of anti-CSP and anti-SE IgG antibodies suggesting why adults are also at risk of malaria in areas of unstable malaria transmission.

The association of IgG antibodies to CSP and LSA-1 not tested in this study with protection from *P. falciparum* and disease (John *et al.*, 2005) and the striking low or absence of high-level antibodies in the study area, suggests that these responses may be important in protection against infection and disease. The idea that immune response to CSP is important for development of clinical immunity against malaria is supported by the success of CSP-based RTS,S vaccine in inducing protection from clinical malaria due to *P. falciparum* in children. Indeed, the first results of phase 3 trial which was launched in 2009 in seven African malaria-endemic countries; Gabon, Ghana, Kenya, Malawi, Burkina Faso, Tanzania and Mozambique showed that RTS,S reduced the risk of children experiencing clinical malaria and severe malaria by 56% and 47% respectively (WHO, 2013). Therefore, the low levels of IgG antibodies to CSP and schizont extract in the area

of low and unstable malaria transmission reported in the present study could be part of the reason for impaired clinical immunity in adults.

5.5 Relationship between gender and antibody prevalence and levels to schizont extract and circumsporozoite protein

Very few studies have attempted to relate IgG antibody responses to *P. falciparum* antigens with gender or sex of the subjects. A study conducted in Nigeria documented that antibody levels to CSP in Nigerian children were higher in male than female children of the same age group (Olalubi *et al.*, 2012) but could not give an explanation. Another study in the Republic of Korea reported that the IgG antibody prevalence to *P. vivax* CSP was higher in male than female adult inhabitants and associated it to exposure where men who work more in the fields are more exposed than females who usually work less in the fields (Lee *et al.*, 2003). Similar information is sparse and more so for areas of low and unstable malaria transmission. The low prevalence of high level IgG antibodies to the two antigens may explain in part why malaria outbreak is indiscriminative of gender in the areas of low malaria transmission. The association of IgG antibodies responses to *P. falciparum* with gender is not understood and is subject to further investigation.

CHAPTER SIX: CONLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. The prevalence of IgG antibodies to both CSP and crude schizont extract was generally low (6.7% for both antigens). There was no significant difference between the prevalence of IgG antibodies to the two *Plasmodium falciparum* antigens (P=0.2050)

ii. There was a correlation between the levels of IgG antibodies to CSP and crude schizont extract. However the levels of IgG antibodies were generally low for both antigens and were not significantly different.

iii. The prevalence of IgG antibodies to both CSP and crude schizont extract increased with age and were generally very low. However, there was no significant difference in IgG antibodies levels among individuals of different age groups. On the other hand, the prevalence of IgG antibodies to the two *Plasmodium falciparum* antigens was higher in males than their female counterparts though not significantly different. The levels of IgG antibodies to CSP and crude schizont extracts between males and females were not significantly different and were generally very low.

Based on the results, the two hypotheses "There are no differences in antibody levels between *Plasmodium falciparum* CSP and schizont extract in individuals living in areas with low unstable malaria transmission" and "Age and gender have no effect on antibody responses to *P. falciparum* antigens in an area of low and unstable malaria transmission" are accepted

6.2 Recommendations

i. The observation that strong correlation occurs between IgG antibody levels to the two antigens, more correlations should be established with more *P. falciparum* antigens in areas of low transmission and vaccine developers should consider multi-antigen vaccines.

ii. The Health Ministries in the countries with epidemic-prone low malaria transmission areas should use several malaria control measures such as vector control and chemotherapy. They should also adopt measures as epidemic preparedness and case management. Furthermore, vaccine developers should fast-track the process in order to save lives in such areas where individuals do not develop partial immunity to malaria with age.

iii. Longitudinal studies should be carried out to cover wider areas involving lager study population over a longer period of time to determine the immune responses even during the period of malaria transmission in areas of low and unstable malaria transmission.

iv. Other *Plasmodium falciparum* antigens such as LSA, AMA-1, EBA-175, MSP-1, GLURP, TRAP and others should be used to assess the possible immune responses among individuals living in areas of low unstable malaria transmission. Other immunological parameters such as cell mediated immunity (CMI) should also be used to assess the possible immune responses in such areas.

v. Although some studies report that males develop higher antibody responses than their female counterparts, there is sparse information on gender and malaria. This

therefore calls for extensive studies to establish the relationship between gender and immune responses to malaria infection both in endemic areas and areas of unstable transmission.

vi. It would also be important to determine if there are naturally resistant individuals in epidemic prone areas and further determine their genetic polymorphism that confers the resistance.

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APPENDICES

APPENDIX I: **Laboratory protocols**

a) Edta Plasma Collection

Supplies

- EDTA Blood Collection Tubes (BD vacutainers catalog # 366450)
- Centrifuge with swinging bucket rotor
- 15 ml polypropylene conical tubes (Fisher, cat #05-538-53D)
- Sterile cryovials with writing surface (Fisher cat #05-669-57)
- 2ml, 5ml and 10ml pipettes (Fisher cat #13-678-11C, 13-678-11D, 13-678-11E)
- Disposable transfer pipettes (Fisher cat #13-711-20)
- Automatic pipette aid
- Small ice bucket

Plasma Separation Procedure

1. After collection, gently mix the blood by inverting the tube 8 to 10 times. Store vacutainer tubes upright at 4°C until centrifugation. Blood samples should be centrifuged within four hours of blood collection.
2. Centrifuge blood samples in a horizontal rotor (swing-out head) for 10 to 20 minutes at 1100-1300 g at room temperature.
3. After centrifugation, plasma layer will be at the top of the tube. Mononuclear cells and platelets will be in a whitish layer, called the "buffy coat", just under the plasma and above the red blood cells (additional processing of these cell fractions is optional).
4. Carefully collect the plasma layer with an appropriate transfer pipette without disturbing the buffy coat layer. If more than one tube is collected, pool the plasma

samples from both tubes into a 15 ml conical tube and mix. Pipette the plasma into appropriate sized aliquots in labeled cryovials. Close the caps tightly and place on ice. This process should be completed within 1 hour of centrifugation.

5. Check that all aliquot vial caps are secure and that all vials are labeled. Place all aliquots upright in a specimen box or rack in an -80°C or colder freezer. All specimens should remain at -80°C or colder prior to shipping. The samples should not be thawed prior to shipping. (Plasma to be shipped on dry ice)

b) ELISA reagents and procedure

i. ELISA reagents

Carbonate/Bicarbonate buffer (coating buffer)

- 0.16g Na_2CO_3
- 0.29g $NaHCO_3$
- In 80cm^3 distilled water and adjust the pH to9.6
- Make up the solution to 100cm^3

NB: always prepare fresh coating buffer immediately before use

Phosphate buffered saline (PBS)

- 8.0g Sodium chloride
- 0.2g Potassium chloride
- 0.15g Disodium hydrogen phosphate
- 0.2g Potassium dihydrogen phosphate
- Dissolve in 800cm^3 of distilled water and make up to 1000ml

PBS-Tween 20 (0.05%v/v)

- Mix 0.25ml Tween 20 with 500cm^3 of PBS.
- Incubate Tween 20 at 37°C for 30 minutes before use because it is very viscous
- Dispense using automatic pipette with disposable tips

Blocking Buffer (5% (wt/v) non-fat milk powder inPBS)

- Dissolve 2.5g non-fat milk powder in PBS while stirring or rotating for 30 minutes
- If high background is experienced 1% milk could be applied for both blocking and antibody dilution

Antibody diluents

- Prepare 5% (wt/v) non-fat powdered milk in PBS immediately before use
- Dilute to 1% (wt/v) by adding PBS while stirring

Diethanolamine buffer (for use with *P*-nitrophenyl phosphate)

- 97ml Diethanolamine
- 100mg $MgCl_2.6H_2O$
- 1M HCl
- Place about 600ml of distilled water into a beaker
- Add diethanolamine and $MgCl_2$ while stirring

- Add 200ml of water to elute any undissolved diethanolamine and adjust pH with HCl
- Store at 4^{oC} in brown bottle or covered with aluminium foil

***P*-Nitrophenyl phosphate (substrate)**

- 1mg/ml p-Nitrophenyl phosphate in diethanolamine buffer
- Prepare immediately before use and keep in dark bottles until used in ELISA

Stop solution (3M Sodium hydroxide)

- 30g NaOH in $250cm^3$ distilled water in plastic beaker and store in plastic container before use

ii. ELISA procedure

DAY ONE

1. Label the ELISA plate appropriately for each antigen and also with the individual ID. Plan the layout of the plate and allocate sets of duplicate wells to act as blanks for calibration of the ELISA reader. Mark a large arrow on the right hand side of the plate with a felt pen to avoid Disorientation of the plate

2. Prepare coating buffer, thaw one aliquot of the antigen and dilute the antigen to give a concentration of 10µg/ml. Coat the 96-well Thermo Immulon- 4HBX ELISA plate with 50µl using automatic pipette and cover with a plate sealer and incubate overnight at $4^{o}C$.

DAY TWO

1. Prepare PBS-Tween 20 and blocking buffer (non-fat milk powder in PBS). Remover the sealer and allow to attain room temperature.

2. Wash the plate 3 times with washing buffer (0.05% PBS-Tween 20) inverting the contents over the sink or using ELISA machine washer. Blot the plate dry using towel or soft tissue paper

3. Add 50µl of blocking buffer to each well and replace the plate sealer. Incubate for 1 hour at room temperatue.

   While plates are blocking, thaw the samples, write out hard copy plate template. Carry out serial dilution of the sample starting with highest concentration to the lowest concentration (1:50, 1:100, 1:200, 1:500 and 1:1000) and obtain a working concentration.

4. Discard the blocking buffer, wash three times and blot the plate

5. Add the standards first and then proceed to add samples according to the template set-up. Cover the plate with the sealer and incubate for two hours at room temperature

6. Prepare the detection antibody (secondary antibody) after titrating to working concentration eg1:1000 in the blocking buffer as reagent diluents.

7. After 2 hours of incubation with samples and standards, wash the plate 3 times in washing buffer. Blot dry and add secondary antibody using automatic pipette, seal the plate and incubate for 2 hours at room temperature.

8. Remove the enzyme substrate buffer from the freezer 15 minutes prior to the end of incubation with secondary antibody and cover with aluminium foil prepare the substrate (p-nitrophenyl phosphate) at 1mg/ml of the buffer (diethanolamine buffer)

9. Remove and discard the plate sealer and wash the plate six times and add 50µl of the substrate to each well, place the plate in the dark and read after exactly 30 minutes

   Absorbance is measured at 405nm

10. If the plate cannot be read immediately, add 50µl of 3M NaOH to prevent futher enzyme activity.

    Once stopped plates must be read immediately. Otherwise, they will quickly start to precipitate and thus skew the readings on the plate reader.

11. Before reading the plates wipe the bottoms thoroughly and inspect the wells for bubbles.

12. Results: only the intensity of colour is measured by the reader and is directly proportional to the amount of antibody bound on the immobilized antigens on the plate. Calculate the mean values of the duplicates.

APPENDIX II: INFORMED CONSENT AGREEMENT

a) INFORMED CONSENT AGREEMENT IN ENGLISH

UMN/KEMRI Consent for Human Investigational Studies
Study Title: Malaria transmission and immunity in highland Kenya
Principal Investigator: Chandy John
Consent form: Site-wide blood sample collection
Study Number:___-____-____

Purpose: This is a malaria immunology study looking at the various methods the body uses to defend itself against malaria. Dr. Chandy John from University of Minnesota (UMN) in the USA and his colleagues at the Kenya Medical Research Institute (KEMRI) are asking your permission for you/your child to participate in this research study. This study is sponsored by the National Institute of Allergy and Infectious Diseases, USA. We believe that the body has ways to protect itself against malaria. The goal of this research study is to understand how the body protects itself against malaria. We can do this by examining the blood from you/your child in the laboratory. We are collecting samples from all consenting people in the entire community (Kipsamoite and Kapsisiywa). Information gained from this study will help researchers design laboratory tests that measure malaria protection. This information may be helpful for future malaria vaccine studies that may be conducted in this area.

Procedure: We request a small blood sample from you/your child. We would like obtain a single sample from you this year. If we test again in future years, we will again request consent from you. We will be obtaining blood by finger prick sampling, and we will obtain approximately 10-20 drops of blood (0.5 -1.0 mL). We will use a sterile lancet to prick your/your child's finger after clearing it with an alcohol swab, and drip the blood into a sterile tube. Taking this amount of blood from you/your child should not cause any harm to you/your child. Blood will be transported to the UMN/KEMRI laboratory in Kisumu. Tests done in the laboratory will tell us how well your/your child's body is protecting itself against malaria (i.e. cytokine & antibody responses). There are no expenses involved in participating in the study. Your participation can end if you decide to end it or if the study decides to end it. We anticipate the participation of ~5800 people in Kipsamoite and Kapsisiywa in this study. You will be informed promptly if information becomes available that may be relevant to your willingness to participate.

Genetic studies: We also would like to store part of your/your child's blood at the UMN/KEMRI laboratory to do genetic studies. The studies we will do currently include only testing for genes belonging to the malaria parasite. This is important to see what strains of malaria parasite are in the community. Future studies may include genes belonging to you/your child, such as HLA type or other genes that are thought to protect you against malaria. If we get any results from the lab studies that may affect the health of you/your child, we will inform you. We will request permission for any genetic studies not described here from the Institutional Review Board (IRB) at UMN and the KEMRI Scientific Steering Committee (SSC) and Ethical Review Committee (ERC).

If you do not agree to have your/your child's blood stored for genetic testing, we will destroy the sample after we have tested it for the type of malaria parasite. If, at any time you wish to withdraw your agreement, please contact Mr. Joseph A. Okweso and we will destroy the sample. If you do not wish to have your/your child's blood stored for genetic testing, you/your child may still participate in this study. You/Your child will still be examined for defenses against malaria. If you/your child has malaria, we will still treat you/your child.

Long-term storage and future studies: I agree for UMN/KEMRI to store my/my child's blood for futures studies of factors that may protect against malaria. I understand that if any test results are found that are important for my/my child's health, UMN/KEMRI will try to report this to me, if possible. This testing may be done in UMN labs in Kisian, Kenya or in the labs of UMN in Minneapolis, Minnesota. I understand that I have the right to withdraw my agreement to use my/my child's blood for future research anytime and for any reason. If I withdraw my agreement to use my/my child's blood for future research testing, the samples will be destroyed. I may also ask that my/my child's blood not be used for certain types of testing. To do this, I may tell Mr. Peter Odada Sumba, the UMN/KEMRI field supervisor, of my request and he will tell the study people at UMN/KEMRI. I understand that the UMN IRB and KEMRI SSC and ERC must approve any future testing not described here.

If you agree, circle "YES". If you do not agree, circle "NO" YES NO
Participant/Parent or Guardian Signature*_____ Date _____
Witnessed by_____ Date_____
*A parent can sign, or verbally state his/her consent in the presence of a witness who will then sign.

Risks and Benefits: There are minimal risks to having your/your child's blood drawn. The risks include bleeding, pain, bruising and possible infection. All of these are uncommon events that have occurred in very few adults or children previously studied by this research group. If you are pregnant, there are no additional risks to your fetus. The benefits of having your/your child's blood drawn are free testing for malaria parasites. If malaria parasites are found and you/your child has malaria symptoms, such as fever and headache, you/your child will be offered the drugs currently recommended by the Kenya Ministry of Health for the treatment of uncomplicated malaria. The Clinical Officer at the health centre will give you the appropriate amount of medication to treat you/your child, and the study will cover payment of any clinic treatment expenses for malaria. A few days after treatment, the field assistant assigned to your area will inquire about your/your child's recovery progress to make sure the drugs are working properly.
During the course of this study, if you or your child develops disease from malaria, you or your child should be seen at the health center, where you will be provided with treatment following the Kenya National Health Guidelines for the treatment of uncomplicated malaria. For adults and children greater than 5 kg, Artemether-Lumefantrine (CoArtem®) is the current treatment. Artemether-Lumefantrine has been shown to be very effective for treating uncomplicated malaria even if the parasites are resistant to other antimalarial drugs. The side effects of this drug may include dizziness

and fatigue, loss of appetite, nausea, vomiting, abdominal pain, myalgia, racing heart beat, trouble sleeping headache, rash and aching joints. These side effects are extremely rare. For children weighing less than 5 kg, the first line treatment is Amodiaquine. The side effects of this drug may include nausea, vomiting, skin rash or itching. If CoArtem® has been approved for treatment of children under 5 kg, this option will be provided. Neither of these drugs should be taken by women who are pregnant or nursing. If you are pregnant, you will be given oral quinine, as per Kenya National Guidelines. The side effects of quinine include ringing in the ears, dizziness, nausea and vomiting. These side effects are fairly common but stop after the medication is stopped. Rare side effects from oral quinine include low blood sugar and heart rhythm abnormalities.

Confidentiality: The results of the studies using your/your child's blood will be assigned a study number to preserve confidentiality. A database linking you or your child's personal identifiers to the study number will be kept by the principal investigator and relevant key personnel. Only study personnel and study monitors, auditors and institutional review boards will be allowed access to the medical information collected in this study.

Summary of your rights as a participant in a research study: Your/your child's participation in this research study is voluntary. Refusing to participate will not alter your/your child's usual health care or involve any penalty or loss of benefits to which you or your child are otherwise entitled. If you decide to enroll yourself or your child in the study, you may withdraw yourself or your child at any time and for any reason. If information generated from this study is published or presented, your and your child's identity will not be revealed. Under some circumstances, the sponsor of the study will pay for injuries resulting directly from being in the study. If you want information about those circumstances or if you think you have suffered a research related injury, let the study physicians know right away. If you or your child experiences physical injury or illness as a result of participating in this research study, contact The Director of the Center for Vector Biology and Control Research (CVBCR) at KEMRI in Kisumu at PO Box 1578 at 057-2022924/22923 or Mr. Joseph A. Okweso at 057-2022989, 0721-257220 or 0733-280116.

Contact Information: _____ has described to you what is going to be done, the risks, hazards, and benefits involved, and can be contacted at _____. Further information with respect to illness or injury resulting from a research procedure as well as a research subjects' rights is available from KEMRI/National Ethical Review Committee (ERC), PO Box 54840, Nairobi at 020-722541 or The Director of KEMRI, PO Box 54840, Nairobi at 020-722541. If you have any questions about this study, you may also speak to The Director of CVBCR, KEMRI in Kisumu at 057-22924. Dr. John's designate, Mr. Joseph A. Okweso can be contacted at CVBCR, KEMRI, PO Box 1578 in Kisumu at 057-2022989, 0721-257220 or 0733-280116.

Signature: Signing below indicates that you have been informed about the research study in which you voluntarily agree to enroll yourself/your child; that you have asked

any questions about the study; and that the information given to you has permitted you to make a fully informed and free decision about your/your child's participation in the study. By signing this consent form, you do not waive any legal rights, and the investigators are not relieved of any liability they may have. You can withdraw yourself/your child from this study at any time. You will be offered a copy of this consent form, and it will be provided to you if you would like one.

Printed Name of Participant

_____

Date_____

Signature of Participant or Child's Signature if this form is used to obtain assent
If Participant is a minor or legally incompetent adult.

_____ Date_____

Parent or Legal Guardian signature
Relationship to Participant _____ Date_____

Signature of Person Obtaining Consent

_____

Printed Name of Person Obtaining Consent

(Must be study investigator or individual who has been designated in the Checklist to obtain consent.)

_____ Date_____

Signature of Principal Investigator (Affirming subject eligibility for theStudy and that informed consent has been obtained.)

b) **INFORMED CONSENT AGREEMENT IN KALENJIN**

UMN/KEMRI Fomitab chametab chii eng chigilet.
SOMATNET KO: Oindap chepligonet ak kimnatet ne imuch koboryo ak chepligonet eng emet nemi barak eng Kenya
CHIGININDET NEO: Chandy John
FOMITAB 4: Chigiletab cheptigonit eng korik

STUDY NUMBER_-___-____-__

Kerjinet (purpose): Nitok ko somanet ne kere oratunwet che terchin imuch koboisie borto koboryo ak cheptigonit. Daktari Chandy John nebunu University of Minnesota (UMN) eng USA ak biik cheterotigei tugul eng Kenya Medical Research Institute (KEMRI) kotochin inye/lakheg'ung eng chigilanitok. Chiginoni konyoru toretet kong'ete cheteleltoljin ng'alekab mionwokik chebo America (National Institute of Allergy and Infectious Diseases, USA). Kimang'u kele mitei oratinwek che imuch ko kirngta borto cheptigonet. Chigilanitok komachei konai ole imuch koboryo borto ak cheptigonit. Akosiketestai ak chigilani kemechei kechigil korotikuk/ lakweg'ung. Kimenu korotik eng biik tugul che keyan chemitei emoni (Kipsamoite ak Kapsiswa). Ng'ang'utik che kinyoru eng chigilanitok kotoreti kipsomaninik kotoi oratinwek chekikirintoe cheptigonit. Ng'ang'utichu ko imuch kotoretech eng kerichekab cheptigonet eng betusiek chemitei tai eng emoni.

Tetutik (Procedure): eng chiginoni ketesetai kechigini kutikab cheptigonit eng korotikuk/ lakweg'ung ye ibita orowek oeng agoi tun kobek kenyisiek somok ak kebeberta (3½ years). Eng chiginoni kinemu korotik eng mornet. Ngot kiyan chiginoni kobo komonut ibitu eng emoni eng kinyisiek somok ak kebeberta chemi tai. Bokomonut mising kinemu korotik yeibata arowek oeng'u amu kimachei kenai ole kenyoru/ lakweg'ung cheptigonit angot ngotko mekosei mionindo age tugul. Chiginoni kotorotech kenai kimnotetab borto koboryo ak cheptigonit. Ngot kitinyei/ lakweg'ung kutikab cheptigonit ak kabarunoikab cheptigonit , konyoin daktari nemitei hospitalinitok. Kiliboni konyosiet age tugul nebunu cheptigonit.
Kisomin/ lakweng'ung kogeny kinemu korotik eng eut yeibata arowek lo (6 months). Imong'u kole kinemu korotik konyil tisab eng kenyisiek somok ak kebeberta. Kinemu korotik eng eung'ung/ lakweng'ung. Kinemu keberta anan kijiget agenge (2-5 ml) eng lakweng'ung. kinemu korotik che legit kijiket 3-4 (15-20 ml) eng biik che echen. Kiibe korotik agoi ole kiciginei eng UMN-KEMRI kisumu. Chiginoni koborwech kimnotetab bortong'ung koboryo ak cheptigonit (cytokine and antibody responses, responding cell type , haemoglobin level)
Kinemu korotik eng mornet konyil 21 ak konyil 7 eng chiginosiek 28 chebo kenyisiek somok ak kebeberta.

Ngot kiyan chiginoni kebwoni oling'ung yeibata betusiek tisabu agoi tuun kobek kenyisiek somok ak kebeberta. Kitebsenin ngotko mi miondo age tugul ne kobiit eng goi ng'ung. Kitebsenin ngot komi brtut age tugul ne kiru sang kobate goi ng'ung. Ngot

kitinyie koborunoikab cheptigonit koimuch itebaei chetoretech eng chiginet konyokonem korotik si kochigin ngot komi cheptigonit si koyaen daktari.

Chiginetab terjinet (Genetic studies): Kimachei got kegonor kebebertab korotikuk/lakweng'ung eng ole kichgilei eng UMN-KEMRI si keboisie kechigin ole tergintoi kutik ab chepgonit. Bo komonut mising kenai kelei mitei kutik ab cheptigonit cheunei emoni. Eng betusiek chebwoni ko imuch kechigin ole itergnei/ lakweng'ng kou HLA anan ko ole toreteten teritang'ung eng kiringtab cheptigonit. Got kinyoru eng chiginoni lomut age tugul eng inye/bortong'ung anan ko lakweng'ung kemwoun. Kisomei boroindo eng kit age tugul nimagimuch kemwa eng yu che intonjin ng'alekab chigilet eng UMN nebo University nebo Mnneapolis,MN ak cheintonjin ngalekab chigilet (Scientific steering committee, SSC) ak ngatutik (Ethical review committee ERC) eng KEMRI.
Ngotko meyani kegonor korotikuk/lakweng'ung sikechigil oletergintoi biik, ke nyere ye kaketar ke chigini kutik chetergin chebo cheptigonit. Nye imach istagei ang chiginonitak ko imuch inyoru Peter Odada Sumba. Ngot ko nemache kekonor korotikuk/lakweng'ng si kechigin loe tertoi bich ko imuch kora itestai ak chiginonitak. Inye/ lakweng'ng ketesei tai kechigini kimnotet an bortongung korboryo ak cheptigonit. Ngot kitinyei/lakweng'ung cheptigonit kinyoi.

Konoretab korotik ak chiginet nemi tai (Long-term storage and future studies): Keyan ane kogonor UMN-KEMRI korotikyu/lakwenyu si koboisie eng betusiek chemi tai kochigin ole kirintoi borto cheptigonit. Kanai ale walutik alak tugul chebo komonut eng ane/ lakwenyu chebunu chiginoni komwowo UMN-KEMRI. Chigiloni koimuch keyai eng ole kichigile eng UMN Kisian, Kenya anan ole kichigile eng UMN, Minneapolis, MN. Kaanai ale amuchi aet kechigin korotikyu/ lakwenyu eng sait age tugulkouye kamach. Ng'ot kasie kechigin korotikyu anan chebo lakwenyu eng betusiek chebwoni, keng'emei korotik alak tugul che kianemu. Amuch asom kimokiboisien korotikyu/ lakwenyu eng chiginet age tugul. Ye amachei aistogei eng chiginoni amuch asom toretet kong'ete Joseph A. Okweso sikomwachi biik che chigini eng UMN-KEMRI. Kaanai ale UMN IRB ak KEMRI SSC ak ERC koimuch koteleltoe/kosirto chiginet age tugul netun kioe nemakimwa eng yu.

Ngot kiyan isir "YES". Ngot iyesie isir "NO" YES NO
Saii nebo sigindet/Telelindet* _____ Tarigit _____

Baornatet Kong'ete _____ Tarigit _____

*Imuch kondeno sai sikindet, anan komwa kotenyi sikonde sai baorintet.

Lomutik ak keljinet (Risks and benefits): Mitei lomutik chemengechen ye kinemu korotik. Lomutichu koboichin, korot korotik tutigin, ng'wanindo, sirunet/kobwa borto. Chuto tugul kikobiit eng biik cheng'ering chikikegin. Ng'ot kinyigis/tamano, komomitei asanet age tugul. Keljinet ne mi kogakinem korotik ak kechigil kuutikab cheptigonit (maleria) ko kanyaiset nebo buch ng'otindoi chito kabarunoikab cheptigonit anan kenyor kutukab cheptigonit eng korotik. Inyoin dactari (clinical officer) kusubge ak ng'atutik

chebo kanyosiet eng serekali nebo Kenya. Konin daktari (clinical officer) nemitei hospitalinito kerichek che inyoein/ lakweng'ung ak kiliboni kerichek tugul che kakinyaenin. Nyekokerin daktari eng betusiek cheng'ering'en si kosich konai ngot kobois kerichek anan mobois kuyekimachei.
Ngot kinyoru/ lakwengung cheptigonit yetestai chiginoni, kinyoin eng hospitali nebo serekali kosubge ak ng'atutik chebo moni chebo konyosietab cheptigonit. Eng biik che echen ak lakok che tindoi nyigisindo nesirei kiloisiek 5 (5kg), keboisien Artemether-Lumefantrine (Coartem®). Artemether-Lumefantrine ko kericheck che inyoi cheptigonit ne moimuch konya kerichek alak. Koborunoik chebitu ngiyaenen kerichechu kou. Ikosei kowisis borto tugul, matemachei amitwokik, ing'ung'u , wasegei moet, ng'wanindo eng banyek, birei muguleldo mising, kobet ruondo, amin metit ak kosir borto. Ngot kishirto serikali keboisien CoArtem eng lagok chetindoi nyigisindo nerekune kiloisiek 5 (5kg) keboisie. Makinyosie kerichechu oeng eng gwonyik che nyigisen ananchetakorertos kina lagok. Kinyoe quinine gwonyik che nyigisen. Koborunoik chebitu kinyaenen quinine kou, kowokjo itik, kowisisit borto, ilombosen ak ing'ung'u. koborunoichu kobitu yetiboisie kerichek akoteleltos yekitelel kerichek. Kobarunoik chematabitu kinyosie kerichechu kou korek sugaruk ak kobir mugulelto eng oret nemonyolchilnot.

Tuget (Confidentialty): Walutik che kinyor eng cheginetab korotikuk/lakwengung keboisie nambosiek simotok kaineng'ung. Imuch konai kaineng'ung neo ne indonjin cheginoni ak chetoretigei. Biik che kionji koro ng'olio ak tugul nebunu chiginoni kou; kipsomaninikab chiginoni ak biik cheteletojin, cheteleltojin robinik ak biik cheintoijin ng'alekab chiginosiek.
Imantang'ung eng chiginoni: kaanai ale kagonugei/ lakwenyu eng chiginoni eng chamenyu. Kaaguiye ale eng kabwatenyu ami anan ko mami eng chiginoni ko memuchi kowal ribetab sobenyu/ lakwenyu. Kaanai ale amuch aistogei/ lakwenyu eng chinoni sait age tugul ne gamach.Ung'at kainengung/ lakwengung ye kiborostoi temukak chiginoni eng ematunwek tugul anan eng siruttik alak tugul. Biik che kionji koro ng'olio ak tugul nebunu chiginoni kou; kipsomaninikab chiginoni ak biik cheteleltojin, cheteleltojin robinik ak biik cheintoijin ng'alekab chiginosiek. Ngot inyoru/lakweng'ung lomut age tugul eng chiginoni inyoru ne indoji ng'alekab chiginisiet eng Kisumu (Director of Centre of Vector Biology and Control Research, KEMRI Kisumu) eng sandukutab posta 1578 ak simet 057-2022924/2022923 anan Joseph A. okweso eng 057-2022989, 0721-257220, anan, 0733-280116.

Ole inyorchin toretet:_____ kakonetin tukuk tugul che testai, lomut alak tugul , anan keljinet nebitu, ak amich anyor eng _____
Ngot koit lomut alak tuguleng chiginoni, ko imuch inyoru imantang'ung eng KEMRI/National Ethical Review Committee (ERC), sandukutab posta 54840, Nairobi eng 020-722541. Ngot komi ng'olio age tugul, ko imuch inyoru ne indoji ng'alekab chiginisiet eng Kisumu (Director of Centre of Vector Biology and Control Research, KEMRI Kisumu) eng simet 057-2022924/2022923 anan Joseph A. okweso eng sandukutab posta 1578 ak 057-2022989, 0721-257220, anan, 0733-280116.
Sai: ngindene sai ng'ony yu koboru kole kakemwoun akobo chiginoni ak keyan eng chameng'ung/ lakweng'ung; kaiteb kaimut age tugul ne itinyie agobo chiginoni; ak kaanai ale ng'alek tugul chigakimwowo ko kaanai ak kayan chiginoni ang magenyu. Ye

keyan chiginoni komaistoi imantanyu anan toretet ne imuch anyor anan koisto chignik eng imatangwai. Imuch aistogei/lakwenyu eng kasarta age tugul. Kigoni fominito agenge ngot kimachei.

_____

_____

Siretab kainetab chito ne teshingei somanani

_____ Tarik _____

Sei nengung ana iteben siiyet* nebo chito netesienkei somsnani anan sei nebo lakwet ngotko fimini nkeboisie eng chamchinetab chito ne mingin. Ngotko chitone tesiengei ko mingin ana ko chito neo ne memuchegei.

_____ Tarik _____

Sei nebo sigindet ko ribindet

Tiliandit ak netesingei _____

*Ngotko inendet ne teshingei somanani, sigendet anan ribindet komemuchi kosoman anan kosir, konyolu komi baoriat ne maguregei kiy ak inentet ye king'alalei chamchinoni. Ye kaketar kesomani ak kinet agobo chamchinet eng netesingei somanet, sigindet anan ribindet. Ye kakoyan eng kutit, koyanji lakwet kotesyigei somanani ak koyan kosir anan koteben konyolu konde sei baoriat ak kosir tarikit. Ngonde sei baoriat koboru kole ng'alek chemi fomitab chamchinet ko kakinet ak koguyo sigindet anan ko ribindet.

_____ Tarik/sait_____

Sei nebo chito ne baoriani

_____ Tarik _____

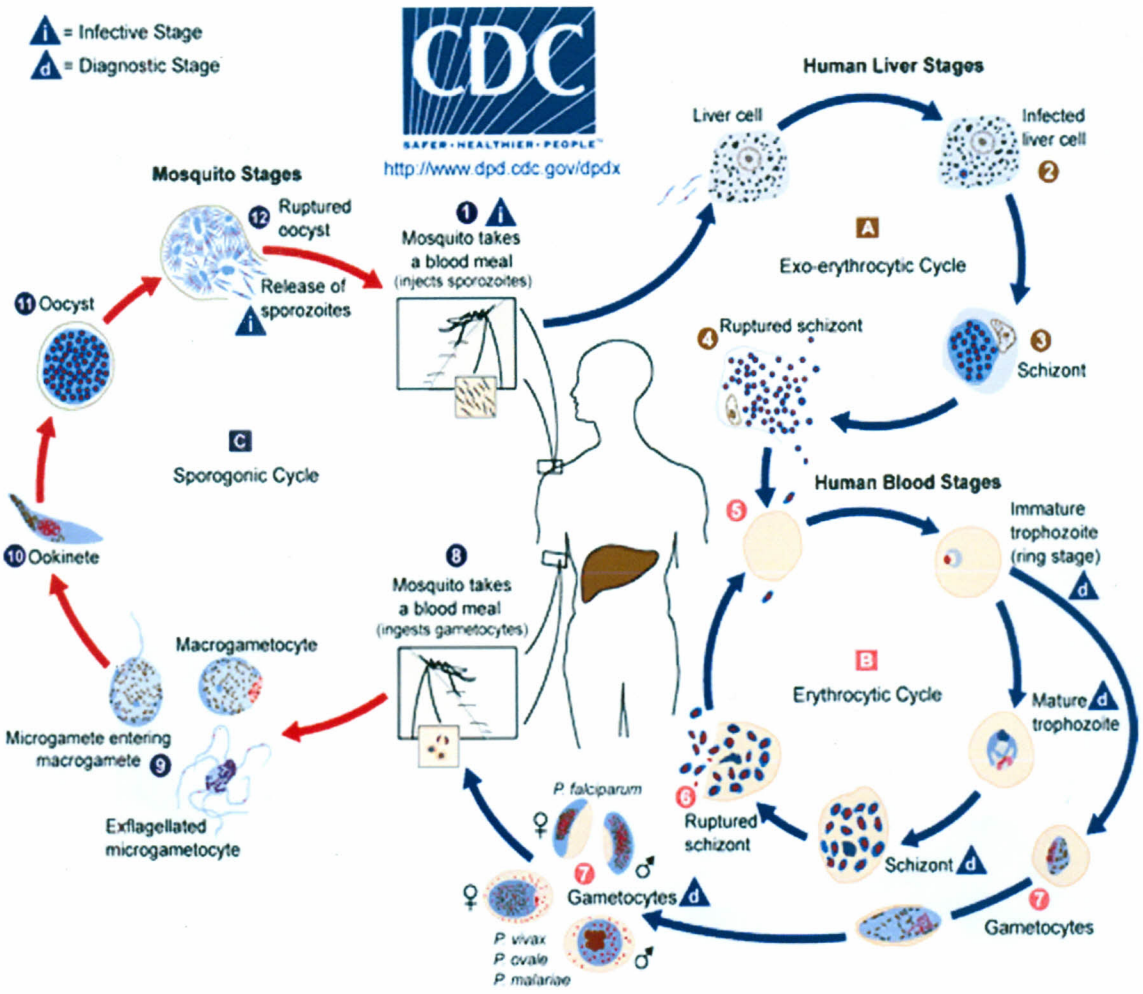
Sei nebo inendet ne ibei chamchinoni kaineta inendet ne ibei chamchinoni

(Nyolu koek inendet ne teleljin ana ko inendet nekikechamchi koib chamchinoni)

_____ Tarik _____

Sei nebo chito ne indojin chikiloni (Kabor kole inendet ne kisomani ko iyanat eng somanani ak kobor kole kakenyor chamchinet kobun inendet).

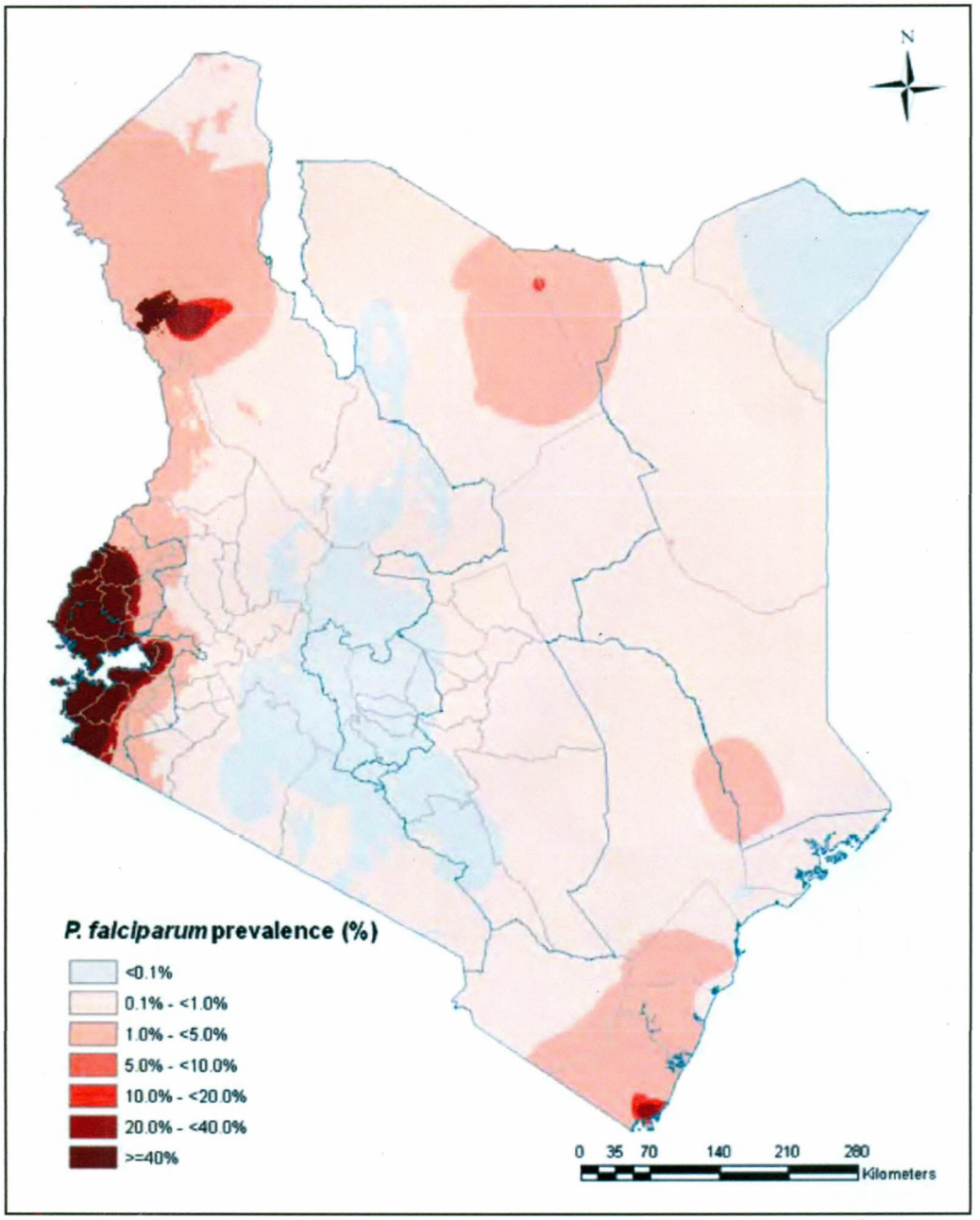
APPENDIX III: Detailed lifecycle of malaria parasite



Adopted from CDC 2006.

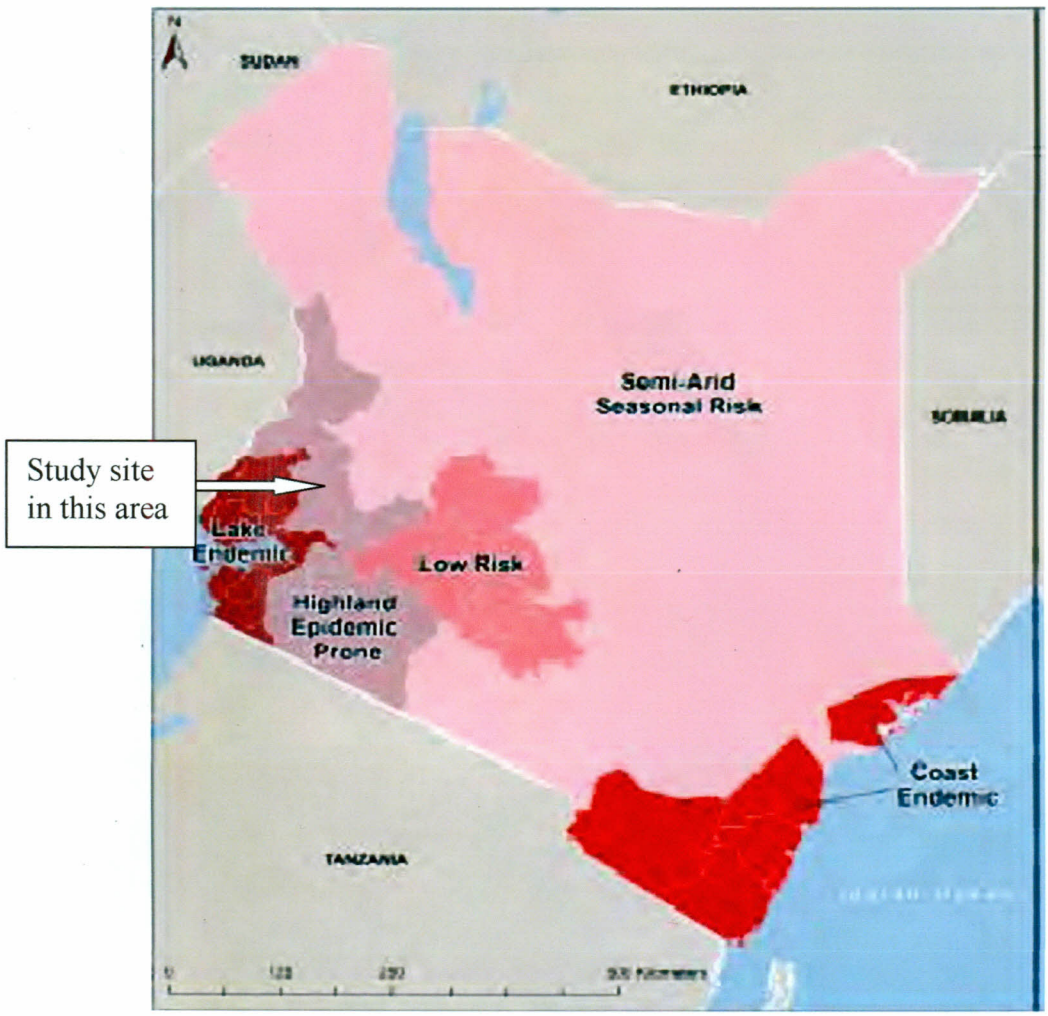
APPENDIX IV: MAPS

a) Map showing prevalence of *Plasmodium falciparum* in Kenya



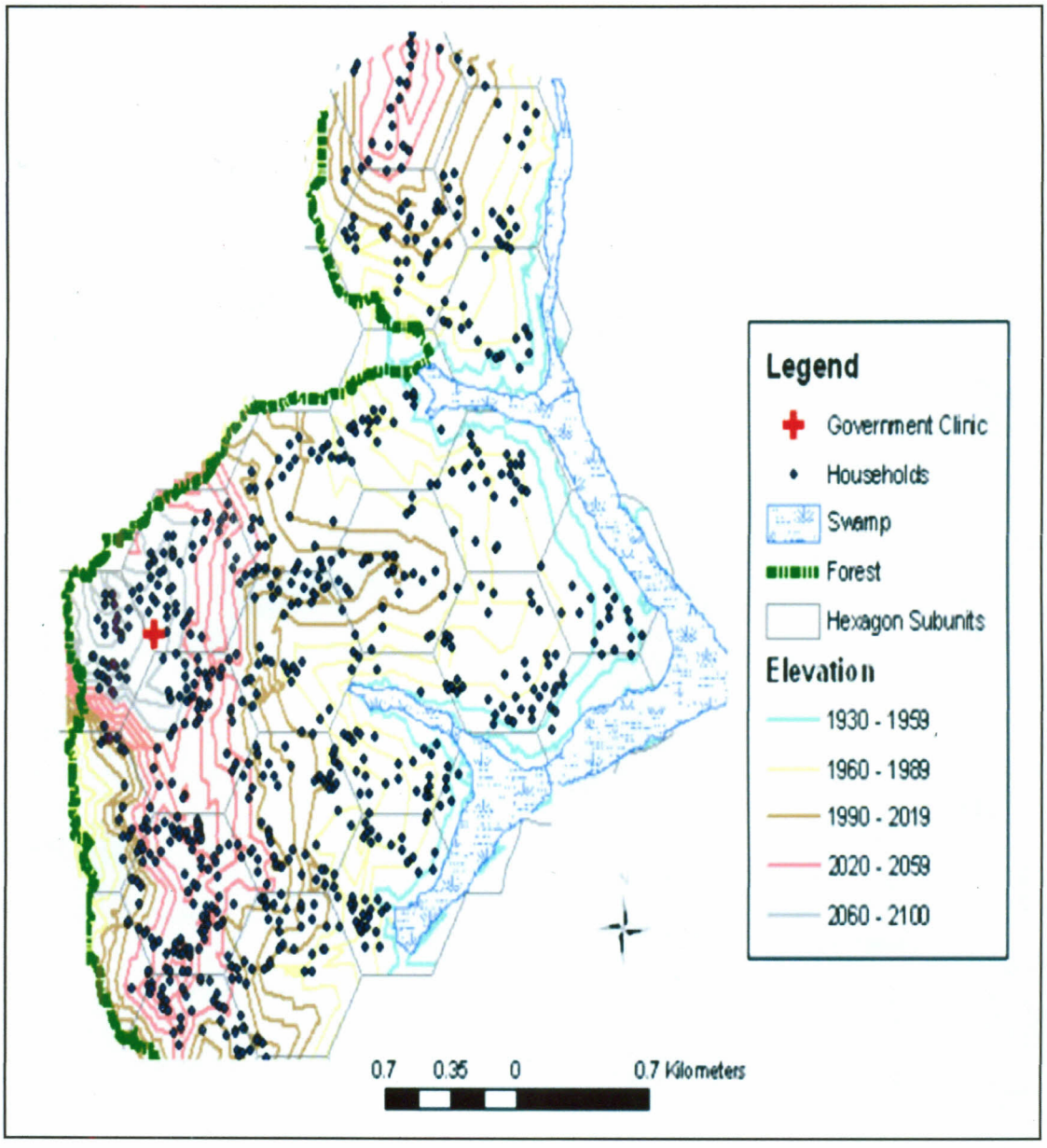
Adopted from Noor *et al.* (2009).

b) **Malaria Epidimiological Zones in Kenya**



Adopted from DOMC (2011)

c) Map of Kipsamoite, Nandi North



Adopted from Kacey *et al.*, (2006)

APPENDIX V: Twelve approaches to a malaria vaccine

1. Sporozoite subunit vaccination, especially with the CS protein: e.g. RTS,S in adjuvant.
2. Irradiated sporozoite or genetically attenuated sporozoite immunization either by mosquito bite or using injected purified sporozoites.
3. Immunization with DNA and/or viral vectors to induce T cells against the liver-stage parasites, or to target other life cycle stages.
4. Use of whole blood-stage malaria parasites as immunogens.
5. Use of protein in adjuvant vaccines to reduce the growth rate of blood-stage parasites.
6. Use of protein (or long peptide) in adjuvant vaccines to induce antibody-dependent cellular inhibition (ADCI) of blood-stage parasites.
7. Use of peptide-based vaccines, mainly against blood-stage parasites—e.g. SPf66, PEV3a.
8. Development of anti-disease vaccines based on parasite toxins—e.g. GPI-based.
9. Immunization with parasite adhesion ligands such as PfEMP1.
10. Use of parasite antigens, such as the Var2 protein, preferentially expressed in the placenta to prevent malaria in pregnancy.
11. Immunization with sexual stage parasite antigens as transmission-blocking vaccines.
12. Use of mosquito antigens as transmission-blocking vaccines

Adopted from Hill, 2011

APENDIX VI: Letter of ethical approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **March 19th, 2013**

TO: **DR. CHANDY C JOHN**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. JOHN VULULE,**
THE DIRECTOR, CGHR,
KISUMU

FORWARDED
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH
25/3/2013

Dear Sir,

RE: **SSC PROTOCOL No. 939 (*ANNUAL RENEWAL – FINAL APPROVAL*):**
MALARIA TRANSMISSION AND IMMUNITY IN HIGHLAND KENYA

Thank you for sending to Dr George Ayodo to attend the 213th ERC meeting held on 19th March 2013 at the KEMRI Headquarters Campus to discuss your request for annual renewal.

The Committee was of the considered opinion that the responses given at the meeting were satisfactory and therefore grants approval for continuation with the study. This approval is effective from **19th March 2013** for a period of one year.

Authorization to conduct this study will automatically expire on **18th March 2014**. If you plan to continue with the study beyond this date please submit an application for continuation approval to the ERC secretariat by **3rd March 2014.**

You may proceed with the study

Sincerely

MR. AMBROSE RACHIER,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE