GENETIC DIVERSITY AND POPULATION STRUCTURE OF *PLASMODIUM FALCIPARUM* FROM FOUR EPIDEMIOLOGICAL LOCATIONS IN MALAWI

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GENETIC DIVERSITY AND POPULATION STRUCTURE OF *PLASMODIUM FALCIPARUM* FROM FOUR EPIDEMIOLOGICAL LOCATIONS IN MALAWI

By

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DECLARATION

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ABSTRACT

In malaria-endemic regions, *Plasmodium falciparum* (*P. falciparum*) infection is characterized by extensive genetic/antigenic diversity. Describing this diversity provides important information about the local molecular epidemiology of infecting *P. falciparum* parasites. Intriguingly, one of the major obstacles to the development of an effective malaria vaccine has been the genetic polymorphisms exhibited by *P. falciparum* genes encoding targets of human immune system. This situation has necessitated the development of polyvalent vaccines with wide antigenic coverage that would increase the likelihood of vaccine efficacy that covers wide geographical areas of malaria endemic countries.

Limited reports are available on the population genetic diversity and structure of *P. falciparum* in Malawi, and this is of particular concern as the country has put in place several interventions to combat the disease.

The primary aim of the research project was to determine the genetic diversity and population structure of *P. falciparum* isolates and comparing complexity from four different epidemiological settings in Malawi using msp-2 gene polymorphisms. Samples were collected from four epidemiological locations in the north, centre and southern regions of Malawi.

The diversity and genetic differentiation of *P. falciparum* populations were analyzed based on the highly polymorphic block 3 msp-2 gene. One hundred and twenty patient samples who presented with signs and symptoms of malaria and who had

microscopically confirmed *P. falciparum* infection were enrolled in the study after they had satisfied the inclusion criteria. Parasite DNA was extracted from the blood spot on to filter paper and analyzed by genotyping the msp-2 gene using allele-specific nested PCR.

A total of 28 msp-2 block 3 fragments, defined by the size and the allelic types were detected in the 102 patients. The length variants of the PCR product ranged from 240basepairs (bp) to 450bp for the K1/FC and 410bp to 780bp for the 3D7/IC allelic families. Isolates of the 3D7 alleles were predominant in the population (59%), compared to isolates of the K1/ FC27 alleles (41%) and for 3D7 and K1 most of the isolates were monoclonal infections. In comparisons between the sites, we observed the highest prevalence of mixed infection in Mwanza (46.7%) followed by Dwangwa (23.3%) compared to Bolero (16.7%) and Mitundu (16.7%). The difference in prevalence of mixed infections between Mwanza and the other sites was statistically significant (p=0.041). There was also a non-significant trend towards a higher mean genotype number per isolate in the children aged >5 years compared to those below 5 years of age.

These data suggest differences in prevalence rates of mixed infections in different geographical/epidemiological settings in Malawi. Further studies are needed to confirm, with larger sample sizes, the observation of a non-significant trend towards higher multiclonality of infection in older children in malaria endemic areas of Malawi

Key words: Population structure, genetic diversity, merozoite surface protein-2, DNA extraction, epidemiology, Malaria, allele-specific, polymerase chain reaction (PCR), *Plasmodium.*

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACT	-	Artemisinin based Combination Therapies
AL/LA	-	Artemether + Lumefantrine
AMA	-	Apical Membrane Antigen
BMP	-	Blantyre Malaria Project
Bp	-	basepair
CD4	-	Cluster of Differentiation 4
CD36	-	Cluster of Differentiation 36
CDC	-	Center for Disease Control
CIDR	-	Cysteine-rich inter domain region
COMREC	-	College of Medicine Research Ethics Committee
CSA	-	Chondroitin Surface Antigen
CSF	-	Cerebral Spinal Fluid
CSP	-	Circumsporozoite Protein
DBP	-	Duffy Binding Protein
DBS	-	Dried Blood Spot
DFID	-	Department For International Development
DHO	-	District Health Office
DNA	-	Deoxyribonucleic Acid
dNTP	-	Deoxyribonucleotide Tryphosphate
EDTA	-	Ethylenediaminetetraacetic Acid
FRTI	-	Faculty Research, Technology and Innovation
GCLP	-	Good Clinical Laboratory Practice

GLURP	-	Glutamate-rich Protein
HIV	-	Human Immunodeficiency Virus
HRP-2	-	Histidine Rich protein-2
ICEMR	-	International Centers of Excellence in Malaria Research
IgG	-	Immunoglobulin G
IPTp	-	Intermittent Preventive Treatment in Pregnancy
IRS	-	Indoor Residual Spraying
ITBN	-	Insecticide Treated Bed Nets
ITN	-	Insecticide Treated Mosquito Nets
kDa	-	KiloDalton
LA	-	Lumefantrine-Artemether
LD	-	Linkage Disequilibrium
MI	-	Multiple Infection
MIM	-	Multilateral Initiative for Malaria
MESA	-	Mature parasite-infected Erythrocyte Surface Antigen
MLW	-	Malawi-Liverpool Wellcome Trust
MRA/MR4	-	Malaria Research and Reference Reagent Resource Centre
msa-2	-	Merozoite Surface Antigen 2
msp-1	-	Merozoite Surface Protein-1
msp-2	-	Merozoite Surface Protein-2
NMCP	-	National Malaria Control Program
NMMU	-	Nelson Mandela Metropolitan University

NMMUHEC	-	Nelson Mandela Metropolitan University Human Ethics Committee
ND	-	No Date
NSO	-	National Statistical Office
OPD	-	Out Patient Department
PCR	-	Polymerase Chain Reaction
Pf	-	*Plasmodium falciparum*
*Pf*EMP-1	-	*Plasmodium falciparum* Erythrocyte Membrane Protein
PMI	-	Presidential Malaria Initiative
RBC	-	Red Blood Cell
RESA	-	Ring stage Surface Antigen
RNA	-	Ribonucleic Acid
SCH	-	Single –clone haplotype
SNP	-	Single-nucleotide polymorphism
SOP	-	Standard Operation Procedure
SP	-	Sulphadoxine-Pyrimethamine
TRAP	-	Thrombospondin-related Adhesion Protein
UNC	-	University of North Carolina
USA	-	United States of America
VSA	-	Variant Surface Antigen
WHO	-	World Health Organisation

CHAPTER 1: INTRODUCTION

1.1. Background information

Malaria is a mosquito-borne infectious disease caused by a eukaryotic protist of the genus *Plasmodium*. *Plasmodium falciparum* is the most virulent of the four species of the parasites which cause malaria in humans (Kiwanuka, 2009). While progress is being made in reducing malaria vector and disease prevalence in Malawi, malaria still remains one of the major causes of morbidity and mortality, especially in children under five years old (PMI, 2010, 2012). The disease has been responsible for over 40% of hospitalization of children under-five years and 40% of all hospital deaths (WHO, 2002) and remains a significant public health burden in the country, with around 4.5 million cases every year for its population of around 13 million (Medicines for Malaria Venture, 2010).

In spite of giant steps made in the fight against malaria over the last century, reports indicate that malaria is still a major cause of death in the tropics. It is estimated that there are about 250 million cases of malaria annually, 86% of which are reported in Africa (WHO, 2008a). Malaria is responsible for about 1 million deaths every year, 85% of which are of children under 5 years of age (WHO, 2009). Carter & Mendis (2002), estimates that malaria may have been responsible for between 2% to 5% of the deaths in the twentieth century and approximately 10% of deaths in the early part of the 21st century.

Malaria is the most important tropical disease and one of the major threats to human health in the world (Bereczky *et al.,* 2005). Malaria has been the leading cause of human mortality and this is still the case presently, despite having been eradicated in many temperate zones. About 40% of the world's population lives in endemic areas (Wiser, 2009), where this poses a serious barrier to economic progress in many developing countries including Malawi.

Efforts to help control malaria are ongoing. The President's Malaria Initiative (PMI) working with the National Malaria Control Programme (NMCP) outlined four key interventions with the goal of halving the burden of malaria in 70 percent of the at-risk populations in sub-Saharan Africa and these include Intermittent Preventive Treatment for pregnant women (IPTp**)** as well as improved diagnosis and treatment (WHO, 2008b). In addition to the two strategies already outlined by the ministry earlier, the Government of Malawi commenced the implementation of a new treatment policy from Sulphadoxine-Pyrimethamine (SP) to Artemether + Lumefantrine (AL) as the first line drug due to its co-formulation in December 2007 (WHO, 2009). As a preventive measure, the country has also adopted indoor residual spraying (IRS) and the use of Insecticide Treated Mosquito Nets (ITNs) (WHO, 2009).

Despite the current control efforts discussed in the preceding paragraph, malaria still remains a burden. Genetic diversity displayed by *P. falciparum* field isolates, the occurrence of variant forms of the parasite at different frequencies in different geographic areas, and the complexity of infection represent major obstacles for the effective vaccine-

based and drug-based control of malaria (Irawati, 2011). Incidentally, early evidence of resistance to artemisinins, the most important class of antimalarials, is now confirmed, having manifested as delayed parasite clearance times in the western region of Cambodia on the border with Thailand (WHO, 2010). Additionally, there is some early evidence that resistance to artemisinins may also be emerging on the Myanmar-Thailand border as well. This is the region where resistance to earlier antimalarial drugs emerged and then subsequently spread throughout much of the world as it did with antimalarial drugs such as chloroquine in the 1960s and 1970s (WHO, 2010). Antimalarial drug resistance is now generally acknowledged to be one of the greatest threats to our ability to "Roll Back Malaria." Resistance to antimalarial drugs is resulting in avoidable morbidity, mortality, and associated financial losses. 'Alternative measures are urgently needed now to reduce the current and future burden of disease' (Yeung *et al*, 2004) and development of an effective malaria vaccine may be especially vital in ultimately combating the disease.

'Vaccines are often the most cost-effective tools for public health' (Clements &Griffiths, 2002). They have historically contributed to a reduction in the spread and burden of infectious diseases and have played a major role in previous elimination campaigns for smallpox and the ongoing polio and measles initiatives (Andre *et al.*, 2008). Yet no effective vaccine for malaria has so far been developed. Other studies are exploring this intervention now by undertaking a phase III clinical trial for the world's most clinically advanced vaccine candidate known as RTS,S, (UNC School of Medicine-Centre for Infectious Diseases-USA, 2010). However, the development of an effective malaria vaccine has not been easy, in part because people can be simultaneously infected

with several parasite strains. These often carry different variants (alleles) of the genes encoding antigens, which means that the actual parasite proteins might differ from the ones used/represented in the vaccine construct (Takala *et al*., 2007).

At first sight, 'today's global malaria vaccine portfolio looks promising' with 47 new vaccine candidates: 31 in preclinical development, narrowing down to 16 in clinical trials. However, this apparently healthy global portfolio is deceptive according to the following argument by Moran and colleagues (2007): "rather than reflecting a high level of early innovation with subsequent careful prioritization and down-selection of candidates (leading to fewer but better candidates), the shape of today's global vaccine portfolio is rather the unwitting product of scientific, technical and policy settings that generate and promote vaccine candidates somewhat indiscriminately", with the vast majority subsequently failing in clinical trials. It is therefore important to understand the distribution and natural dynamics of vaccine antigen polymorphisms in endemic populations when preparing for vaccine efficacy trials of vaccines against malaria and other genetically variable pathogens.

Molecular epidemiological studies of malaria parasites will guide vaccine design and provide information that is needed to measure and interpret population responses to vaccines, both during efficacy trials and after introduction of vaccines into the population (Takala *et al*., 2007). Data from such studies may provide insights into the selective forces (e.g., immunity) acting on antigen genes. One of the major obstacles to the development of an effective malaria vaccine is the genetic polymorphism of many of the

genes in natural parasite populations that otherwise would be promising vaccine candidates. In fact, it may be generally the case that the proteins of pathogens that are the best targets of immune responses are also the most polymorphic because of immune selective pressure (Duan *et al.*, 2008). This necessitates the development of polyvalent vaccines with wide antigenic coverage and that covers a wide geographical area. The challenge is to see variability of the vaccines in relation to the geographical area hence the need to limit the vaccines to certain geographical areas in order to counter the parasite polymorphism. The genes, merozoite surface proteins 1 and 2 (msp-1 and msp-2) are widely used to study the allelic diversity and frequency of *P. falciparum* which are most commonly correlated with the level of transmission in the area under study (Atroosh *et al.*, 2011). Information on antigenic coverage, prevalence and dynamics of vaccine antigen polymorphisms in a population being targeted for malaria vaccines will provide informed choices about which merozoite surface protein-1 (msp-1) and merozoite surface protein-2 (msp-2) haplotypes to include in future vaccine formulations, and will allow more accurate interpretation of the efficacy of current formulations of msp-1 and msp-2 based vaccines being tested in clinical trials (Takala *et al*., 2007). Thus knowledge of the genetic structure of malaria parasites in relation to the evolution of parasite virulence will be useful in designing efficacious anti-malarial vaccines (Khatoon *et al*., 2010).

As malaria continues to be one of the leading causes of morbidity and mortality, there is a paucity of information on genetic diversity and population structure of *P. falciparum* in Malawi (Dzinjalamala, 2006). As observed from one study in Mali, molecular epidemiology studies provides information needed to accurately measure and

interpret population responses to malaria vaccines in clinical trials of vaccine efficacy. Molecular epidemiologic studies for malaria also provides insight into which msp-1 and msp-2 polymorphisms that may be most relevant to cross-protective immunity and thus informs vaccine design (Takala *et al*. 2007). Against this backdrop, knowledge of the genetic structure of parasite is useful for understanding the evolution of parasite virulence, designing anti-malarial vaccines and assessing the impact of malaria control measures. Given the polymorphic nature of vaccine antigens for *P. falciparum* and other genetically diverse pathogens, it is important to monitor pathogen populations before, during, and after introduction of vaccines to determine vaccine efficacy at the molecular level and to detect potential vaccine-induced changes in the pathogen population that could compromise vaccine efficacy.

1.2 Aim and objectives of the study

The primary aim of the research project was to determine the genetic diversity and population structure of *P. falciparum* isolates and comparing complexity from different epidemiological settings in Malawi using msp-2.

1.2.1 Specific objectives of the study

The overall objective of the study project was to characterize the population structure of *P. falciparum* based on the highly polymorphic block 3 msp-2 gene. The research project would specifically:

- Determine the presence and number of msp-2 allelic and sub-allelic families in isolates from four selected study sites.

- Calculate the *P. falciparum* genetic complexity by site and compare the complexity between the four study sites.

CHAPTER 2 : LITERATURE REVIEW

2.1 Malaria Distribution

Malaria exists in about 100 countries though it has a worldwide distribution (Bereczky, 2005; WHO-World Malaria Report, 2011). It is found in Sub-Saharan Africa and to a lesser extent in South Africa, South East Asia, the Pacific Islands, India, Central and South America, as shown in figure 2.1 below. *Plasmodium falciparum* is the predominant specie in most endemic areas, with the exception being India and South America where *Plasmodium vivax (P. vivax)* is more common. *Plasmodium ovale (P. ovale)* is mainly found in West Africa whilst *Plasmodium malariae (P. malariae)* is commonly found together with either *P. falciparum* or *P. vivax* (Ashley, McGready, Proux & Nosten, 2005).

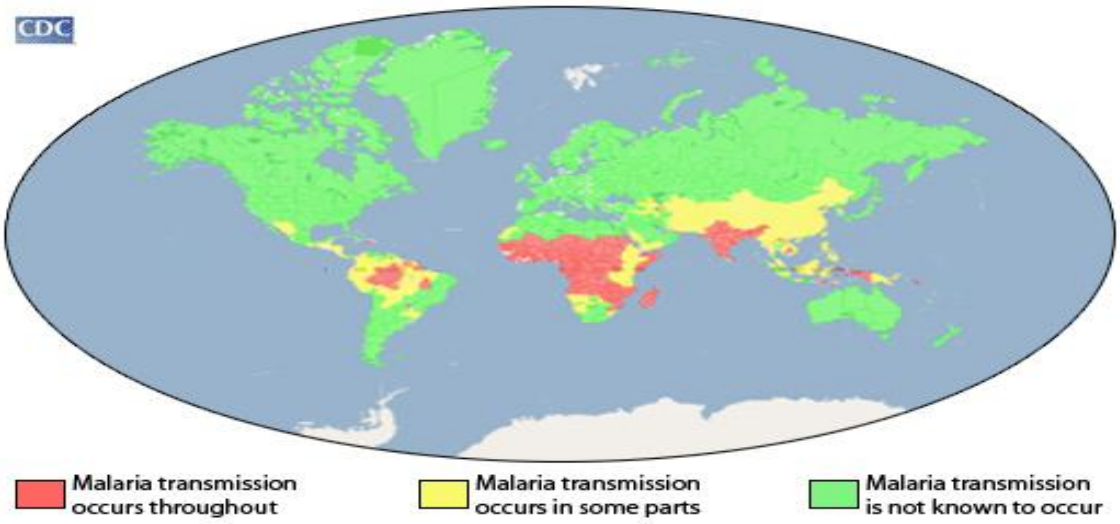

Figure 2.1 World Map showing Malaria distribution (copyright from CDC, 2008)

2.2 Malaria and sampling Locations in Malawi

In Malawi, malaria remains the most important public health concern and is the leading cause of mortality especially amongst infants under the age of five. Malawi is a landlocked country located in the Southern African region and has a population of approximately 13.6 million people (NSO, 2008, Malawi population data sheet, 2012). It is bordered by Zambia to the northwest, Tanzania to the northeast, and Mozambique on the east, south and west. The country is separated from Tanzania and Mozambique by Lake Malawi and it is over 118,000 km^2 (45,560 sq. mi; as shown in figure 2.2 and 2.3 below). These countries are also in malaria endemic regions of sub-Saharan Africa (see world malaria map, figure 2.1).

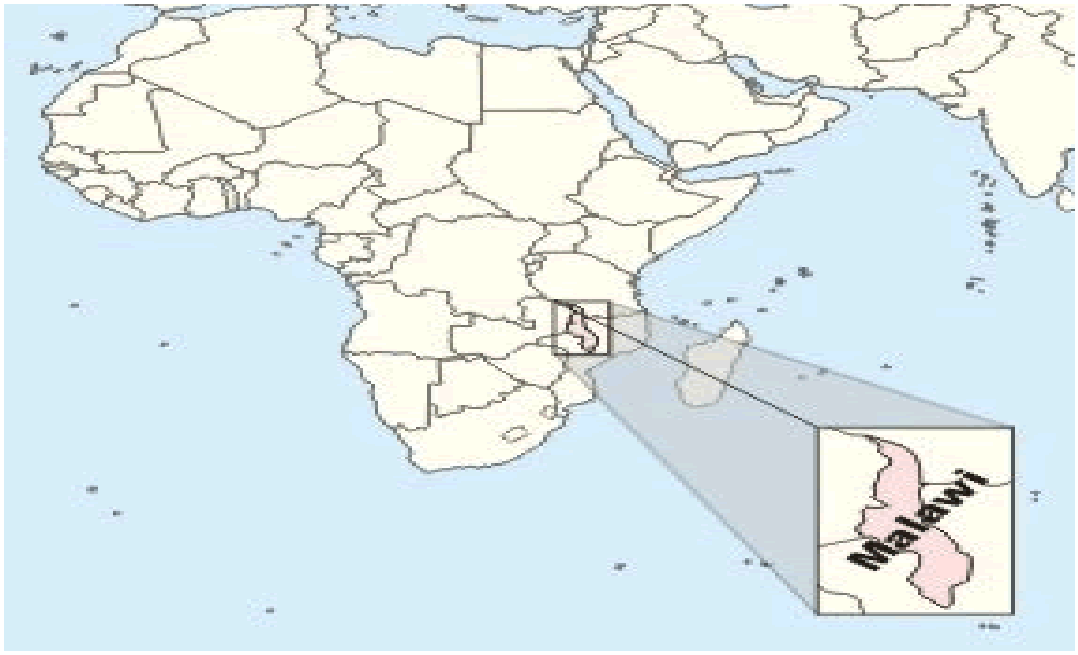

Figure 2.2 African map showing the location of Malawi (copyright from nationsonline, 2013).

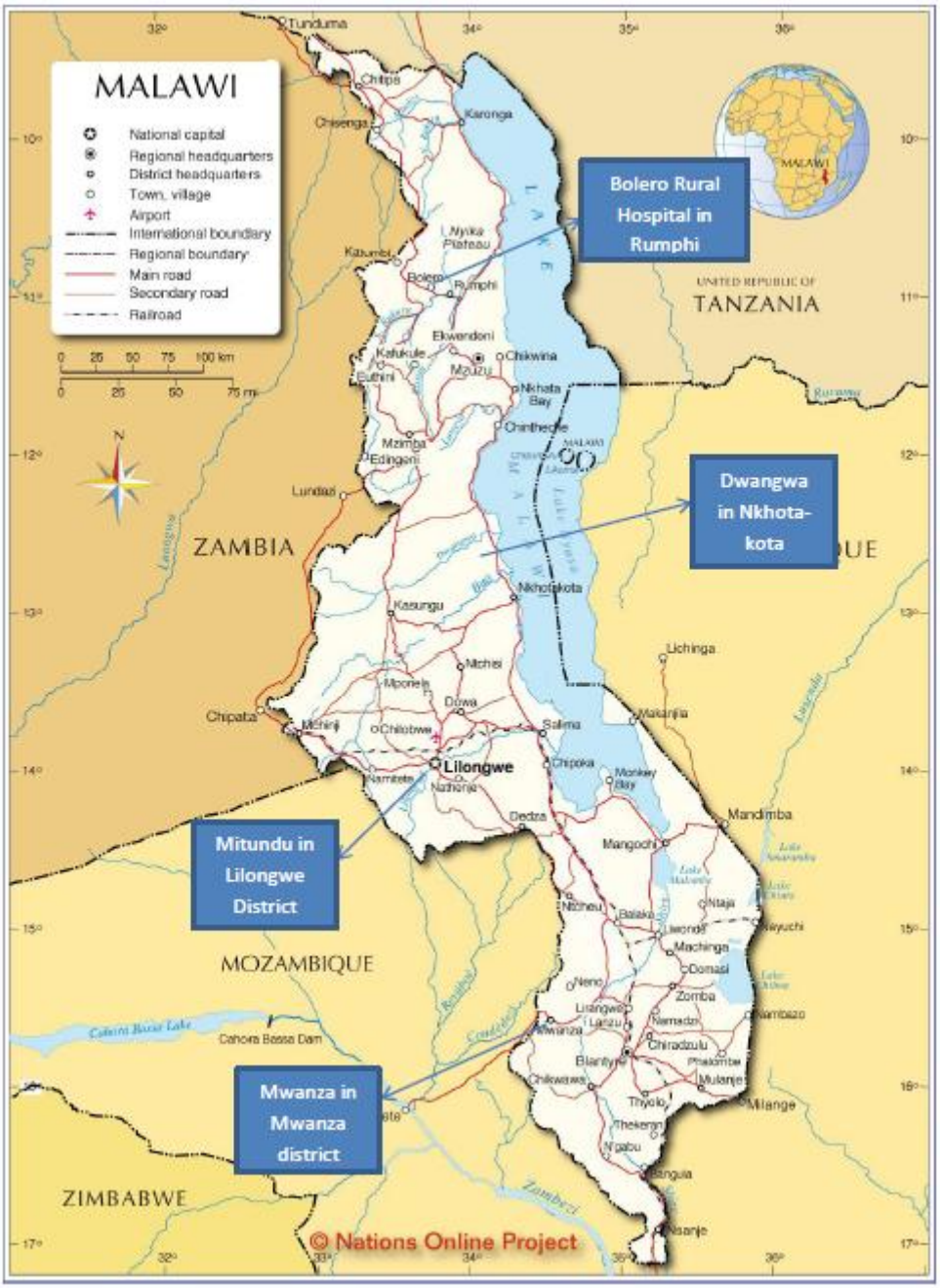

Figure 2.3: Map of Malawi showing districts with study sites (adapted from nationsonline, 2013)

2.3 *PLASMODIUM*

Malaria is a mosquito-borne infectious disease of humans and other animals caused by parasitic protozoans (a type of unicellular microorganism) of the genus *Plasmodium*. The parasite belongs to the domain Eukaryota, kingdom Chromalveolata, superphylum Alveolata, phylum Apicomplexa, class Aconoidasida, order haemosporidia, family Plasmodiidae and genus *Plasmodium* (Baun, 2006). Commonly, the disease is transmitted via a bite from an infected female anopheles mosquito, which introduces the organisms from its saliva into a person's circulatory system. In the blood, the protists travel to the liver to mature and reproduce. Malaria causes symptoms that typically include fever and headache, which in severe cases can progress to coma or death. As already elucidated, the disease is widespread in tropical and subtropical regions in a broad band around the equator, including much of Sub-Saharan Africa, Asia, and the Americas. Malaria is prevalent in tropical and subtropical regions because rainfall, warm temperatures, and stagnant waters provide habitats ideal for mosquito larvae.

Five species of *Plasmodium* can infect and be transmitted by humans. The vast majority of deaths are caused by *P. falciparum* and *P. vivax*, while *P. ovale* and *P. malariae* cause a generally milder form of malaria that is rarely fatal. The zoonotic species *P. knowles*, prevalent in Southeast Asia, causes malaria in macaques but can also cause severe infections in humans (WHO, 2010). Some forms of the *Plasmodium* parasite are shown in the blood smear in figure 2.4 below:

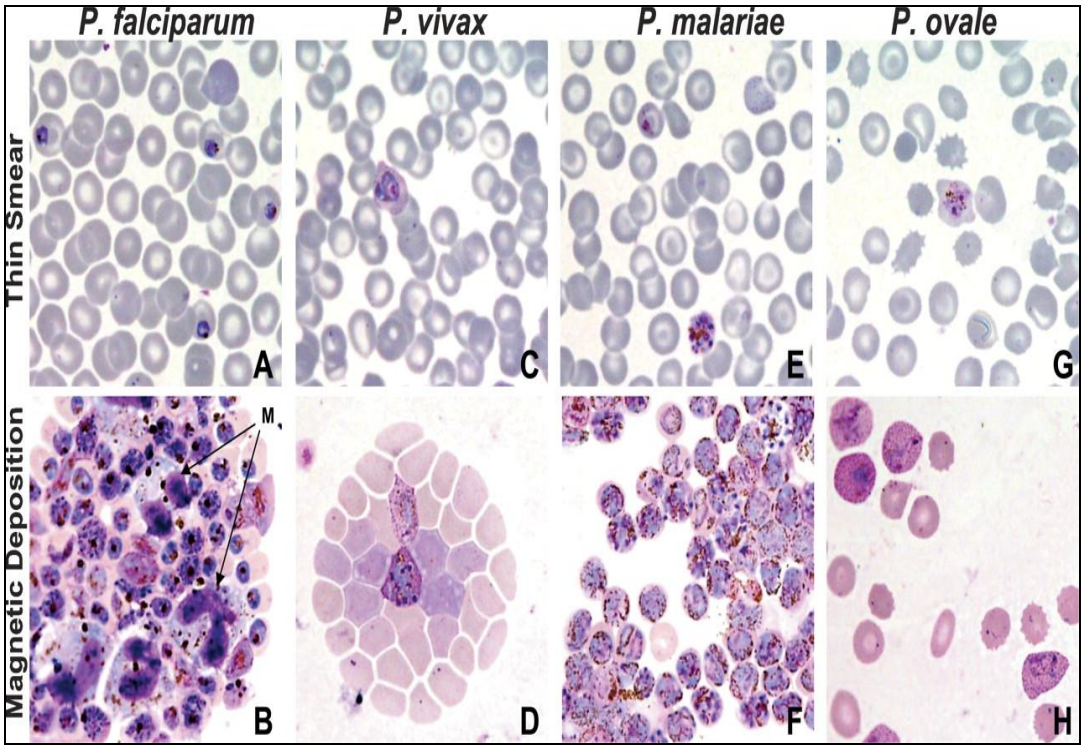

Figure 2.4: *Plasmodium* in blood (adapted from CDC, 2011)

2.4 *Plasmodium* Species

There are many species of *Plasmodium* infecting mammals. However, four different species of Plasmodium are known to cause malaria in humans. These include *Plasmodium falciparum, Plasmodium vivax, Plasmodium malaria* and *Plasmodium ovale*. In Malawi, the most common species available is *P. falciparum*, however, mixed infections with *P. malaria* and *P. ovale* has been reported, raising the possibility of multiple *Plasmodium species* being co-transmitted (Bruce *et al*., 2008).

2.4.1 *Plasmodium vivax*

The predominant malaria parasite in many parts of the world is *Plasmodium vivax*. This species is found almost everywhere malaria is endemic and is the only one which has a range extending into the temperate regions, but is most commonly found in southern Asia and central America. It causes the second most serious malaria. Black Africans tend to resist *P. vivax* infection, because the Duffy blood group antigen is rare in this population and this is the erythrocyte molecule to which the *P. vivax* merozoites bind (Markell & Voge, 1981; Knell, 1991).

2.4.2 *Plasmodium malariae*

Plasmodium malariae occurs primarily in those subtropical and temperate areas where other species of malaria are found and generally has a much lower incidence than *P. vivax* and *P. falciparum*. As described by Markell and Voge (1981), in the thick smear trophozoites of *P. malariae* do not assume the ameboid, comma or swallow forms as seen in other species, but due to their compact nature they usually appear as small dots of nuclear material as a rounded or slightly elongated mass of cytoplasm.

2.4.3 *Plasmodium ovale*

This species has been known since 1922 and seems widely distributed in tropical regions, especially the West African coast, South America and Asia. The morphological feature which led to the establishment of *P. ovale* as a separate species, an ovoid shape of many of the infected erythrocytes has been found to be variable. The parasite is not ameboid as *P. vivax* and the nuclei in all stages are larger than corresponding stages of that species (Markell & Voge, 1981).

2.4.4 *Plasmodium falciparum*

Severe malaria is usually caused by *P. falciparum* (often referred to as *falciparum* malaria). Symptoms of *falciparum* malaria arise 9–30 days after infection (Bartoloni & Zammarchi, 2012). *Plasmodium falciparum* causes malignant tertian malaria and is almost entirely confined to the tropics and subtropics causing serious infection compared to all other species. Morphologically, there are certain differences with other *Plasmodium* species. The gametocytes of *P. falciparum* are elongated or sausage shaped in contrast to the spherical or ovoid gametocytes of other species (Markell & Voge, 1981). The infection is acute and the parasites tend to stick to endothelial cells causing blockage and cerebral damage, often resulting in death. In severe *falciparum* malaria, the proportion of erythrocytes parasitized may rise to 30% or more, causing anemia (Cox, 1993).

2.5 MALARIA PATHOGENESIS

All of the pathology of malaria is due to parasites multiplying in erythrocytes. The primary attack of malaria begins with headache, fever, anorexia, malaise, and myalgia. The primary pathological effects of any malaria infection are the result of hemolysis of infected and uninfected erythrocytes, the liberation of the metabolites of the parasite and the immunological response of the host to this antigenic material. The various species differ in their ability to infect erythrocytes. Merozoites of *Plasmodium vivax* and *Plasmodium ovale* are able to invade only reticulocytes, whereas those of *Plasmodium malariae* are limited to the senescent cells nearing the end of their lifespan. However, *Plasmodium falciparum* is able to invade all ages of erythrocytes (Markell & Voge,

1981). Rupture of the infected erythrocytes brings on the malarial paroxysm. Lysis of numerous uninfected cells during paroxysm, plus enhanced phagocytosis of normal cells in addition to the cell remnants and other debris produced by schizogony, leads to both anemia and enlargement of the spleen and liver (Wiser, 2009). There may be nausea, vomiting, and diarrhoea. Such symptoms are not unusual for an infectious disease and it is for this reason that malaria is frequently called "The Great Imitator." Then, depending on the species, the paroxysms tend to assume a characteristic periodicity. In *P. vivax, P. ovale* and *P. falciparum* the periodicity is 48hr and for *P. malariae* the periodicity is 72 hours. The fever spike may reach up to 41^{O}C and corresponds to the rupture of the red cell as merozoites are released from the schizont-infected cell. Anaemia is the most immediate pathologic consequence of parasite multiplication and destruction of erythrocytes and there can also be suppression of red cell production in the bone marrow**.** Anaemia is most frequently observed in young children and pregnant women and can be detected in acute as well as repeated malaria infection (Mendez, Flemming & Alonso, 2006). Extra vascular and intravascular hemolysis of both infected and uninfected erythrocytes play a role: changes in surface proteins on infected erythrocytes lead to increased clearance of these cells (Mohan, Dubey, Ganguly & Mahajan, 1995), while non-infected erythrocytes are destroyed in the spleen during acute infection. This leads to hemolysis and depletion of iron stores (Dondorp *et al*., 1999). *Falciparum* infections are more severe and when untreated can result in a death rate of 25% in adults.

Bone marrow suppression also plays an important role in the pathogenesis of malarial anaemia. The normal response to hemolytic anaemia is enhanced secretion of

erythropoietin, leading to the stimulation of erythropoiesis. But this mechanism is defective in patients with malaria. During acute infection, abnormalities are seen in erythroid progenitors (Abdalla & Wickramasinghe, 1998). Abnormal production of erythrocytes (dyserythropoiesis) is observed in chronic infection (Abdalla, Weatherall, Wickramasinghe & Hughes, 1980).

Cerebral and other organ damage are mediated through interactions between infected erythrocytes and host receptors on the blood vessel wall, resulting in adherence and sequestration of infected erythrocytes in the post capillary venules, obstruction of the blood flow and subsequent tissue damage due to lack of oxygen (Medana & Turner, 2006). Characterized by coma and or seizures, there is occasional brain edema and elevated intracranial pressure. Postmortem samples from children who died from cerebral malaria showed activation of endothelial cells (with increased regulation of intercellular cell adhesion molecule-1[ICAM-1]) and macrophages (with elevated macrophage scavenger receptor and sialoadhesin) and disruption of endothelial intercellular junctions (zona occludens-1 [ZO-1], occudin and vinculin) in vessels containing sequestered parasitized erythrocytes. No leakage of plasma proteins (fibrinogen, C5b-9 and IgG) into the brain parenchyma was seen, suggesting that the brain-blood barrier remains intact. However, there were elevations in cerebral spinal fluid (CSF) albumin taken prior to death, which may indicate brain-blood barrier permeability (Ing, Segura, Thawani, Tam & Stevenson, 2006). Figure 2.5 below is the schematic representation of the complex pathophysiology of *P. falciparum*. Complications, the result of what has been called the pathology cascade includes renal insufficiency, renal failure, pulmonary edema, neurologic symptoms and severe hemolytic anemia. In the pregnant female *falciparum*

malaria may result in stillborn, lower than normal birth weight, or abortion. Non-immunes and children may develop cerebral malaria, a consequence of the mechanical blockage of microvessels in the brain, or organ infarcts, due to sequestration of infected red cells via protuberances called knobs.

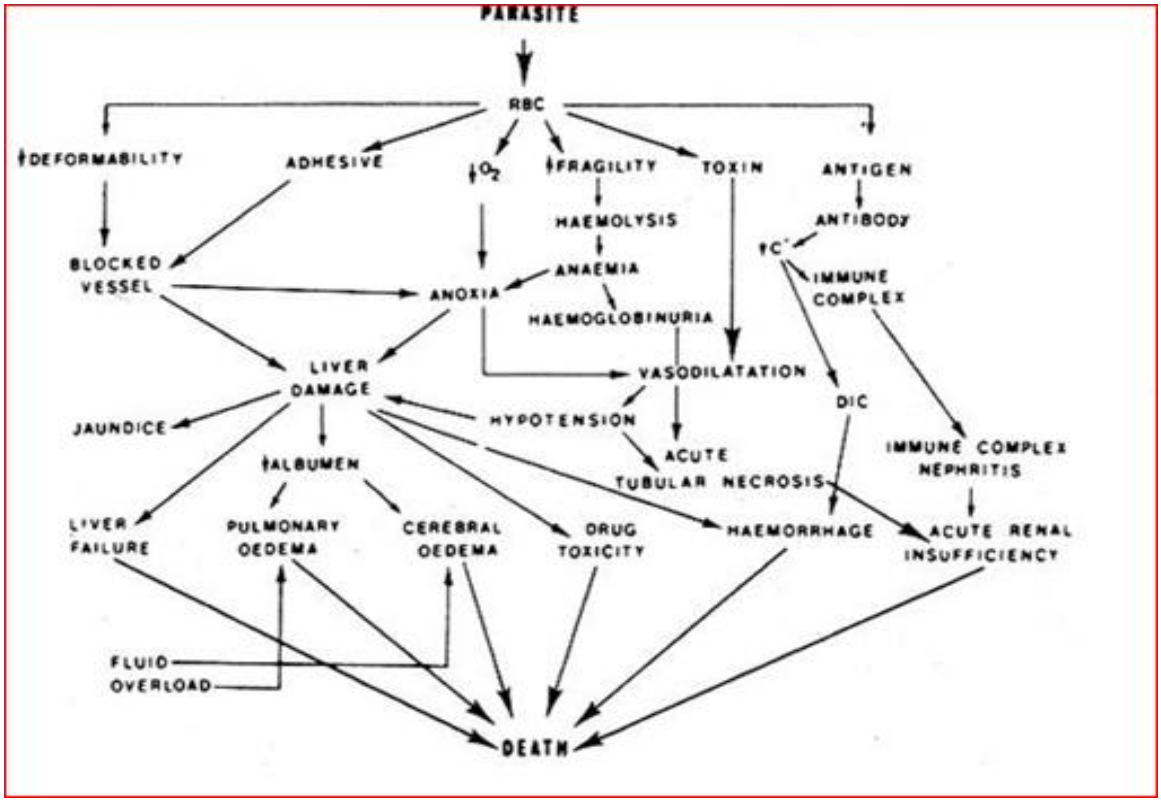

Figure 2.5 The complex pathophysiology of *P. falciparum* infection. (Adapted from Hall, A.P., Transactions of Royal Society Trop. Med. 71:367-379 (1977).

The pathogenesis of malaria therefore involves a cascading interaction between parasite and red cell membrane products, cytokines and endothelial receptors, leading to inflammation, activation of platelets, hemostasis, a procoagulant state, microcirculatory dysfunction and tissue hypoxia, resulting in various organ dysfunctions manifesting in severe malaria (Nolan *et al*., 2006).

2.6 CELLULAR AND MOLECULAR BIOLOGY OF *PLASMODIUM*

Malaria is a complicated syndrome determined by both parasitic and human factors. A unique feature of the malaria parasite is its intracellular lifestyle. Due to its intracellular location, the parasite has an intimate relationship with its host cell which can be described at the cellular and molecular levels. In particular, the parasite must enter the host cell, and once inside, it modifies the host cell (Waters & Janse, 2004).

2.6.1 Host Cell Invasion

Malaria parasites are characterized by a set of organelles found in some stages of the parasite's life cycle. These organelles, collectively known as apical organelles because of their localization at one end of the parasite, are involved in interactions between the parasite and host. In particular, the apical organelles have been implicated in the process of host cell invasion. Three distinct invasive forms have been identified: sporozoite, merozoite and ookinete (Wiser, 2009).

Merozoites rapidly (in approximately 20 seconds) and specifically enter erythrocytes. This specificity is manifested both for erythrocytes as the preferred host cell type and for a particular host species, thus implying receptor-ligand interactions**.** Erythrocyte invasion is a complicated process which is only partially understood at the molecular and cellular levels as explained by Gratzer & Druzewski (1993). Four distinct steps in the invasion process can be recognized:

1. Initial merozoite binding.

2. Reorientation and erythrocyte deformation.

3. Junction formation.

4. Parasite entry.

2.6.1.1 Merozoite Surface Proteins and Host-parasite Interactions

The initial interaction between the merozoite and the erythrocyte is probably random collision and presumably involves reversible interactions between proteins on the merozoite surface and the host erythrocyte. Several merozoite surface proteins have been described. The best characterized is merozoite surface protein-1 (msp-1). Circumstantial evidence implicating msp-1 in erythrocyte invasion includes its uniform distribution over the merozoite surface and the observation that antibodies against msp-1 inhibit invasion (Holder et al. 1994). In addition, msp-1 does bind to band 3 (Goel, Li, Liu, Chishti & Oh, 2003). However, the role for msp-1 in invasion has not been definitively demonstrated. Similarly, the circumsporozoite protein (CSP) probably plays a role in targeting sporozoites to hepatocytes by interacting with heparin sulfate proteoglycans (Sinnis & Sim, 1997).

Another interesting aspect of msp-1 is the proteolytic processing that is coincident with merozoite maturation and invasion (Cooper, 1993). A primary processing occurs at the time of merozoite maturation and results in the formation of several polypeptides held together in a non-covalent complex. A secondary processing occurs coincident with

merozoite invasion at a site near the C-terminus. The non-covalent complex of msp-1 polypeptide fragments is shed from the merozoite surface following proteolysis and only a small C-terminal fragment is carried into the erythrocyte. This loss of the msp-1 complex may correlate with the loss of the 'fuzzy' coat during merozoite invasion. The C-terminal fragment is attached to the merozoite surface by a GPI anchor and consists of two EGF-like modules. EGF-like modules are found in a variety of proteins and are usually implicated in protein-protein interactions. One possibility is that the secondary proteolytic processing functions to expose the EGF-like modules which strengthen the interactions between merozoite and erythrocyte. The importance of msp-1 and its processing are implied from the following observations:

- Vaccination with the EGF-like modules can protect against malaria.
- Inhibition of the proteolytic processing blocks merozoite invasion.

The exact role(s) which msp-1 and its processing play in the merozoite invasion process are not known (Wiser, 2009).

2.6.1.2 Re-orientation and Secretory Organelles

After binding to the erythrocyte, the parasite reorients itself so that the 'apical end' of the parasite is juxtaposed to the erythrocyte membrane. This merozoite reorientation also coincides with a transient erythrocyte deformation. Apical membrane antigen-1 (AMA-1) has been implicated in this reorientation. AMA-1 is a trans-membrane protein localized at the apical end of the merozoite and binds erythrocytes. Antibodies against AMA-1 do not interfere with the initial contact between merozoite and erythrocyte thus suggesting that AMA-1 is not involved in merozoite attachment. But antibodies against

AMA-1 prevent the reorientation of the merozoite and thereby block merozoite invasion (Mitchell, Thomas, Margos, Dluzeuski & Bannister, 2004).

Specialized secretory organelles are located at the apical end of the invasive stages of apicomplexan parasites. Three morphologically distinct apical organelles are detected by electron microscopy: micronemes, rhoptries, and dense granules (Carruthers & Sibley, 1999). Dense granules are not always included with the apical organelles and probably represent a heterogeneous population of secretory vesicles.

The contents of the apical organelles are expelled as the parasite invades, thus suggesting that these organelles play some role in invasion. Experiments indicate that the micronemes are expelled first and occur with initial contact between the parasite and host (Carruthers & Sibly, 1997). An increase in the cytoplasmic concentration of calcium is associated with microneme discharge (Carruthers & Sibley, 1999), as is typical of regulated secretion in other eukaryotes.

The rhoptries are discharged immediately after the micronemes and the release of their contents correlate with the formation of the parasitophorous vacuole. Dense granule contents are released after the parasite has completed its entry and therefore, are usually implicated in the modification of the host cell. For example, RESA is localized to dense granules in merozoites and is transported to the host erythrocyte membrane shortly after merozoite invasion (Culvenor, Day & Anders, 1991). However, subtilisin-like proteases, which are implicated in the secondary proteolytic processing of msp-1, have also been

localized to *Plasmodium* dense granules. If msp-1 processing is catalyzed by these proteases, then at least some dense granules must be discharged at the time of invasion (Blackman *et al.,* 1998; Barale *et al.,* 1999).

2.6.1.3 Specific Interactions and Junction Formation

Following merozoite reorientation and microneme discharge, according to Wiser (2009) a junction forms between the parasite and host cell. Presumably, microneme proteins are important for junction formation. Proteins localized to the micronemes include:

- EBA-175, a 175 kDa 'erythrocyte binding antigen' from *P. falciparum.*
- DBP, Duffy-binding protein from *P. vivax.*
- SSP2, *Plasmodium* sporozoite surface protein-2. Also known as TRAP (thrombospondin-related adhesive protein).
- CTRP, circumsporozoite- and TRAP-related protein of *Plasmodium* found in the ookinete stage. Of particular note are EBA-175 and DBP which recognize sialic acid residues of the glycophorins and the Duffy antigen, respectively.

These parasite proteins are probably involved in receptor-ligand interactions with proteins exposed on the erythrocyte surface. Disruption of the EBA-175 gene results in the parasite switching from a sialic acid-dependent pathway to a sialic acid-independent pathway, indicating that there is some redundancy in regards to the receptor-ligand interactions (Reed *et al.,* 2000).

Comparison of sequences of EBA-175 and DBP reveal conserved structural features. These include trans-membrane domains and receptor-binding domains as shown in figure 2.6 below. The receptor-binding activity has been mapped to a domain in which the cysteine and aromatic amino acid residues are conserved between species (blue area in figure 2.6 below). This putative binding domain is duplicated in EBA-175. The topography of the trans-membrane domain is consistent with the parasite ligands being integral membrane proteins with the receptor-binding domain exposed on the merozoite surface following microneme discharge (Adams *et al.*, 1992).

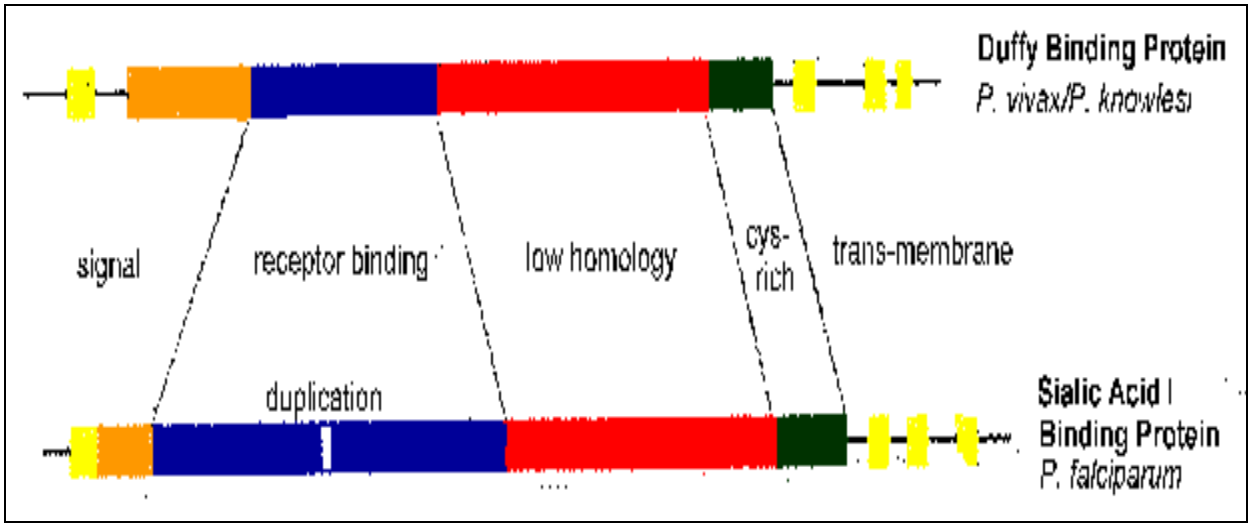

Figure 2.6: Receptor binding activity (Wiser, 2009)

The other microneme proteins in the 'TRAP' family have also been implicated in locomotion and/ or cell invasion. All of these proteins have domains that are presumably involved in cell-cell adhesion, as well as N-terminal signal sequences and transmembrane domains at their C-termini (Tomley & Soldati, 2001).

In summary:

- an electron-dense junction (arrow in figure 2.7 below) forms between the apical end of the merozoite and host erythrocyte membrane immediately after reorientation;
- tight junction formation and microneme release occur at about the same time; and
- proteins localized at the micronemes bind to receptors on the erythrocyte surface.

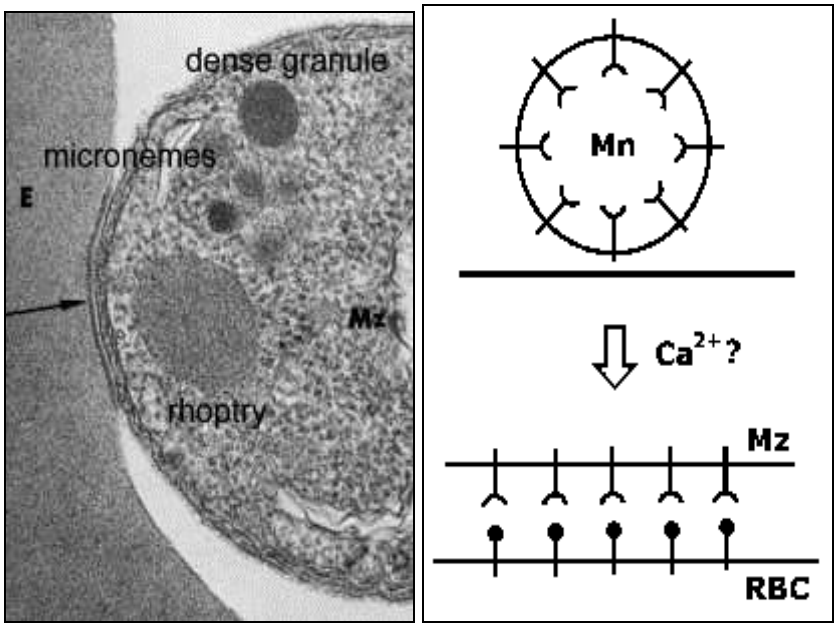

Figure 2.7 Micrograph showing binding activity

These observations suggest that the junction represents a strong connection between the erythrocyte and the merozoite which is mediated by receptor-ligand interactions. Junction formation may be initiated by microneme discharge which exposes the receptor-binding domains of parasite ligands. This mechanism for initiating a tight

host-parasite interaction is probably similar in other invasive stages of apicomplexan parasites (Adams *et al.,* 1992; Tomley & Soldati, 2001).

2.6.1.4 Parasite Entry

Plasmodium actively invades host cells and entry is not due to uptake or phagocytosis by the host cell. This is particularly evident in the case of the erythrocyte which lacks phagocytic capability. Furthermore, the erythrocyte membrane has a 2-dimensional submembrane cytoskeleton which precludes endocytosis. Therefore, the impetus for the formation of the parasitophorous vacuole must come from the parasite (Wiser, 2009).

Erythrocyte membrane proteins are redistributed at the time of junction formation so that the contact area is free of erythrocyte membrane proteins. A merozoite serine protease which cleaves erythrocyte band 3 has been described (Braun-Breton & da Silva, 1993). Due to the pivotal role band 3 plays in the homeostatis of the submembrane skeleton, its degradation could result in a localized disruption of the cytoskeleton. An incipient parasitophorous vacuolar membrane (PVM) forms in the junction area. This membrane invagination is likely derived from both the host membrane and parasite components and expands as the parasite enters the erythrocyte. Connections between the rhoptries and nascent PVM are sometimes observed. In addition, the contents of the rhoptries are often lamellar (i.e. multi-layered) membranes and some rhoptry proteins are

localized to the PVM following invasion, suggesting that the rhoptries function in PVM formation (Sam-Yellowe, 1996).

Ookinetes lack rhoptries and do not form a parasitophorous vacuole within the mosquito mid-gut epithelial cells. The ookinetes rapidly pass through the epithelial cells and cause extensive damage as they head toward the basal lamina (Han, Thompson, Kafatos & Barillas-Mury, 2000; Ziegler & Dvorak, 2000). Similarly, sporozoites can enter and exit hepatocytes without undergoing exoerythrocytic schizogony. Those parasites which do not undergo schizogony are free in the host cytoplasm, whereas those undergoing schizogony are enclosed within a PVM (Mota *et al.,* 2001). These observations suggest that the PVM is needed for intracellular development and is not necessary for the process of host cell invasion. As the incipient parasitophorous vacuole is being formed, the junction between the parasite and host becomes ring-like and the parasite appears to move through this annulus as it enters the expanding parasitophorous vacuole (Wiser, 2009).

Apicomplexan parasites actively invade host cells and entry is not due to uptake or phagocytosis by the host cell. In addition, the zoites are often motile forms that crawl along the substratum by a type of motility referred to as ‘gliding motility’. Gliding motility, like invasion, also involves the release of micronemal adhesins, attachment to the substratum, and a capping of the adhesins at the posterior end of the zoite. One difference between gliding motility and invasion is that the micronemes must be continuously released as the organism is moving. Thus, gliding motility does not involve

this relatively small moving junction, but a continuous formation of new junctions between the zoite and the substratum. In addition, the adhesins are cleaved from the surface of the zoite as the adhesins reach the posterior of the zoite and a trail of the adhesive molecules are left behind the moving zoite on the substratum. However, the mechanism of motility and invasion are quite similar and thus, during invasion the parasite literally crawls into the host cell through the moving junction. In addition, some apicomplexans use this type of motility to escape from cells and can traverse biological barriers by entering and exiting cells (Han, Thomson, Kafatos & Barillas-Mury, 2000; Ziegler & Dvorak, 2000; Wiser, 2009).

Cytochalasins inhibit merozoite entry, but not attachment. This inhibition suggests that the force required for parasite invasion is based upon actin-myosin cytoskeletal elements. The ability of myosin to generate force is well characterized (e.g. muscle contraction). A myosin unique to the Apicomplexa has been identified and localized to the inner membrane complex. This myosin is part of a motor complex which is linked to the adhesins (Kappe, Buscaglia, Bergman, Coppens & Nussenzweig, 2004).

Members of the TRAP family and other adhesins have a conserved cytoplasmic domain. This cytoplasmic domain is linked to short actin filaments via aldolase. The actin filaments and myosin are oriented in the space between the inner membrane complex and plasma membrane so that the myosin propels the actin filaments toward the posterior of the zoite. The myosin is anchored into the inner membrane complex and does not move. Therefore, the trans-membrane adhesins are pulled through the fluid lipid bi-layer of the

plasma membrane due to their association with the actin filaments. Thus the complex of adhesins and actin filaments are transported towards the posterior of the cell. Since the adhesins are either complexed with receptors on the host cell or bound to the substratum, the net result is a forward motion of the zoite. When the adhesins reach the posterior end of the parasite they are proteolytically cleaved and shed from the zoite surface (Iyer, Gruner, Renia, Snounou & Preiser, 2007; Baum, Gilberger, Frischknecht & Meissner, 2008).

2.6.1.5 Host Erythrocyte Modification

Once inside of the erythrocyte, the parasite undergoes a trophic phase followed by replicative phase. During this intraerythrocytic period, the parasite modifies the host to make it a more suitable habitat. For example, the erythrocyte membrane becomes more permeable to small molecular weight metabolites, presumably reflecting the needs of an actively growing parasite.

According to Wiser (2009), another modification of the host cell concerns the cytoadherence of *P. falciparum*-infected erythrocytes to endothelial cells and the resulting sequestration of the mature parasites in capillaries and post-capillary venules. This sequestration likely leads to microcirculatory alterations and metabolic dysfunctions which could be responsible for many of the manifestations of severe *falciparum* malaria as described in pathogenesis. The cytoadherence to endothelial cells confers at least two advantages for the parasite:

1. A microaerophilic environment which is better suited for parasite metabolism.
2. Avoidance of the spleen and subsequent destruction.

2.6.1.6 Knobs and Cytoadherence

A major structural alteration of the host erythrocyte is electron-dense protrusions, or 'knobs', on the erythrocyte membrane of *P. falciparum*-infected cells. The knobs are induced by the parasite and several parasite proteins are associated with the knobs (Deitsch & Wellems, 1996). Two proteins which might participate in knob formation or affect the host erythrocyte sub-membrane cytoskeleton and indirectly induce knob formation, are the knob-associated histidine rich protein (KAHRP) and erythrocyte membrane protein-2 (*Pf*EMP2), also called mature parasite-infected erythrocyte surface antigen (MESA). Neither KAHRP nor *Pf*EMP2 are exposed on the outer surface of the erythrocyte, but are localized to the cytoplasmic face of the host membrane as shown in figure 2.8 below. Their exact roles in knob formation are not known, but may involve reorganizing the sub-membrane exoskeleton.

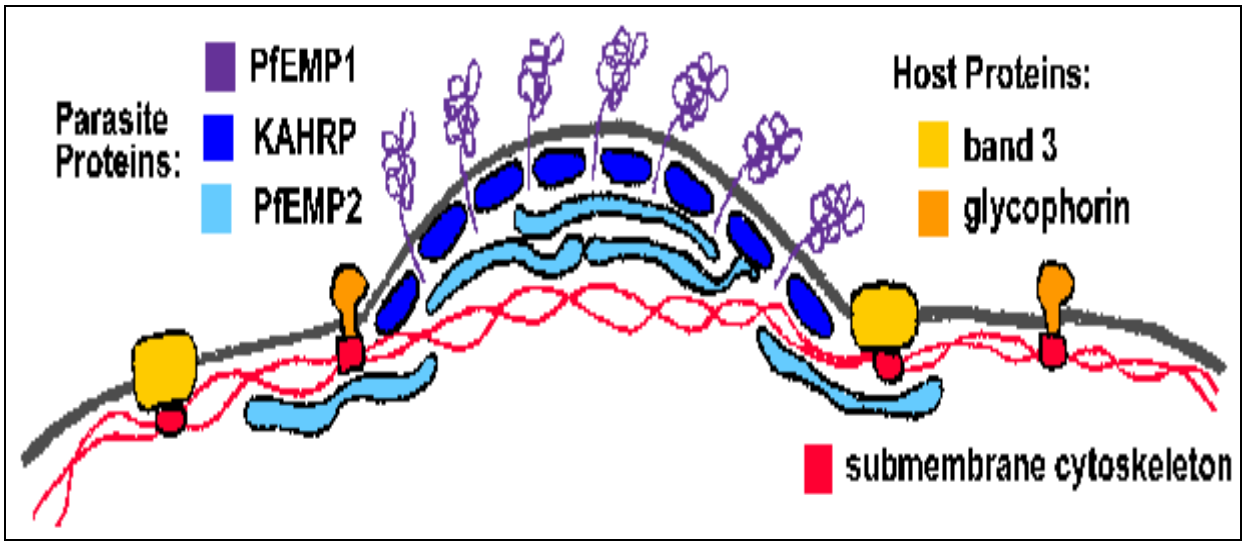

Figure 2.8 Knob formation (Wiser, 2009)

The knobs are believed to play a role in the sequestration of infected erythrocytes since they are points of contact between the infected erythrocyte and vascular endothelial cells, and parasite species which express knobs exhibit the highest levels of sequestration. In addition, disruption of the KAHRP results in loss of knobs and the ability to cytoadhere under flow conditions (Crabb et al. 1997). A polymorphic protein, called *Pf*EMP1 (figure 2.9), has also been localized to the knobs and is exposed on the host erythrocyte surface. The translocation of *Pf*EMP1 to the erythrocyte surface depends in part on another erythrocyte membrane associated protein called *Pf*EMP3 (Waterkeyn *et al.*, 2000). *Pf*EMP1 probably functions as a ligand which binds to receptors on host endothelial cells. Other proposed cytoadherence ligands include a modified band-3, called pfalhesin (Sherman, Crandall, Guthrie & Land, 1995), sequestrin, rifins and clag9 (Craig & Scherf, 2001).

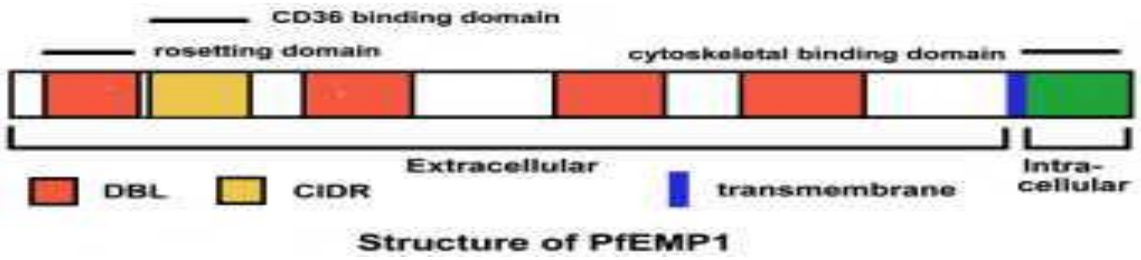

Figure 2.9 Structure of PfEMP1 (Smith, Gamain, Baruch & Kyes, 2001)

*Pf*EMP1 is a member of the *var* gene family. The 40-50 *var* genes exhibit a high degree of variability, but have a similar overall structure as shown in figure 2.9 above (Smith *et al.* 2001). *Pf*EMP1 has a large extracellular N-terminal domain, a trans-membrane region and a C-terminal intracellular domain. The C-terminal region is conserved between members of the *var* family and is believed to anchor *Pf*EMP1 to the erythrocyte sub-membrane cytoskeleton. In particular, this acidic C-terminal domain may interact with the basic KAHRP of the knob as well as spectrin and actin (Waller, Cooke, Nunomura, Mohandas & Coppel, 1999; Oh *et al*. 2000).

The extracellular domain is characterized by 1-5 copies of Duffy-binding like (DBL) domains. These DBL domains are similar to the receptor-binding region of the ligands involved in merozoite invasion discussed above. The DBL domains exhibit a conserved spacing of cysteine and hydrophobic residues, but otherwise show little homology. Phylogenetic analysis indicates that there are five distinct classes (designated as a, b, g, d, and e) of DBL domains. The first DBL is always the same type (designated a) and this is followed by a cysteine-rich inter-domain region (CIDR). A variable number

of DBL in various orders makes up the rest of the extracellular domain of *Pf*EMP-1 (Smith, Gamain,Baruch & Kyes, 2001).

2.6.1.7 Endothelial Cell Receptors

Several possible endothelial receptors (table 2.1 below) have been identified by testing the ability of infected erythrocytes to bind in static adherence assays (Beeson & Brown, 2002).

Table 2.1: Endothelial Receptors (adapted from Beeson & Brown, 2002)

Possible receptors identified by in vitro binding assays
CD36
Ig Superfamily
ICAM-1
VCAM-1
PECAM-1
Chondroitin Surfate A
Heparan Surfate
Hyaluronic Acid
E-selectin
Thrombospondin
Rosetting Ligands • CR1 • Blood group A Ag • Glycosaminoglycan

One of the best characterized among these is CD36, an 88 kDa integral membrane protein found on monocytes, platelets and endothelial cells. Infected erythrocytes from most parasite isolates bind to CD36 and the binding domain has been mapped to the CIDR of *Pf*EMP1 as shown in table 2.2 below. However, CD36 has not been detected on endothelial cells of the cerebral blood vessels and parasites from clinical isolates tend to adhere to both CD36 and ICAM1. ICAM1 is a member of the immunoglobulin superfamily and functions in cell-cell adhesin. In addition, sequestration of infected erythrocytes and ICAM1 expression has been co-localized in the brain (Turner *et al*., 1994; Craig & Scheif, 2001).

Table 2.2: Binding Receptors (adapted from Craig & Scheif, 2001)

Binding Phenotypes	
Binding	**Phenotypes**
CIDR	CD36
DBLa	Rosetting
DBLb	ICAM-1
DBLg	CSA

Chondroitin sulfate A (CSA) has been implicated in the cytoadherence within the placenta and may contribute to the adverse effects of *P. falciparum* during pregnancy.

The role of some of the other potential receptors is not clear. For example, adherence to thrombospondin exhibits a low affinity and cannot support binding under flow conditions. Binding to VCAM1, PECAM1 and E-selectin appears to be rare and questions about their constitutive expression on endothelial cells have been raised. However, cytoadherence could involve multiple receptor/ligand interactions (Reeder *et al.*, 1999).

Rosetting is another adhesive phenomenon exhibited by *P. falciparum*-infected erythrocytes. Infected erythrocytes from some parasite isolates will bind multiple uninfected erythrocytes and *Pf*EMP1 appears to have a role in at least some rosetting. Possible receptors include complement receptor-1 (CR1), blood group A antigen, or glycosaminoglycan moieties on an unidentified proteoglycan (Rowe, Moulds, Newbold & Miller, 1997; Reeder *et al.*, 1999).

The different types of DBL domains and CIDR bind to different endothelial cell receptors. For example, DBLa, which comprises the first domain, binds to many of the receptors associated with rosetting. The binding of the CIDR to CD36 may account for the abundance of this particular binding phenotype among parasite isolates (Craig & Scherf, 2001: Smith, Gamain,Baruch & Kyes, 2001).

2.6.1.8 Antigenic variation

The encoding of the cytoadherence ligand by a highly polymorphic gene family presents a paradox, in that receptor/ligand interactions are generally considered highly

specific. Selection for different cytoadherent phenotypes result in a concomitant change in the surface antigenic type (Biggs *et al*., 1992). Similarly, examination of clonal parasite lines revealed that changes in the surface antigenic type correlated with differences in binding to CD36 and ICAM1. For example, the parental line (A4) adhered equally well to CD36 and ICAM1, whereas one of the A4-derived clones (C28) exhibited a marked preference for CD36. Binding to ICAM1 was then reselected by panning the infected erythrocytes on ICAM1. All three parasite clones (A4, C28, C28-I) exhibited distinct antigenic types as demonstrated by agglutination with hyper-immune sera (Roberts *et al.,* 1992).

The expression of a particular *Pf*EMP1 will result in a parasite with a distinct cytoadherent phenotype and this may also affect pathogenesis and disease outcome. For example, binding to ICAM-1 is usually implicated in cerebral pathology. Therefore, parasites expressing a *Pf*EMP1 which binds to ICAM1 may be more likely to cause cerebral malaria. In fact, higher levels of transcription of particular *var* genes are found in cases of severe malaria as compared to uncomplicated malaria (Rottmann *et al.,* 2006).

Similarly, a higher proportion of isolates which bind to CSA are obtained from the placenta as compared to the peripheral circulation of either pregnant women or children (Beeson *et al.,* 1999). Furthermore, placental malaria is frequently associated with higher levels of transcription of a particular *var* gene which binds CSA (Duffy *et al.,*

2006). This phenomenon is not restricted to the placenta in which there is a dominant expression of particular *var* genes in the various tissues. This tissue specific expression of particular *var* genes implies that different tissues are selecting different parasite populations based on the particular *Pf*EMP1 being expressed on the surface of the infected erythrocyte (Montgomery *et al.,* 2007).

Although sequestration offers many advantages to the parasite, the expression of antigens on the surface of the infected erythrocyte provides a target for the host immune system. The parasite counters the host immune response by expressing antigenically distinct *Pf*EMP1 molecules on the erythrocyte surface. This allows the parasite to avoid clearance by the host immune system, but yet maintain the cytoadherent phenotype. This antigenic switching may occur as frequently as 2% per generation in the absence of immune pressure (Roberts *et al.,* 1992). The molecular mechanism of antigenic switching is not known. Only a single *var* gene is expressed at a time (i.e., allelic exclusion). The non-expressed genes are kept silent by proteins which bind to the promoter region. A gene can become activated by repositioning to a particular location in the nucleus and is associated with chromatin modification. This expression spot can only accommodate a single active gene promoter. Thus the *var* promoter is sufficient for both the silencing and the mono-allelic transcription of a *Pf*EMP1 allele (Voss *et al.,* 2006).

2.7 GENETIC DIVERSITY OF *P. FALCIPARUM*

Genetic diversity is one of the prominent features of *P. falciparum* infections. Natural infections often contain mixtures of several genotypes and the human and

mosquito hosts are exposed to heterogeneous parasite populations. *Plasmodium falciparum* population diversity is commonly assessed by polymerase chain reaction (PCR)-based typing of the highly polymorphic parasite merozoite surface proteins 1 and 2 (msp-1 and msp-2) (Zakeri *et al.*, 2005). The extent of genetic diversity in an area, measured as the total number of allelic variants and the mean number of concurrent genotypes per infection, is related to the level of malaria endemicity (Babiker *et al*., 1997; Zwetyenga *et al*., 1998).

It has been observed that attempts to generate protective blood-stage immunity to *P. falciparum* by vaccination in humans have met with limited success to date (Goodman & Draper, 2010). A key feature in human malaria epidemiology is parasite diversity, in terms of species or within species populations (different genotypes) (Arez *et al*., 2003). Thus, as observed by PATH (2011), the development of malaria vaccines is complex for several reasons. Firstly, the size and genetic complexity of the parasite mean that each infection presents thousands of antigens to the human immune system. Understanding which of these can be a useful target for vaccine development has been complicated, and to date at least 40 promising antigens have been identified. Secondly, the parasite changes through several life stages even while in the human host, presenting a different subset of molecules for the immune system to combat at each stage. Thirdly, the parasite has evolved a series of strategies that allow it to confuse, hide, and misdirect the human immune system (Todryk & Hill, 2007). The malaria parasite develops through several phases in the human body and the mosquito vector (Annex 1) and employs mechanisms to evade the immune system. Immunity to severe disease is acquired relatively quickly

but immunity to milder disease and asymptomatic infection requires much greater exposure. This means that designing a vaccine for the malaria parasites is a greater technical challenge than for some of the simpler viruses, such as smallpox and measles for which a single natural infection provides lifelong immunity (DFID, 2010). To this effect, vaccine design or formulation, the selection of appropriate vaccination strategy and consideration of the immunological profile of the target population at initial steps of vaccine design are critical to achieve optimal efficacy (Todryk & Hill, 2007).

Presently, vaccines for all phases of parasite development are under investigation. Vaccines that target the pre-erythrocytic stages usually aim to prevent infection. Blood-stage vaccines aim to reduce or eliminate the parasite once a person has been infected and thus prevent disease. Potential vaccines that prevent transmission, that target specific conditions such as malaria in pregnancy, and which target specific *Plasmodium* species, are also in development (DFID, 2010). This approach of targeting single phases of the parasite development in vaccine development is what is causing the trials for malaria not to work as the parasite change through the stages. A multi-component, multi-stage vaccine that may combine pre-erythrocytic, blood stage and/or transmission blocking components may work to provide effective immunity. Most licensed vaccines generate antibodies against extracellular pathogens, which can be accurately measured and often correlate with protection. Such vaccines comprise whole inactivated microorganisms or, increasingly, parts (or subunits) of microorganisms with appropriate adjuvant (Todryk & Hill, 2007). Deciphering the biological bases for the

efficacy of existing vaccines may give us some clues regarding optimal development strategies (Lambert, Liu & Siegrist, 2005).

The epidemiology of malaria shows considerable geographical variation. Difficulties in differentiating strains and to some extent species in field samples had previously hindered the study of relationships between *Plasmodium* populations and epidemiological characteristics (Bousema & Drakeley, 2011). The recent use of PCR has helped to overcome these difficulties. Polymorphic genes within a parasite species have been used as genetic markers, thus providing a means to assess the composition of the parasite population. Results from investigations using PCR assays have demonstrated that *P. falciparum* populations are highly complex (Snounou *et.al*., 1999).

It has been demonstrated that the genetic structure of the *P. falciparum* populations can be determined by PCR amplification of polymorphic regions of 4 antigen genes: merozoite surface protein 1(msp-1), merozoite surface protein 2 (msp-2), the glutamate-rich protein (glurp) and the 24-Single Nucleotide Polymorphism bar coding assay (24SNP). In a study done in Pakistan and Thailand (Snounou *et al*., 1999), the overall population structure of the parasites associated with patent malaria infections was observed to remain relatively stable over time although there was a high degree of diversity characterizing these isolates.

2.8 Merozoite Surface Protein 1

The *P. falciparum* malaria parasite is the causative agent of malaria in the tropics. Merozoites, one of the extracellular developmental stages of this parasite, expose at their surface the merozoite surface protein-1 complex (msp-1), which results from the proteolytic processing of a 190–200 kDa precursor (Pan *et al*., 1998). Msp-1 is one of the promising vaccine candidates and people naturally exposed to *P. falciparum* develop antibodies against msp-1(Irawati, 2011).

Merozoite surface protein-1 is highly immunogenic in humans and numerous studies suggest that this protein is an effective target for a protective immune response (Shi *et al.,* 2007; Rosa *et al.*, 2006; Pan *et al.*, 1999). Although its function is unknown, there are indications that it may play a role during invasion of erythrocytes by merozoites. The parasite-derived msp-1 gene, is ~5000 base pair (bp) long, (Pan *et al*, 1998) and in a number of independent studies as reported by Irawati, (2011) and Kiwanuka (2009) sequence comparisons showed that the entire msp-1 gene could be divided into 17 blocks that are variable, conserved, or semi conserved. In five blocks (1, 3,5,12, and 17), the sequences are conserved. In seven blocks (2, 4, 6,8,10, 14, and 16), the sequence show extensive diversity, while in the remaining blocks (7, 9, 11, 13, and 15), the sequences are semi conserved. Variations in sequences of variable regions are dimorphic (K1/Wellcome or MAD20) in nature with the exception of the trimorphic-encoding region in block-2, which has a third version (RO33) found in natural isolates. Naturally acquired antibodies react more frequently against variable rather than conserved msp-1 blocks and are specific for one of the major version of variable blocks.

2.9 Merozoite Surface Protein 2

Merozoite Surface Protein-2 (msp-2) also known as merozoite surface antigen 2 (msa-2), is a glycoprotein expressed on the surface of merozoites, the stage of parasite that invade the RBCs. Merozoite Surface Protein-2 of *P. falciparum* is another leading candidate antigen for the development of a subunit malaria vaccine. The msp-2 gene, codes for a merozoite surface polymorphic glycoprotein that has been widely studied as one of the major vaccine candidates. The sequencing of DNA has shown that a single copy of msp-2 gene has conserved N- and C- terminal domains (blocks 1 and 5), two non-repetitive variable regions (blocks 2 and 4), and a polymorphic central region (block 3) containing variable numbers of tandem repeats, which also vary in sequence and length (fig. 2.10 below) (Kiwanuka, 2009; Ferreira *et al*, 2007). Merozoite Surface Protein-2 has a central repetitive region flanked by unique variable domains and conserved N- and C-terminal domains with two very distinct allelic classes, though these two classes are thought to have diverged much more recently than the msp-1 classes. As with msp-1, the 5' and 3' regions show much lower levels of diversity (Amodua *et al*., 2008). The msp-2 alleles generally fall into two allelic types, FC27 and 3D7, which differ considerably in the dimorphic structure of the variable central region, block 3 (Kang *et al.,* 2010).

Figure 2.10 Schematic representation of the msp-2 of *P. falciparum*. The conserved domains (blocks 1 and 5) are shown as open boxes, the non-repetitive dimorphic domains (blocks 2 and 4) as black boxes, and the central polymorphic repeats (block 3) as a stripped box (taken from Ferreira *et al*, 2007)

2.10 Population Structure of *P. falciparum*

Populations exposed to *Plasmodium* infection develop genetic mechanisms of protection against severe disease. The clinical manifestation of malaria results primarily from the lysis of infected erythrocytes and subsequent immune and inflammatory responses (de Mendonça *et al.*, 2012). The genetic diversity of *P. falciparum* within a single host can reach up to about 10 genotypes at a given time but the mean number in a population is about 2-4 in most studies (Bereczky, 2005). Further, Bereczky (2005) extrapolates that the number of genotypes within an infection is a result of interaction between several factors which may include intensity of malaria transmission in the area, individual's exposure to infective mosquitoes, natural and acquired immunity as well as chemoprophylaxis or recent treatment.

The genetic diversity and population structure of *P. falciparum* has been extensively studied in various parts of the world. Microsatellite markers revealed a

spectrum of population structures in *P. falciparum* in that strong linkage disequilibrium (LD), low genetic diversity, and high levels of geographical variation are observed in regions of low transmission, while random association among loci, high genetic diversity, and minimal geographical differentiation are observed in regions of Africa and Papua New Guinea, where transmission is intense (Anderson *et al*., 2009). In the Philippines, the levels of genetic diversity and the effective population sizes are similar to those reported in the mainland of Southeast Asia or South America in that in the low transmission area, there was a low level of genetic diversity and a strong LD while in the high malaria transmission areas, there was a high level of genetic diversity and a weak LD when the single-clone haplotype (SCH) was used in the multilocus LD analysis. On the other hand, when the unique haplotypes were used in multilocus LD analysis, no significant LD was observed in the low transmission and high transmission populations. Both populations were therefore estimated to have an epidemic structure (Iwagami *et al*., 2009). In Pakistan, South Asia, a similar pattern has been reported despite potential substantial regional variations and increases in *P. falciparum* incidence thereby representing a low transmission setting as previously believed (Ghanchi *et al*., 2010).

2.11 Vaccine Efforts other than msp-1 and msp-2

By their very nature, parasites are more complex organisms than bacteria and viruses, with more complicated structures and life cycles. The distinct developmental stages involved in the life cycle present numerous opportunities for targeting antigens, thus potentially eliciting an immune response. Thus vaccines that target other

developmental stages of the parasite other than the blood stage have been developed to elicit immune response. The pre-erythrocytic vaccines are intended to stop the parasite lifecycle from progressing from the sporozoite or liver stage. They aim to generate antibody responses against sporozoites, and thus prevent the infection of hepatocytes or T cells against the antigens expressed by infected hepatocytes, which prevent merozoite release by killing infected hepatocytes or interfering with parasite development (Todryk & Hill, 2007). The vaccine that is currently most advanced in development is the RTS,S vaccine in an AS02 adjuvant developed by GlaxoSmithKline Biologicals. This vaccine comprises portions of the circumsporozoite protein (CSP), containing antibody and T-cell epitopes. This vaccine is believed to act mainly through anti-sporozoite antibodies, but perhaps also through T cells that target infected hepatocytes (Bejon *et al*., 2013). Other vaccines include FP9/MVA, ME-Trap simian adenovirus/MVA, LSA-1-AS02 and AdHu35 all of which target the hepatocytes (Todryk & Hill, 2007).

Some more candidate vaccine efforts not based on msp-1 and msp-2 are the transmission blocking vaccine. These vaccines aim to induce antibodies against antigens that are expressed by the sexual stage of the parasite, for example gametocytes, and thus stop their subsequent combination in the mosquito gut and development into infective sporozoites. The main antigens assessed as vaccine candidates are the surface antigens Pfs25, Pfs28, Pfs48/45 and Pfs230. The intention is to protect communities from infection, rather than the individual, but active clinical development of this approach is still awaited (Todryk & Hill, 2007).

2.12 Vaccine Efforts utilizing msp-1 and msp-2

A number of strategies have been adopted in the pursuit of making a successful malaria vaccine including the use of blood stage antigens which would primarily target the merozoite and prevent its replication through the asexual blood cycle of the parasite. In this regard, merozoite surface proteins have gained a lot of attention by the malaria vaccine fraternity for development as subunit vaccines (Chauhan, Yazdani & Gaur, 2010). These vaccines aim to reduce infection, rather than to eliminate it, in order to protect against clinical and particularly severe disease. Efficacy trials tend to take place in the field because an experimental challenge model for erythrocytic infection is not widely used.

An example of erythrocytic vaccine is a vaccine known as combination B comprising of msp-1 and msp-2 with *P. falciparum* ring-stage-infected erythrocyte surface antigen (RESA) in Montadine ISA720 (Todryk & Hill, 2007). A Phase IIb trial in 5–9-year old children in Papua New Guinea elicited a 62% reduction in parasite density (in the subgroup not treated with anti-malarial drugs) but no reduction in disease incidence (Genton *et al*., 2003). Interestingly, the vaccine also had an impact on the strain of msp-2 identified in breakthrough infections. A 42 kDa fragment of msp-1 in AS02A has been assessed in Phase I and II field trials in Kenya and Mali (Thera *et al*., 2006), but without any clear efficacy so far. Several formulations of another erythrocytic antigen, AMA1, in an adenovirus vector in various adjuvants and in virosomal formulations as a domain III cyclized peptide, are currently in clinical trials. The latter formulation has recently been evaluated in a Phase IIa challenge study in the United Kingdom. A long

synthetic peptide derived from msp-3, containing B- and T-cell epitopes, has been tested in Burkina Faso, and it generated long-lasting antibodies that show *in vitro* activity. The same study also observed that the variability and antigen-switching properties of *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) make it a difficult candidate to focus on for a vaccine, despite the fact that anti-PfEMP-1 antibodies have a role in natural immunity (Sodiomon *et al*., 2006).

Antibody responses to polymorphic parasite antigens of *P. falciparum* are associated with protective immunity to malaria. Although merozoite surface protein 1 (msp-1) is a leading candidate vaccine antigen for blood-stage malaria, its efficacy in clinical trials has been limited in part by antigenic polymorphism and potentially by the inability of protein-in-adjuvant vaccines to induce strong cellular immunity (Goodman & Draper, 2010). As said before, the malaria parasite presents a difficult and changing target immunologically. Different types are targeted at different stages of the parasite life cycle (Moran *et al*., 2007). The focus for most vaccine candidates has been on the induction of antibodies against merozoite antigens and msp-1 in particular (Holder, 2009). However, the induction of antibodies to the 42-kDa portion of msp-1 (msp-142) appeared to be insufficient to provide protective immunity in humans in one study (Ogutu, *et al*., 2009).

Plasmodium falciparum is the most virulent of the four species of the parasites which cause malaria in humans. Though covering a large geographical area, the parasites exhibit a regional clustering of their antigens which accounts for the loss of protective

immunity in individuals who move between regions (Borrmann & Matuschewski, 2011). This places further restrictions on vaccine design. To optimize the economic case for any vaccine, the formulation must have sufficient geographical coverage to make investment in its development viable. The *P. falciparum* parasite throughout its lifecycle actively evades or utilizes host immune responses to its benefit. Low parasite numbers initially act to reduce its visibility to the immune system (VandenBerg, 2009). The intracellular, hepatocyte life stage only allows slow feeding of parasite antigen to the cytotoxic cells which are essential in clearing infection. Upon emergence from the hepatocyte, thousands of merozoites are enveloped in host cell membrane components making them indistinguishable from host cells to the immune system, finally bursting out from the protective host provided cloak in the lung, to infect erythrocytes (Hafalla, Silvie & Matuschewski, 2011). This causes the vast majority of vaccine candidates to still fail either in the laboratory or at first clinical trial (Moran *et al*., 2007).

This study project proposed the use of msp-2 to evaluate the genetic diversity and population structure of *P. falciparum* because firstly, as reported by Bereczky (2005) in agreement with Polley *et al.*, (2005) msp-2 has been widely studied as one of the major vaccine candidates and the PCR amplification of this gene is widely used in molecular epidemiologic studies and drug trials to determine the number and types of parasite genotypes of *P. falciparum* infections. The highly polymorphic, single-copy msp-2 gene has been found to be the most informative single marker frequently used to assess mean number of genotypes per infection, i.e., multiplicity of infection. In addition, this genetic marker was chosen because reports from Happi (Happi *et al.*, 2004 and 2006) and

Snounou (Snounou *et al.*, 1999) have further demonstrated that msp-2 is the best and most reliable marker to evaluate diversity and complexity of *P. falciparum* infections in both pre-treatment and post-treatment isolates because it showed more clones than other markers (msp-1 or glutamate-rich protein). Based on the ensuing discussion of the research findings that msp-2 gene is one of the most polymorphic antigens, it was selected to characterize *P. falciparum* populations in Malawi.

CHAPTER 3 : RESEARCH METHODOLOGY

3.1 Research Design

The research study used the cross sectional research design to evaluate and analyze *P. falciparum* genetic diversity and its population structure in selected epidemiological sites in Malawi. The study used prospectively collected filter paper dried blood spot (DBS) samples from the WHO/TDR funded project entitled 'Molecular Surveillance for antimalarial drug resistance in Malawi' Principle Investigator (PI): Fraction Dzinjalamala, Project ID Number: A80688, College of Medicine Research Ethic Committee (COMREC) Approval Number: P.08/09/819. The DBS were prepared, after obtaining informed consent from patients seeking routine clinical care for malaria-suggesting symptoms in public health facilities in four districts in Malawi namely; Bolero in the Northern region district of Rumphi, Dwangwa (Nkhotakota district) and Mitundu (Lilongwe district) in the Centre and Mwanza district hospital (Mwanza district) in the Southern region as shown in figure 2.3 above. These sites were selected because they are sentinel sites used by the National Malaria Control Program (NMCP) as representative of the malaria situation in Malawi. All four hundred samples were screened for malaria using the standard thick blood smear. Out of the four hundred patient samples collected from the four sites (100 per site) thirty patient microscopy confirmed malaria positive samples from each site were selected for the study. Enrolment criteria included the following:

- All ages at the four sentinel sites
- Outpatients presenting with signs and symptoms of malaria
- Ability to give consent or assent.

The participants went through the routine clinical and laboratory procedures as stipulated by the Ministry of Health and Population in Malawi in the diagnosis of Malaria. In brief, the procedure is as follows:

- When the participants reported to the clinic, they were seen by clinicians who ordered a malaria blood film if malaria was suspected.
- Informed Consent (appendix 1 & 2) in terms of adults and assent (appendix 3 & 4) in terms of children was administered to these participants in order to collect a blood spot on a filter paper. In this case, when a finger prick was made for thick blood smear, a blood spot was also collected on filter paper. The thick blood film was used as a laboratory result in the diagnosis of malaria for these participants by the clinician. The blood spots on the filter papers were air dried at ambient temperature away from sunlight, packaged in desiccant-filled plastic pill-bags and sent to the International Centre for Excellence in Malaria Research (ICEMR) Molecular Core Laboratory located in Biochemistry Department of the College of Medicine, University of Malawi.

Other demographic data such as age, body temperature, parasite and gametocyte density and antimalarial drug/medication history were also collected. Figure 3.1 below is the flow chart outlining procedures followed during the study, from enrolment to sample processing and testing:

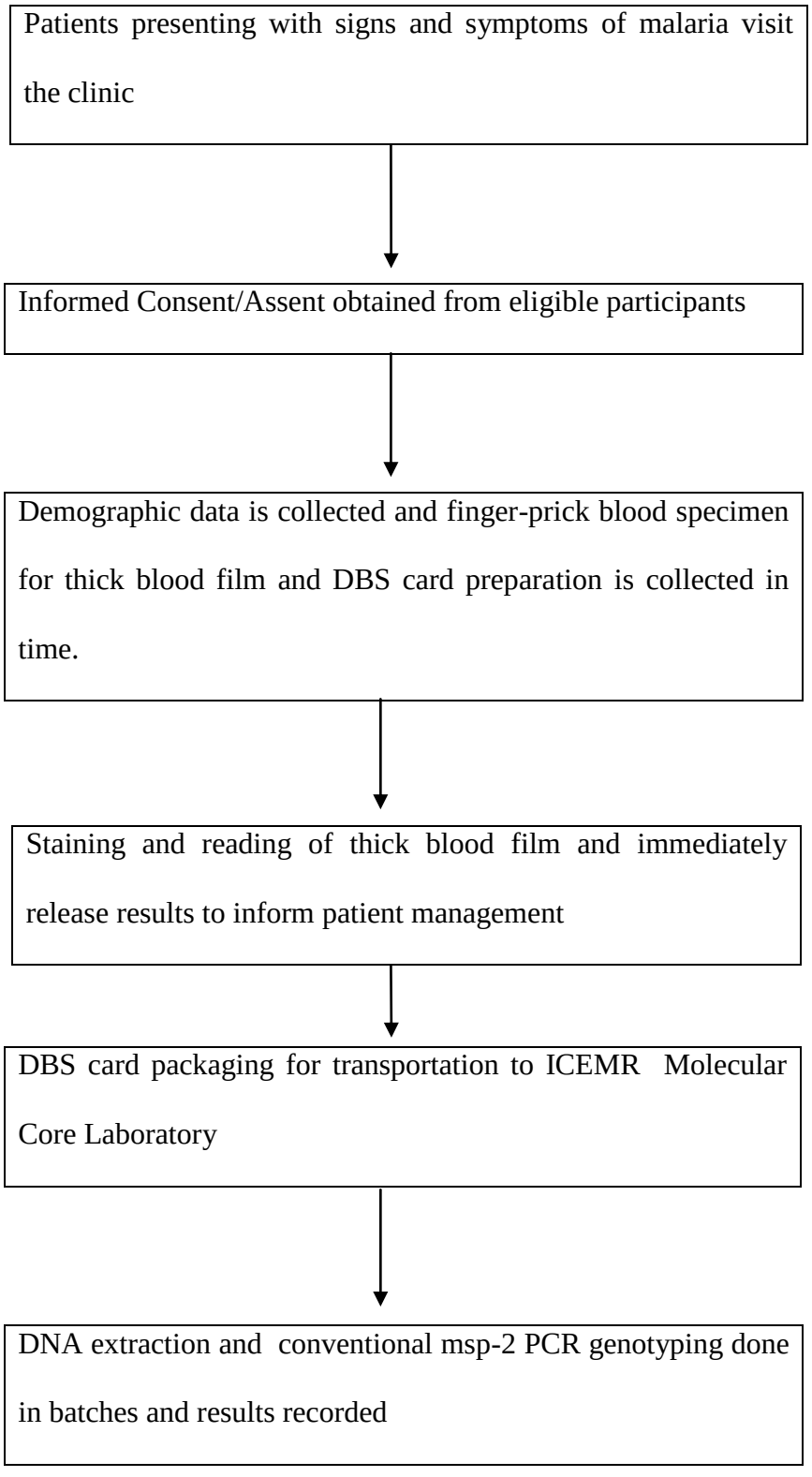

Figure 3.1 Flow chart outlining study procedures

3.2 Ethical Consideration

The study proposal was submitted to and approved by the Nelson Mandela Metropolitan University Human Ethics Committee (NMMUHEC), Ethics clearance Number: H12-HEA-BTR-003. The ethics committee of the University of Malawi, College of Medicine Research Ethics Committee (COMREC), approved the project under the Molecular surveillance for antimalarial drug resistance study project, clearance Number P.08/09/819 (Appendix 5&6). The study was also conducted in accordance with The Belmont Report (1979) following Good Clinical Practice (GCP) and Good Clinical Laboratory Practice (GCLP).

3.3 Sample Size Determination

A total of 30 specimens from each site were randomly selected out of the four hundred samples that were collected for the research project. In order not to arbitrarily choose a sample size, a formula below was used to calculate the sample size for the research project. The following simple formula (Daniel, 1999) was used:

$$n = \frac{Z^2 P (1-P)}{d^2}$$

where n = sample size,

Z = Z statistic for a level of confidence,

P = expected prevalence or proportion (in proportion of one; if 20%, P = 0.2), and

d = precision (in proportion of one; if 5%, d = 0.05).

Using the formula and a sample size calculator (Naing, Winn & Rusli, 2006) a sample size of 385 was arrived at. Due to constraints in reagent availability, this sample size was reduced to approximately one third of the total to make an average of 120 samples, 30 in each of the four study sites.

3.4 LABORATORY PROCEDURES

Procedures followed in this study are outlined below.

3.4.1 Microscopy

Microscopy remains the gold standard for malaria diagnosis as recommended by the WHO. Routine procedures available at the hospital laboratory for the diagnosis of malaria using microscopy were followed. Thick blood smears were used for all slides that were collected in order to aid the clinicians to accurately diagnose the participants. It was again important to confirm a *P. falciparum* infection as the genotyping methods for msp-2 are based on this species of *Plasmodium*. Blood smears were air dried and stained with Field stain A and B. A slide was considered negative if no asexual parasites were seen after examination of 100 fields. Parasite density was estimated by counting the number of asexual parasites per 300 leukocytes while gametocytes were estimated by counting them per 500 leukocytes. Counts were converted to number of parasites/gametocytes per microlitre by assuming a standard leukocyte count of 8000 per microlitre (Blantyre

Malaria Project Laboratory Manual/ Malawi Liverpool-Wellcome Trust Manual PAR.004 Version 1, 2011).

3.4.1.1 Test summary

This is a useful method for rapid presumptive identification of malaria parasites. The method shows adequate staining of all stages including morphology. With thick preparations the end of the smear closest to the edge of the slide that was draining, must be examined. The edges of the film will also be enhanced, compared to the center where the film may be too thick or cracked (Blantyre Malaria Project Laboratory Manual/ Malawi Liverpool-Wellcome Trust Manual PAR.004 Version 1, 2011).

3.4.1.2 Test Procedure

1. Prepare a blood thick smear film on a microscope slide at a 45˚angle.

2. Allow the smear to air dry.

3. Hold the slide with the dried thick smear facing downwards and dip in Field Stain B for 1 to 3 seconds, allow the smear to be wholly dipped in the stain.

4. Drain the excess stain by touching a corner of the slide against the side of the container.

5. Wash gently in clean water for about 3 seconds and gently agitate to drain off excess water.

6. Dip the slide in Field Stain A for 2 seconds.

7. Drain off excess stain.

8. Wash gently in clean water for 3 seconds.

9. Wipe the back of the slide clean and place it upright in a draining rack for the film to air dry.

10. Read the slide on the microscope, first using the 40x objective lens to check the quality of the slide, then with the 100x oil immersion objective to check the presence or absence of the parasites (Blantyre Malaria Project Laboratory Manual/Malawi Liverpool-Wellcome Trust Manual PAR.004 Version 1, 2011).

3.5 DNA Preparation

Deoxyribonucleic acid (DNA) is an informational molecule encoding the genetic instructions used in the development and functioning of all known living organisms and many viruses. Along with Ribonucleic Acid (RNA) and proteins, DNA is one of the three major macromolecules that are essential for all known forms of life. Genetic information is encoded as a sequence of nucleotides (guanine, adenine, thymine, and cytosine) recorded using the letters G, A, T, and C. Many types of samples can be analyzed for nucleic acids by PCR which is very versatile. Most PCR methods use DNA as a target,

rather than RNA, because of the stability of the DNA molecule and the ease with which DNA can be isolated. By following a few basic rules, problems can be avoided in the preparation of DNA for the PCR (Veilleux, N.D). DNA Extraction is the removal of DNA from the cells or viruses in which it normally resides. Extraction of DNA is often an early step in many diagnostic processes used to detect bacteria and viruses in the environment as well as diagnosing disease and genetic disorders (Rice, N.D). The essential criteria for any DNA sample are that it contains at least one intact DNA strand encompassing the region to be amplified and that any impurities are sufficiently diluted so as not to inhibit the polymerization step of the PCR reaction. PCR involves preparation of the sample, the master mix and the primers, followed by detection and analysis of the reaction products (Veilleux, N.D)

3.5.1. Methanol-Water Extraction

The study used the simple and inexpensive methanol-water extraction method to elute the DNA for PCR processing. Immediately following the extraction, parasite DNA in the samples was quantified on a nanodrop (Nanodrop ND-1000 UV/VIS Spectrophotometer, Labtech International, UK) to establish that the amount of DNA in each of the samples was enough for genotyping.

3.5.2 Methanol-Water Extraction Procedure

- Preset the water bath/heating block to 98-100˚C.

- Cut filter (DBS) paper into small squares (approx. 3mmX3mm) and carefully place into a microcentrifuge tube.
- Make sure to wipe the scissors clean with 70% ethanol between each sample.
- Add 125µl of methanol and leave for about 10-15min at room temperature.
- Pour off or decant off the methanol and leave the tube open on its side to dry the filter paper pieces.
- Add 65µl of PCR grade water and boil at 97-100˚c for 15-20min with intermittent vortexing every 5min.
- Spin in a microcentrifuge for a few seconds to settle the liquid that has collected at the cover.

5µl of this DNA is used in each PCR reaction. Store at 4˚C for short term storage or at -20˚C for long term (Chris Plowe's laboratory manual, University of Maryland; ICEMR-Molecular Core Laboratory Manual, Version 1.0, 2011).

3.6 Polymerase Chain Reaction

PCR has rapidly become one of the most widely used techniques in molecular biology and for good reasons: it is a rapid, inexpensive and simple means of producing relatively large numbers of copies of DNA molecules from minute quantities of source DNA material even when the source DNA is of relatively poor quality (Veilleux, N.D.).

3.6.1 Genotyping of P. falciparum parasites

Nested PCR methods were adapted as described in Methods in Malaria Research by Moll, Ljungström, Perlmann, Scherf & Wahlgren, (2008). Briefly, in the first

amplication reaction oligonucleotide primer pairs, which hybridized to conserved sequences flanking the repeat polymorphic regions of the msp-2 genes, were used. The product of this first reaction was then used as a DNA template for the separate second amplification reactions in which allele-specific oligonucleotide primers were used to recognise polymorphic sequences contained within the DNA fragment (i.e. the msp-2 gene) amplified in the first reaction.

The msp-2 allele-specific oligonucleotide primers used in the second round of amplification specific to the following msp-2 allelic families: FC27, and IC. The K1 laboratory strain was used as positive control for the FC27 msp-2 allelic family whereas the 3D7 laboratory strain was used for the IC msp-2 allelic family.

Genotyping of *P. falciparum* infections at the msp-2 locus permits characterization of distinct *P. falciparum* subpopulations present in an isolate. As said earlier, the genetic marker msp-2 was chosen due to its extensive polymorphism in size and sequences. The different msp-2 allelic families (KI/FC27 and 3D7/IC) were detected with allele-specific primers in a second nested PCR.

3.6.1.1 Equipment

Micropipettes and micropipette filtered tips

Veriti Thermal Cycler (ABiosystems)

Apparatus for agarose gel electrophoresis

Gel documentation camera and UV transilluminator

Refrigerator (4˚C) and freezer (-20 ˚C) for reagent and DNA storage

Microcentrifuge

3.6.1.2 Materials and Reagents

Invitrogen *Taq* polymerase (store at -20˚C) with appropriate buffer (store at 4˚C)

MgCl2 stock solution (often provided by enzyme supplier)

dNTP (Invitrogen): a working solution with a concentration of 5 mM for each of dATP, dCTP, dGTP and dTTP (store at -20˚C)

Oligonucleotide primers (sequences are given below): a working solution with a concentration of 2.5 μM for each oligonucleotide primer (store at -20˚C)

Loading buffer (Invitrogen)

50 mM Tris (pH 8.0)

75 mM EDTA (pH 8.0)

10x TBE buffer:

1x TE (1 M Tris, 1M boric acid, 50 mM EDTA, pH 8.3)

Agarose (Invitrogen Ultrapure)

Pure water

Ethidium bromide solution containing 10 mg per mL of water

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood. Ethidium bromide must be stored in the dark at 4˚C.

3.6.1.3 Setting up the first amplification reaction

- Calculations for the total volume of reaction mixture was done as a requirement i.e: 25 μL per reaction x (total number of reactions + 1).

- Then a calculation for the amount of:

  Buffer (final concentration 1x)

  MgCl2 (final concentration 1.5 mM)

  dNTP (final concentration 100 μM)

  Taq polymerase (final concentration 2 units per reaction)

  and water to make up the total volume

- Add, in order, the correct volumes of: water, buffer, MgCl2, "gene specific" oligonucleotide primers, dNTP and the *Taq* polymerase. Mix by a short vortex pulse.
- Aliquot 20 μL of the reaction mixture per labeled tube.
- Add 1 μL of DNA template to each tube (water for the negative controls).
- Place in the thermocycler and run the PCR reaction (Moll *et al*., 2008).

3.6.1.4 Setting up the second amplification reaction

- Bullets 1 – 5 as above.
- 1 μL was removed from the first amplification reaction tube and added to the second amplification reaction tube.
- The tube was then placed in the thermocycler and the PCR reaction was run.

NB: Each second amplification reaction as a requirement was performed with a single allele-specific oligonucleotide pair (Moll *et al*., 2008).

3.6.1.5 Sensitivity and Specificity

- The parameters (cycling parameters and final concentrations) as provided Moll *et al* (2008) had to be altered in order to obtain good PCR optimization and for

optimal sensitivity and specificity. The use of different temperature cyclers and enzymes has an influence on the efficiency of the PCR reaction. Moll *et al* (2008) notes that nested PCR for the 3 genetic markers should be capable of detecting about 10 parasite genomes per microliter of blood.

- Optimization of the PCR conditions requires the preparation of a standard set of genomic DNA templates. These are prepared from dried blood spots. Sensitivity is mainly dependent on the number of cycles, thus these can be increased if needed.
- Specificity is most affected by the annealing temperature and eventually the MgCl2 concentration. A common problem is the generation of two PCR products for the specific second amplification reactions. This is due to carryover of oligonucleotide primers and PCR product from the first reaction, which can be minimized by reducing the oligonucleotide concentrations and/or the number of cycles in the first amplification reaction (Moll *et al*., 2008).

3.6.1.6 Cycling Parameters

Below are the cycling parameters that were used for the first nested PCR

Step 1 95°C for 5 min Initial denaturation

Step 2 94°C for 1 min Annealing

Step 3 58 °C for 2 min Extension

Step 4 72 °C for 2 min Denaturation

Step 5 Repeat Steps 2–4 a total of 25 cycles (Nest 1)

Step 6 72°C for 2 min Final extension

Step 7 the reaction is completed by reducing the temperature to 25°C.

For the second nested PCR, below are the cycling parameters that were used;

Step 1 95°C for 5 min Initial denaturation

Step 2 95°C for 1 min Annealing

Step 3 61 °C for 2 min Extension

Step 4 72 °C for 2 min Denaturation

Step 5 Repeat Steps 2–4 a total of 30 cycles (Nest 2)

Step 6 72°C for 2 min Final Extention

Step 7 the reaction is completed by reducing the temperature to 25°C

3.6.1.7 Minimizing Contamination

The risks of contamination are enormously increased when nested PCR is performed. Thus the transfer of the product of the first amplification reaction to the second amplification reaction mixture should be performed with extreme care. Ideally, as recommended by Moll (Moll *et al*., 2008) setting up the first and second amplification reactions should be performed in a separate room from the one where the gels are migrated. Moreover, the transfer of the template from the first to the second amplification reactions must be performed with a dedicated pipette in yet another room, preferably with filter tips.

3.6.1.8 Analysis of the PCR product

The amplified products from the nested reaction were separated using 2% agarose gel (Invitrogen Ultrapure, UK), and visualized under ultraviolet (UV) trans-illumination. A

100 base pairs (bp) DNA ladder marker (New England Biolabs Inc) was used to determine the size of bands. The size polymorphism in each allelic family was estimated assuming that one band represented one amplified PCR fragment derived from a single copy of msp-2 gene. Positive controls (laboratory *P. falciparum* cloned lines 3D7/IC and K1/FC27 for msp-2) and PCR grade water as negative controls were included in all PCR amplifications.

3.6.1.9 Procedure for Analysis

- Add 1 μL of the loading buffer to the PCR product.
- Load 8 μL of sample on 2% agarose gel (Invitrogen Ultrapure, UK) and migrate (1x TBE buffer).
- Stain the gel in TBE buffer containing ethidium bromide (final concentration 1 μg per mL) for 30 min.
- Destain in TBE or water for 5 min.
- Visualise on a UV transilluminator.

Normal agarose is suitable for the analysis of all 3 genetic markers. However, given the small size of the bands which will result from the amplification of msp-2, and the small variations in the sizes of the different allelic variants, the use of agarose type which give higher resolution is advised. The best results are obtained if the gel is kept cold before and during electrophoresis (Moll *et al*., 2008).

3.6.1.10 Oligonucleotide Sequences

Oligonucleotide primers (England biolabs) were used following the same protocol. The table below (Table 3.1) is a list of sequences for genotyping msp-1&2.

Table 3.1 List of msp-2 genotyping sequences

Primer name	Target gene	Target region/variant	Primer sequence (5' to 3' end)
M2-OF	msp2	msp2 block 3	ATGAAGGTAATTAAAACATTGTCTATTATA
M2-OR	msp2	msp2 block 3	CTTTGTTACCATCGGTACATTCTT
M2-FCF	msp2	FC27	AATACTAAGAGTGTAGGTGCARATGCTCCA
M2-FCR	msp2	FC27	TTTTATTTGGTGCATTGCCAGAACTTGAAC
M2-ICF	msp2	3D7/IC	AGAAGTATGGCAGAAAGTAAKCCTYCTACT
M2-ICR	msp2	3D7/IC	GATTGTAATTCGGGGGATTCAGTTTGTTCG

A primer is a short segment of nucleotides which is complementary to a section of the DNA which is to be amplified in the PCR reaction. Primers are annealed to the denatured DNA template to provide an initiation site for the elongation of the new DNA molecule. Primers can either be specific to a particular DNA nucleotide sequence or they can be "universal." Universal primers are complementary to nucleotide sequences which are very common in a particular set of DNA molecules. Thus, they are able to bind to a wide variety of DNA templates (Veilleux, N.D).

In the first reaction, primers pairs corresponding to conserved sequences spanning the polymorphic regions of the *msp-2* were included. These are M2-OF; 5'-ATGAAGGTAATTAAAACATTGTCTATTATA-3' and M2-OR; 5'CTTTGTTACCATCGCTACATTCTT-3'. Using the product from the first reaction as a template, two separate secondary nested reactions were then performed using specific primers for KI/FC27 and 3D7/IC. These are M2-FCF;

5’AATACTAAGAGTGTAGGTGCARATGCTCCA-3’ and M2-FCR; 5’-TTTTATTTGGTGCATTGCCAGAACTTGAAC-3’ for KI/FC. M2-ICF;5’-AGAAGTATGGCAGAAAGTAAKCCTYCTACT-3’and 5’GATTGTAATTCGGGGGATTCAGTTTGTTCG-3’ for 3D7/IC.

3.7 PCR amplification and Product Analysis

It was proposed that msp-2 will be used to evaluate the genetic diversity and population structure of *P. falciparum*. This genetic marker was chosen because reports from Happi *et al*, (2004 and 2006) have demonstrated that msp-2 is the best and most reliable marker to evaluate diversity and complexity of *P. falciparum* infections in both pre-treatment and post-treatment isolates because it showed more clones than other markers (*msp-1* or glutamate-rich protein).

3.8 Statistical Analysis

Data was entered on an excel spreadsheet and analyzed using STATA (statistics /Data Analysis) Version 11.0. Frequencies were calculated as a ratio of samples found to harbor a particular variant out of the total number of samples collected. Significant differences in the frequencies of msp-2 the allelic families, KI/FC and 3D7/IC or the individual allelic variants within and between study sites were established using Chi-squared test. The same test was also applied to the observed frequencies of single and double infections with selected allelic variants. Expected values were calculated from individual frequencies of each allelic variant. The difference in mean genotype number of

the different msp-2 allelic families between study sites were assessed using the student t test. Note that the denominator for frequency calculations was the total number of samples where variants of a particular marker family were found (Snounou *et al*., 1999).

CHAPTER 4: RESULTS

4.1 Introduction

One hundred and twenty patient samples confirmed by microscopy to have *P. falciparum* malaria parasite, were randomly picked and enrolled in this study as previously described. Most of these participants were between the ages of 3months to 45 years and the age profiles did not significantly differ between the four epidemiological locations. The mean age in the four epidemiological locations is as tabulated below (Table 4.1)

Table 4.1 Demographic characteristics of the study population

Parameter	**Mwanza**	**Mitundu**	**Dwangwa**	**Bolero**
Mean age(95%CI) (Range)	7.9(4.0-11.8)(0.4 to 45)	2.9(2.4-3.4)(0.3 to 5.5)	11.7(7.0-11.4) (0.4 to 50)	4.9 (0.6-9.0) (0.6 to 20)
Average Parasite density	24053	57177	85127	2324
Average Gametocyte density	0	0.35	4.1	3
Average body Temperature	37.4	38.2	39.4	38.4

It was observed that there was no significant mean age difference of the study population between the four study sites. Most of the study populations were children. However, from the four study sites, Mwanza and Dwangwa site had a category of an older age group compared to Mitundu and Bolero site (95%CI:4.0-11.8) (Table 4.1). For samples that had optimized for the msp-2 genotype and those that did not optimize, the trend was the same in that the mean age group was not significantly different amongst the

four study sites with Mwanza and Dwangwa showing to have a trend of an older mean age group(data not shown). In terms of the average parasite density, Mitundu and Dwangwa had a higher average parasite density compared to Mwanza and Bolero. Average body temperature for the clients was higher in Mitundu, Dwangwa and Bolero sites with Mwanza site having a lower average body temperature.

4.2 Microscopy Results

WHO classify malaria microscopy result as the gold standard for malaria diagnosis. Two microscope readers must confirm the result before releasing it to the patient (WHO Malaria Report, 2009). However, with the set-up of the four epidemiological locations, only one reader was available at a time, largely due to the shortage of well-trained microscopists compared to the large number of patients seeking laboratory services at those locations. It was necessary to have microscopy result as this study samples were based on samples that were found to be malaria positive on microscopy.

4.2.1 Interpretation of results

Positive: Presence of the *Plasmodium* parasite in the smear stain the following:

Chromatin of parasite - Dark red

Cytoplasm of parasite - Blue-mauve

Schüffner's dots - Pale red

Negative: Absence of *Plasmodium* parasite in the smear (WHO Basic Malaria Microscopy, 2010).

4.2.2 Grading of Malaria Thick Smear

• **Negative**: the absence of a parasite after counting a minimum of 100 fields using 100x oil immersion objective.

• +: 1-10 parasites counted in all the 100 fields using 100x oil immersion objective.

• ++: 11-99 parasites counted in all the 100 fields using 100x oil immersion objective.

• +++: 1-10 parasites counted per field using 100x oil immersion objective.

• ++++: More than 10 parasites counted per field using 100x oil immersion objective (WHO Basic Malaria Microscopy, 2010).

4.2.2.1 Quality Control

Positive Control: known positive smears were used as positive control.

Negative control: known negative smears were used as negative controls (Blantyre Malaria Project Laboratory Manual/ Malawi Liverpool-Wellcome Trust Manual PAR.004 Version 1, 2011).

As a reference, below are some examples of the positive slides taken from the microscope camera (figures 4.1 and 4.2).

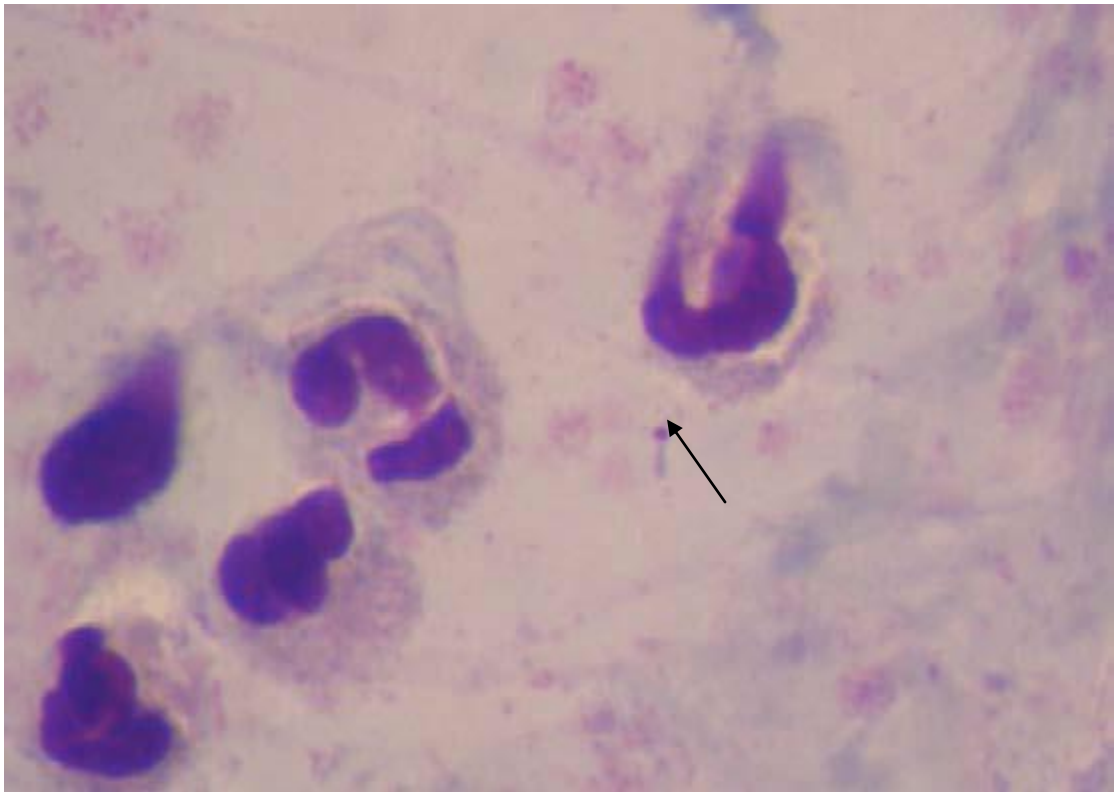

Figure 4.1 Field stained thick malaria blood smear showing low parasitemia

As figure 4.1 above refers, the arrow is pointing at the malaria parasite trophozoite seen on this particular field using 100x oil objective of the light microscope. On the other hand, figure 4.2 below shows a slide with high parasitemia.

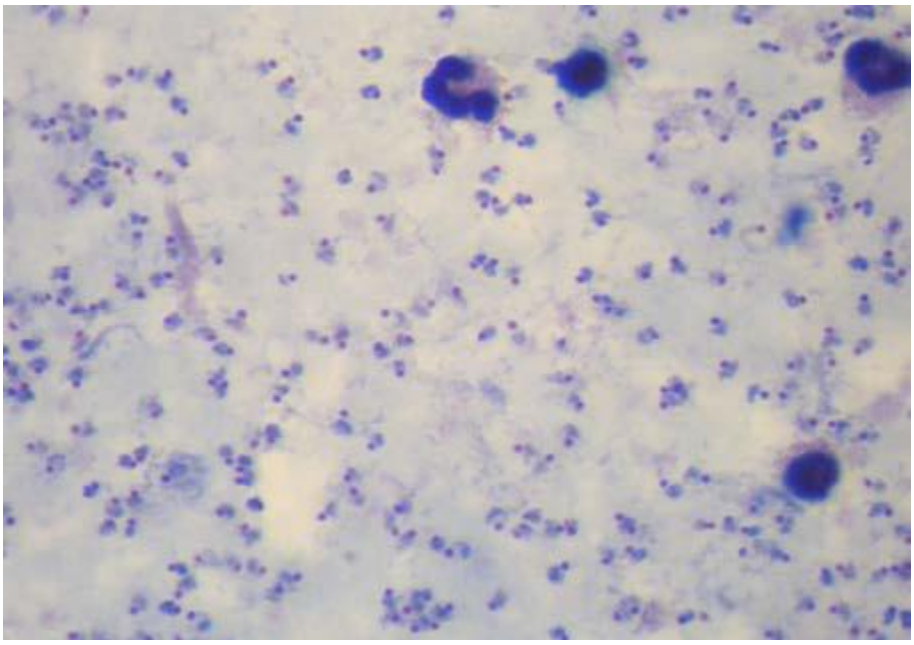

Figure 4.2 Field stained thick malaria blood smear showing high parasitemia

As is the case everywhere in Malawi, the National Malaria Control Program 'plus system' of grading malaria density was used during the examination of the thick blood smear. Correlation of the 'plus system' with the conventional parasite count per micro-litre of blood is tabulated in the Table 4.2 below.

Table 4.2 Correlation between 'Plus System' and the parasite count per micro-litre (µl) of blood (adapted from NMCP Malawi, 2011)

Parasites per ul of blood	**Plus system**
40-400parasites	1+ (1-10 parasites per 100fields)
400-4000 parasites	2+(11-100 parasites per 100 fields)
4000-40 000 parasites	3+ (1-10 parasites per one field)
40 000-400 000 parasites	4+ (11-100 parasites per one field)

4.3 PCR RESULTS

Parasite DNA was extracted from 120 patient samples. These were then genotyped using nested PCR assays based on the amplification of msp-2 as described previously (section 3.6.1). Immediately following the extraction, parasite DNA in the samples was quantified on a nanodrop (Nanodrop ND-1000 UV/VIS Spectrophotometer, Labtech International, UK) and it was established that the amount of DNA in each of the samples was enough for genotyping (average 132ng/ul). Although this average amount of DNA was found in the samples, eighteen (15%) of the 120 samples selected did not optimize in the first msp-2 nested PCR despite being repeated several times and were thus excluded from the secondary msp-2 analysis even though the samples were confirmed being malaria positive by microscopy. A large number of samples that did not optimize in the first Nested PCR came from the Bolero site. The reason for sample refractoriness is unknown although handling at the site and also storage conditions may be suspected to have contributed to DNA degradation.

4.3.1 Msp-2 Fragment Length Polymorphisms

Allelic polymorphism typing analysis displayed the highly polymorphic nature of *P. falciparum* from the different epidemiological setting in Malawi with respect to msp-2. Both 3D7/IC and KI/FC allele types were identified among the isolates from all the four study sites (Table 4.3). A total of 28 msp-2 block 3 fragments, defined by the size and the allelic types were detected in the 102 patients. The length variants of the PCR product were 240–450 bp for the K1/FC and 410–780 bp for the 3D7/IC allelic families. These product size ranges are broadly in agreement with Snounou *et al*., (1999) who found that the product size ranged from 250-550bp for FC and 300-700 for 3D7/IC.

4.3.2 Multiplicity of Infection

The number of msp-2 genotypes per infection for each study site was calculated as the number of alleles found per patient (Table 4.3). The incidence of multiple-clone infections ranged from 20% to 50% at the four different sites with the highest prevalence of mixed infections observed in Mwanza compared to all the other three study sites. This trend was statistically significant (p=0.041).

Table 4.3 Prevalence of monoclonal and multiclonal infection by site

Site	Total Patients	Number of msp-2 alleles		
		1(%)	2(%)	3-4(%)
Mwanza	28	14 (50)	7 (25)	7 (25)
Mitundu	25	20 (80)	3 (12)	2 (8)
Bolero	8	06 (75)	2 (25)	0 (0)
Dwangwa	26	19 (73)	6 (23)	1 (4)

In the table 1-represents monoclonality or the presence of only one clone in an isolate;

2 or 3-4 represents multiclonality or number of clones

4.3.3 Mean Number of msp-2 Genotypes by Site

The number of genotypes from the four sites was analyzed to obtain the mean as summarized in the Table 4.4 below:

Table 4.4 Mean Genotypes number, isolate and ranges by site

Site	Sample size	Mean	SD	Range
Mwanza	28	1.4	0.56	1-3
Mitundu	25	1.2	0.41	1-2
Dwangwa	26	1.3	0.45	1-2
Bolero	8	1.3	0.35	1-2

Comparing the mean genotypes amongst the four sites, it was observed that there was no significant difference in the mean number of genotypes in patients between sites. When stratified by age group, the observed mean genotype number did not differ between children under five years and those that were above five years of age as captioned in Table 4.5 below, although there was a trend of a higher mean genotype number for older children.

Table 4.5 Mean genotypes between <5 and >5 year age

Age category	**Observations**	**Mean**	**S D**	**95% CI**
<5 years	35	1.37	0.55	1.18-1.56
>5years	51	1.96	0.40	1.08-1.31
Combined	86	1.27	0.47	1.17-1.37

4. 3.4 Factors Influencing Multiplicity of Infection

A multiple linear regression analysis was performed to examine the independent influence of covariates on mean genotype number per infection (i.e. covariate influence on multiplicity of infection). The following covariates were considered: Patient age, body temperature, study site, asexual parasite density, and history of previous antimalarial use within the last 28 days. All these covariates had no significant influence on multiplicity of infection in this study. Inclusion of these variables did not generate a significant model compared to the base model (Adjusted R-squared = 0.0662; F = 1.224, p = 0.3548).

CHAPTER 5: DISCUSSION OF RESEARCH FINDINGS

5.1 Microscopy

The WHO recommends that two independent microscopy readers must read the slide before results are issued to the patient (WHO Malaria Quality Assurance Manual, 2008). However, Malawi is faced with an acute shortage of well-trained medical laboratory staff capable of malaria microscopy reading. The number of patients visiting government hospitals and clinics requiring malaria diagnostic services remains constantly high. As a result, only one reader is available at a time, therefore results that are released are only read by one microscopy reader. In this study however, the slides that were read at the study sites were brought to the ICEMR laboratory together with the DBS samples. These slides were reviewed later at random to confirm the malaria microscopy results. However, this reading only identified whether *P. falciparum* was present or not and no parasite density was considered. This may have had an effect on the DNA extraction yield as samples with low parasite density would have yielded low DNA hence could not polymerize in the nested PCR. This lead to such samples being discontinued in the genotyping procedures. The storage of the DBS samples could again have contributed to the degradation of the DNA in the samples as some of them had fewer desiccant packs in the bag.

5.2 Multiplicity of Infection and Incidence of Multiple Clone Infections

Multiplicity of infection did not vary across sites though there was a trend towards a high prevalence of multiple infections in Mwanza. Mwanza borders Mozambique to the west and movement of people is not restricted as most people form

Mozambique seek medical attention at Mwanza District hospital. Taking into account that the malaria control program is not intensive on the other side of the border, it may lead to the high number of multiple infections in Mwanza than the other study sites. This is in tandem with one study (Rehman *et al*., 2011), which found that there was high prevalence of malaria in Mozambique than in Malawi.

Mean number of genotypes reflects the intensity of malaria transmission. This was around one (1) at all study sites meaning there was an average of one parasite genotype per infection. There were more single-clone infections than expected of high transmission settings (Table 4.3). Transmission may be going down, situation closer to lower transmission settings of South East Asia and South America.

This has so many implications e.g.

(i) In association mapping studies: easy to resolve genotype-phenotype relationships because parasite haplotypes can be easily constructed when single-genotype infections are predominant. There is no ambiguity than when multiple alleles are present

(ii) Immunity development – difficult to acquire immunity to malaria as this develops with repeated mosquito bites and immunization against one parasite clone confers protection only against that clone so more clonal inoculations are better. Lower transmission leads to fewer bites per person per year and less immunity

(iii) Less within-host competition because most infections are monoclonal

The percentages (%) of multiple-genotype infections at the four different sites seem to be different as reflected in Table 4.3. (i.e 50% for Mwanza, 20% for Mitundu and 27% for Dwangwa and 25% for Bolero), meaning the population structure is different. This suggests there is greater outcrossing/outbreeding in Mwanza than at other sites, incidence lower than what Nkhoma *et al*, (2012) found in Ndirande (75%) in 2008. This may suggest that *P. falciparum* transmission may be declining or these *P. falciparum* populations are just very different from the Ndirande population.

The biology of *P. falciparum* has an effect on the transmission rate i.e. where transmission is low; there is lower incidence of multiple-clone infections (MIs) e.g. South East Asia where transmission is low compared to Africa where transmission is high. MIs are thought to be predominantly generated by bites from multiple infected mosquitoes (super infection) although single mosquito inoculation may play an important role as well (Nkhoma *et al* 2012). Low incidence of MIs suggests lower rate of outbreeding – situation similar to South East Asia. Having said that, low incidence of MIs could be due to poor discriminatory power of msp-2 genotyping at resolving infection complexity. It is possible that incidence of MIs was underestimated. Therefore, it is well possible that some of the multiple-clone infections were missed. This underscores the importance of using more markers to resolve the DNA fingerprint of parasite malaria infections.

5.3 Multiple Genotypes and Parasite Density

The number of msp-2 genotypes was investigated in relation to the total number of parasites as established by microscopy from the field sites. Infection with different genotypes might lead to the development of genotype-specific or allele-specific immunity. If this occurs, a host would only develop resistance to an immunologically defined genotype, remaining susceptible to others (Gupta *et al*, 1994). This theory predicts that multiple-genotype infections would lead to more rapid development of anti-malaria parasite immunity, since the immune response is exposed to a greater amount of the allelic diversity in one infection. In this study, there was no much correlation between high parasite densities and higher number of genotypes. This is in contrast to one study in Tanzania (Bereczky, 2005) in which it was found that there was a correlation between high parasite densities and higher number of genotypes. The relationship between diversity and density may have been partly affected the results since genotyping was performed in symptomatic patients. As observed by Beresczky (2005) that previous studies reported significant correlation between parasite density and number of msp-2 genotypes in infants and young children, the impact of parasite density on number of genotypes may reflect higher sensitivity of detection but may also reflect unspecific PCR amplication and has to be taken into consideration in the interpretation of genotyping studies.

5.4 Fever and Mean Genotypes

Fever (also known as pyrexia), an inflammatory response is one of the most common medical signs and is characterized by an elevation of body temperature above the normal range of 36.5–37.5 °C (97.7–99.5 °F) due to an increase in the temperature

regulatory set point. This increase in set-point triggers increased muscle tone and chills. As a person's temperature increases, there is, in general, a feeling of cold despite an increase in body temperature. Once the new temperature is reached, there is a feeling of warmth. *Malaria* causes symptoms that typically include *fever* and headache, which in severe cases can progress to coma or death. As observed by Branch *et al*, (2001) it is also possible that high fever, a defining characteristic of clinical malaria, might clear parasite genotypes from an individual. This may explain the findings in our study in which it was found that there was no difference in the mean number of genotypes between those who had and those without fever.

5.5 Effect of Patient Age on Multiplicity of Infection

Studies (Branch *et al*., 2001; Mueller *et al*., 2012) have shown that in malaria-endemic settings, malaria immunity develops with age and with successive parasitaemic episodes such that older children develop some form of immunity against malaria, which does not necessarily prevent malaria infection but limits the severity of the disease. As observed by Branch *et al*., (2001), children born in malaria-holoendemic areas are infected almost constantly, but it takes 3 to 5 years to develop immunity that confers protection against parasitemia and illness. The many allelic forms of asexual blood-stage *P. falciparum* antigens might contribute to this delayed acquisition of immunity. The extent of multiple-genotype infections sheds light on malaria transmission, parasite diversity, and the development of immunity.

In our study, there was no association found between patient age and mean number of parasite genotypes harbored by patients when we compared this metric in children < 5 years old and > 5 years old (Table 4.5). This is in agreement to one study in

Senegal (Vafa *et al.*, 2001) in which MOI was not age-dependent, in the range of two to ten years, but was correlated with parasite density. However in relation to the Senegal study and our study project, some of these observations need to be confirmed including larger sample size with broader age range and using other *msp2* genotyping method. It was noted that there was a trend towards a higher mean genotype for older children in our study though. It was expected that older children would have a lower multiplicity of infection compared to children < 5 years old because the latter have very little or no pre-existing immunity to malaria.

5.6 Biology of *P. falciparum* in Relation to Genotying Methods

Plasmodium falciparum, the causative agent of the most severe form of human malaria, as cited by Nkhoma *et al* (2013) is an obligately sexual hermaphrodite protozoan parasite. Haploid parasites replicate mitotically in the human host, with some parasite cells differentiating into male and female sexual stages (gametocytes). Male and female gametes fuse in the mosquito midgut to form a short-lived diploid zygote (ookinete), which then undergoes meiosis to generate haploid infective stages. Recombination occurs during the brief obligately sexual stage in the mosquito and results in the re-assortment of genes and generation of new parasite genotypes. *P. falciparum* has a mixed mating system. When male and female gametes of the same genotype fuse (self-fertilization), the haploid infective stages generated are unchanged by recombination, while when two genetically distinct gametes fuse (outbreeding), the genome of infective stages is reshuffled. Because blood stage malaria parasites are haploid, we expect to see only one allele per locus if an infection contains a single parasite clone and multiple alleles if more

than one clone is present. Multiple mosquito bites can generate multiple clone infection also called super infection but can be generated by recombination hence resulting in reshuffling and re-assortment of genes. MI carriage assumes that MIs result from bites from 2 or more infected mosquitoes (super-infection).However, recent data question the validity of the super-infection model. Analyses of the component clones within multiple-genotype infections demonstrate that MIs consist predominantly of related parasites that are likely to result from haploid recombinant infective stages inoculated by single mosquito bites rather than superinfection (Nkhoma *et al*., 2012).

Data from other studies (Atroosh *et al*., 2011; Greenhouse *et al*., 2006) show that msp-2 genotyping is poor at resolving infection complexity in terms of multi-clonal infection. This has an effect as countries scale up intervention strategies, transmission will decrease resulting in inbreeding within infections between closely related parasites within the population. If using msp-2 genotyping alone to define infection complexity, there are chances of ending up with the same parasite fingerprint for otherwise closely related but genetically distinct infections in an area. If msp-2 genotyping is said to have low resolution power for infection complexity, we cannot effectively distinguish between re-infections and treatment failures (recrudescences) in antimalarial drug efficacies/effectiveness trials. As such, use of PCR correction of efficacy may not be confounded.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

Malaria remains one of the most important public health problems in Malawi. It is estimated that a large proportion of Malawi's workforce loses about 15-25 days a year due to malaria and families spend about 28% of their yearly income to treat malaria. Malaria is the leading cause of mortality, especially among pregnant women and children under the age of five years (NMCP Malawi, 2011).

A parasitological confirmation of malaria in high-transmission settings is recommended. It improves the differential diagnosis of fever, improves fever case management and reduces unnecessary use of anti-malaria medication. Anti-malaria treatment on the basis of clinical suspicion of malaria should only be considered in situations where a parasitological diagnosis is not accessible and where delayed treatment could be fatal for example in children less than five years old. This consideration is of high significance particularly in vulnerable populations (e.g. children under five years of age, pregnant women, suspected severe malaria cases and in settings with a high prevalence of HIV/AIDS where the patients present with fever or a history of fever and no other obvious cause of the fever is present) in whom the disease can rapidly become fatal (WHO Guidelines for the treatment of malaria, 2010).

Knowledge about *P. falciparum* population structure is critical for both malaria control and elimination. It can provide information about progress made in malaria control and can identify malaria-endemic areas where malaria elimination is potentially realistic. This study provides the first description of *P. falciparum* population structure at

four locations in Malawi. Malaria transmission in Malawi is reported to be high (Mzilahowa *et al.*, 2012) and mainly symptomatic in adults (Dzinjalamala, 2006). However, contrary to our expectations, *P. falciparum* populations studied appeared to exhibit a predominantly clonal population structure as shown by a lower incidence of multiple-clone infections and lower multiplicity of infection than previously reported from other studies. In other words, the observed population structure suggested that the transmission of *P. falciparum* is much lower at sites where sampling of infections was done. This result is not as might be expected from a generalized picture of malaria transmission epidemiology in Malawi. Since we did not do genotyping on malaria negative samples, there may be more asymptomatic parasite carriers than expected. A more thorough investigation of the malaria epidemiology, with population-based malaria prevalence surveys, measures of transmission based on entomological inoculation rates and use of high resolution assay for genotyping parasites would present a better picture of parasite population structure at these four epidemiological settings of Malawi.

As observed by Nkhoma *et al* (2013) the central aim of infectious disease control programs is to reduce the size of circulating pathogen populations. Effective control interventions may result in dramatic changes in parasite population structure. For example, a predominantly outbred parasite population characterized by a high prevalence of multiple-clone infections, low inbreeding and a high multiplicity of infection may change to a largely clonal population structure with a low incidence of multiple-clone infections, low multiplicity of infection and high levels of parasite inbreeding. In this study, we found unusually low parasite genetic diversity at the four sites where we

sampled malaria infections. Malawi is generally thought to have intense levels of malaria transmission that occur throughout the year. Under such conditions, we would have expected to see high levels of multiple-clone infections and a higher multiplicity of infection (number of clones per infection). However, the parasite population structure seen was similar to that of low transmission settings in South East Asia and South America. It is possible that the levels of malaria transmission at the four sites studied have waned considerably, generating a predominantly clonal population structure characterized by a low incidence of multiple-clone infections and multiplicity of infection. It is also possible that msp-2 genotyping methods that we used to characterize the population structure of malaria parasites had lower discriminatory power for infection complexity. In future studies of parasite population structure, it will be better to use higher resolution methods for genotyping malaria parasite infections**.** If sufficient markers are used combining multiple measures of transmission including epidemiological and serological markers and population genetic measures will provide the most effective approach to monitoring transmission decline following intervention.

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ANNEXURE

Annex 1

Life cycle/Infection and biology of malaria and Intervention Stages (adapted from DFID 2010)

Understanding the malaria lifecycle is useful for a full appreciation of the complexities of treatment, prevention and surveillance. For example life cycle biology identifies several points where the malaria parasite can be damaged or destroyed. For example, Artemisinin drugs have the ability to target parasites in the erythrocytic stages which prevents the growth and spread of *Plasmodium.*

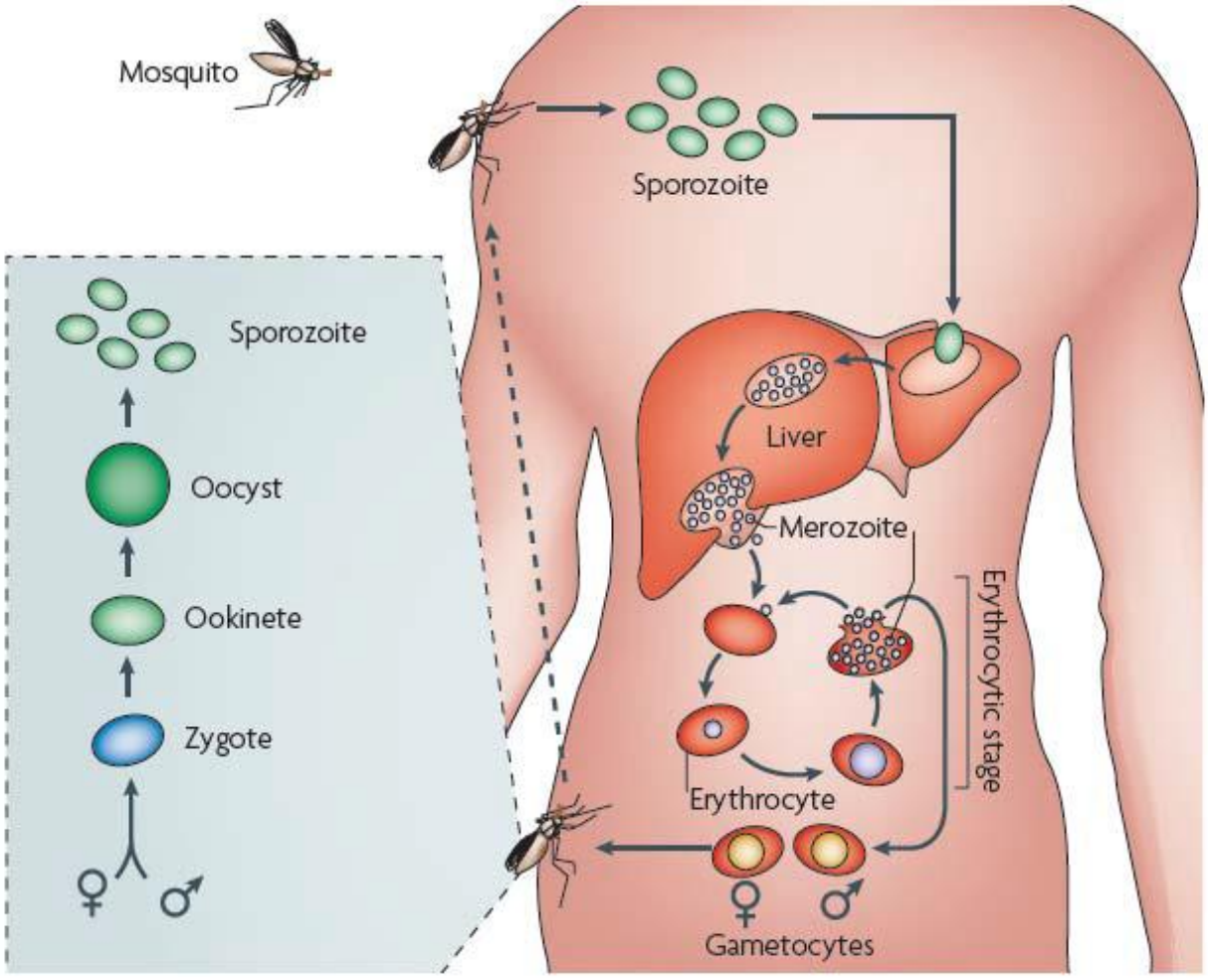
Malaria infects both humans and mosquitoes spending its lifecycle partly in the mosquito and partly in the human host (Figure 1). The mosquito acts as the 'vector' to carry infection from one person to another.

Human stage: When a female *Anopheles* mosquito takes a blood meal on a human, it injects parasites from its salivary glands into the human blood stream. The parasites injected into the human are in their sporozoite form. Sporozoites then enter the liver cells and reproduce. These liver cells eventually rupture and release merozoites into the blood. The human blood stage is when these merozoites invade the red blood cells, reproduce and rupture red blood cells. This is often the stage when clinical features such as fever and chills begin. It is also the stage that is targeted by many antimalarial drugs. Some of the merozoites differentiate into becoming male or female gametocytes.

Mosquito stage: When another *Anopheles* mosquito takes a blood meal from an infected human it will then ingest these gametocytes; microgametocytes (male) and macrogametocytes (female). While in the mosquito gut, the fertilised gametocytes fuse

into a zygote and become ookinetes. Ookinetes which traverses the mosquito gut wall develop into sporozoites-filled oocysts. These oocysts grow, rupture and release more sporozoites. The sporozoites travel up to the mosquito's salivary glands and are injected into the human during another blood meal. Thus, the process begins again.

Figure 1: Life cycle of the *P. falciparum* parasite



It is also important to understand that the life cycle of *P. vivax* is different to that of *P. falciparum*. *Vivax* parasites form a dormant liver stage known as hypnozoites which are

resistant to drugs that target the erythrocytic stages. This makes eradication of *P. vivax* much more difficult using current tools because of the multiple relapses and lack of treatments for the hypnozoite stage.

Mosquito vectors

Malaria is transmitted through the bites of female *Anopheles* mosquitoes. There are over 400 species of *Anopheles* but only about 20 are important as vectors of malaria. All transmission occurs from female mosquitoes which bite between dusk and dawn. There are important differences between vector populations, and these have implications for malaria control. Peak biting times varies by species, which has implications for malaria control- later biting favours ITNs.

Most important vectors bite indoors (endophilic). This favours ITNs and IRS. Where they bite outdoors (exophilic) these control measures are less effective. Most important African vectors are endophilic, but in south-east Asia and Latin America in particular substantial transmission occurs outdoors. It is, however, possible to use nets outdoors for protection from malaria.

Most indoor biting mosquitoes also rest indoors, but not all (endophagic). Where mosquitoes fly in to bite but then fly out IRS is less effective.

Mosquitoes vary considerably in how selective their breeding habits are. Where they only breed in well-defined habitats (e.g. slow-flowing water), they are much more easy to target for larval control. Many African vectors are relatively unselective, making larval control more difficult.

Mosquitoes vary significantly in the probability they will survive a full day- and this has major implications for their efficiency as a vector.

Some anopheline mosquitoes take almost all their blood meals from humans; others take some from humans and some from animals. The higher the proportion from humans the greater their chance of transmitting malaria. Mosquitoes who feed on animals as well as humans may be susceptible to interventions (such as cattle sponging with insecticide) which target the animals.

For malaria transmission to occur there must be sufficient contact between the host and the vector, and the survival of the vector must be long enough for the parasite to complete a life cycle and the vector to become infective. The greater difficulties in controlling malaria in parts of Africa than most of Asia relate to the local vectors, especially *A. gambiae*, *A. aribiensis* and *A. funestus*. They are hardy (so long-lived) and take almost all their blood meals from humans, making them efficient vectors of malaria. They are relatively non-selective in breeding sites, making larval control more difficult. Transmission is most intense when the mosquito prefers to bite humans and in areas where the mosquito is long-lived which allows the parasite to complete its development inside the mosquito. Along with host immunity, the most important factors in malaria transmission are the number (or density) of the mosquito, the life-span of the mosquito and its human biting habits. The *Plasmodium* parasite causes malaria. Of the five species of *plasmodia* affecting humans, *P. falciparum* and *P. vivax* are the most common causes of human malaria. *P falciparum* is by far the most the most common cause of severe illness and death and is the predominant form in Africa. *P vivax* is widespread through Central and South America and Asia but rare in Africa outside Ethiopia. It is less severe

and causes fewer complications than *P. falciparum*, however, it often causes relapses months after the initial infection because it lays down dormant forms (hypnozoites) in the liver which then enter the blood stream months or occasionally years later.[1201] This has implications both for case management and control. The other forms are *P. malariae*, *P. ovale* (similar in many ways to vivax and does occur in Africa), and *P. knowlsei*, a monkey malaria which can infect humans in Oceania. All of these are relatively rare and of limited public health importance compared to *falciparum* (the cause of most mortality) and vivax (the cause of much morbidity outside Africa).

APPENDICES

Appendix 1 Consent form

Title of Research Project: Genetic Diversity and Population structure of *P. falciparum* in Malawi

Principal Investigator: George Paul Selemani

Sponsor :

Study number:_____

Age of participant_____

Medication history in past 2 months_____

Introduction

Malaria is a sickness caused by a very small germ that can get into a person's body when a mosquito bites them. It can cause fevers, headaches, body aches and weakness, and if it is not treated, it can make people very sick. Malaria often affects all people can kill if not treated. This consent form explains a malaria research study you are being asked to join. You have been chosen as a possible participant for this study because the doctor who has examined you thinks you have symptoms of a malaria infection. Please review this form carefully and ask any questions about the study before you agree to join.

WHAT DO WE WANT TO DO?

In the past this country was using SP (also called fansidar) and chloroquine to treat people

suffering from malaria. These drugs are no longer used because they do not cure the malaria.

This is because the parasites that cause malaria can no longer be killed by these drugs as they

have learned to fight these drugs. At the moment this country is using a drug called LA or Lumefantrine-artemether to kill malaria parasites and in that way cure people of their malaria sickness. This drug LA, now works very well. Although things are like this now, we believe that malaria germs may soon learn to fight against LA so that it will also become a useless drug for the treatment of malaria as was the case with the drugs SP and chloroquine. Again, there is a feeling that in order to combat the disease, a vaccine for malaria needs to be introduced. But as reports from other countries are to go by, vaccines for malaria which have been designed have worked in some settings and not in others. This is due to the fact that malaria parasites have been changing their characteristics that the vaccine which works in one area does not work in another. We feel, it is important to start right now looking for differences in the type of malaria that affect different areas and the changes they are undergoing. The aim of this research study is to find out if there are differences in the malaria parasites and how complex these differences are form one area to another. We ask you to take part in this research study to find out if there are such differences in malaria infection and how complex the situation is in Malawi.

HOW ARE WE GOING TO DO OUR WORK?

A small amount of blood (~5 drops) will be taken from your finger onto a glass slide and filter paper. Blood on the glass slide will be used to check if you have malaria parasites in your blood. This is not part of our research—it is necessary to find out if you need to be treated for malaria and how sick you are. If you choose not to take part in this study your blood will be thrown away after diagnosis. But if you agree we shall keep the drop of

blood on a piece of paper so that we can learn about the parasites as said above. We shall ask you to tell us your age or when you were born, whether you took bactrium, or any other drugs used to treat malaria in the last two months.

CAN YOU BE HURT FROM TAKING PART IN THIS STUDY?

There are no foreseeable risks to participants in this study. However, finger sticks can cause a bruise, and very rarely infection or fainting. The methods used to check for the presence of malaria are standard clinical/laboratory practice in all Malawi health care centers. Thus, whether you or your child enrolls into this study or not, a finger prick for malaria diagnosis is needed if you or child exhibits malaria-suggesting symptoms. In that case a trained and licensed healthcare provider will clean your finger with alcohol and use new lancets and needles, so that infection is very unlikely. Usually if fainting occurs, you or your child will receive medical treatment and be observed until you are feeling better according to national guidelines.

WHAT WILL YOU GAIN FROM TAKING PART IN THIS STUDY?

If you accept us to take your blood, you will not gain any money or anything like that. This study will only help us to learn how the malaria parasite is behaving in different areas in Malawi and may increase our understanding of how malaria parasites learn to resist drugs. Again it will provide information on how varied the parasites are spread in Malawi.

YOUR FREEDOM

You are free to refuse or accept participation in this study. You may decide to withdraw your

child's or your consent for participation in our study any time during the procedure described

above or any time during the period of the study from now until end of year 2012. This will not

affect your medical treatment today or any time in the future.

KEEPING YOUR SECRETS

The blood sample collected from you or your child will be given its own study number. Your

name will not be recorded. Your blood will not be subjected to any other tests apart from those described to you here and after this study ends we shall discard and destroy your sample.

WHO CAN YOU TALK TO ABOUT THIS STUDY

Please contact Mr. George Selemani through Dr Fraction Dzinjalamala at the University of Malawi, College of Medicine for any questions or worries you might have about this study. You can call him at +265 8818 70475. The College of Medicine, University of Malawi is located on Mahatma Ghandi road close to Queen Elizabeth Central Hospital. Additional information regarding this research can be obtained from the Secretariat, College of Medicine Research and Ethics Committee if you call at +265 1 877 245/877 291.

Please sign or make your mark below if you agree that you should take part in this study.

Print Name of Subject:_____

_____ _____

Signature or Mark of Subject or Legally Authorized Representative Date

_____ _____

Signature of Person Obtaining Consent Date

_____ _____

Signature of impartial Witness Date

……

……

Participant's Copy

Declaration by or on behalf of Participant

I, the participant and the undersigned	(full names)	Initial
ID number		
OR		
I, in my capacity as	(parent or guardian)	
Of the participant	(full names)	
ID number or fingerprint		
Date		
Give consent to participate in the above mentioned study		

Appendix 2 Informed Consent Form- Chichewa

KALATA YA CHILOLEZO KWA WOFUNA KULOWA MU KAFUKUFUKU

MUTU: KAFUKUFUKU WA KUPIMA KWA TIZIROMBO TAMALUNGO KU MANKHWALA AMALUNGO

WOFUFUZA WAMKULU: George Paul Selemani

WOPEREKA CHITHANDIZO:

Nambala ya afukufuku_____

Zaka zobadwa_____

Mbiri ya mankhwala a malungo pa masiku 30 apitawa_____

MAU OYAMBA

Malungo ndi matenda omwe amayambitsidwa nditizilombo tating'onoting'ono tomwe timalowa m'thupi lamunthu akalumidwa ndi udzudzu. Matendawa amapangitsa kuti munthu adzimva kuzizira, litsipa, kuphwanya m'thupi ndi kufooka. Ngati munthu salandira chithandizo msanga, amadwalika kwambiri. Matendawa amatha kupha, makamaka ana. Chikalata chino chikufotokoza za kafukufuku yemwe tikukufunsani kuti mutengemo mbali. Inu mukhoza kutenga nawo mbali mukafukufukuyi chifukwa chakuti adokotala atatha kukuyezani akuganiza

kuti muli ndi matenda amalungo. Chonde werengani chikalatachi bwino lomwe ndikufunsa mafunso aliwonse omwe mungakhale nawo musanaganize zolowa nawo mukafukufukuyi.

KODI CHOLINGA CHATHU NCHIYANI?

Mmbuyomo dziko lino limagwiritsa ntchito mankhwala a SP (amenenso amatchedwa kuti fansida) ndi kololokwini pofuna kuchiritsa anthu odwala matenda a malungo. Mankhwala awiriwa panopa sakugwiritsidwanso ntchito pa matenda a malungo muno m'Malawi. Izi ziri chonchi chifukwa choti tizirombo toyambitsa matenda a malungo sitikufa mankhwala amenewa akagwiritsidwa ntchito. Tiziromboti tinapima kotero kuti sitingaphedwe ndi mankhwala a SP kapena kololokwini. Pakali pano dziko lino likugwiritsa ntchito mankhwala a LA (Lumefantrine- Aretemether) monga mankhwala ochizira matenda a malungo mwa anthu odwala matendawa. Pakadali pano mankhwala a LA ali ndi mphamvu ndipo akugwira ntchito bwino lomwe pa matenda a malungo. Ngakhale zinthu zili choncho panopa, tili kukhulupilira kuti tizirombo toyambitsa malungo posachedwapa tikhoza kuyamba kupima kotero kuti mankhwala a LA atha kuyamba kulephera kupha tiziromboti monga mmene zinachitikira ndi mankhwala a SP ndi kololokwini. Padakali pano pali maganizo ofuna kupeza katemera wa matenda a malungo. Koma malingana ndi kafukufuku amane anachitika ku maiko ena, katemera ameneyu amatha kugwira ntchito mu madera ena osati enanso. Tikuona kuti ndikofunka kuti tiyambiretu panopa kufufuza ngati tizirombo topima motereti tayamba kale kupezeka mwa wanthu osonyeza zizindikiro za matenda a malungo. Cholinga cha kafukufukuyi ndikufufuza kuti tipeze kuti kodi malungo akusintha bwanji mu madera osiyanasiyana

mu dziko lathu lino. Tikukufunsani inu kuti mulowe mukafukufukuyi kuti tipeze ngati malungo ali osiyanasiyana m'dziko muno.

NDONDOMEKO YA KAFUKUFUKU

Lero magazi apang'ono (madontho asanu) atengedwa kuchokera pachala chanu kuti ayezedwe ngati ali ndi tizilombo ta malungo ndinso kuti aonedwe ngati ali ndi vuto la kuchepa kwa magazi. Zimene zachitikazi simbali imodzi ya kafukufuku wathu ayi komano izi zachitika poti nzofunikila pofuna kudziwa ngati inu muli ndi matenda a malungo kotero kuti musowa kulandira mankhwala a malungo. Ngati inu simusankha kutenga nawo mbali mukafukufukuyi, magaziwa tiwataya. Komano ngati mulora, ife tisunga magazi anu pakapepala kuti tikachotsemo tizirombo tamalungo pofuna kuphunzira za tiziromboto monga tidafotokozera mmwambamu. Tidzakufunsani kuti mutiuze zaka zanu zobadwa kapena chaka nditsiku lomwe mudabadwa. Tidzakufunsani ngati mudamwa mankhwala a bakitirimu (Bactrim) kapena mankhwala ena aliwonse amalungo mumiyezi iwiri yapitayi.

KODI PALI KUOPSYA KWANJI PA INU POLOWA NAWO MUKAFUKUFUKU AMENEYU

Palibe choopsya chenicheni chomwe tikuchiona kuti chingakuchitikireni chifukwa choti mwatenga mbali mukafukufukuyi. Kutenga magazi pa chala kumapangitsa kachilonda kakan'gono ndiponso nthawi zina koma osati kawirikawiri kukomoka kumene. Kachilondaka kamatha kulowedwa ndi tizilombo tina toyambitsa mafinya ngakhale kuti izi sizichitika kawirikawiri. Njira yomwe imagwiritsidwa ntchito kuyeza ngati munthu ali ndi malungo ndi yofanana mzipatala monse m'Malawi muno. Chotero ngakhale mutakana kapena kulora kulowa mukafukufukuyi, kubayidwa pachala kuyenera

kuchitidwa monga mbali yofuna kuyeza magaziwo ndikuwaona ngati ali ndi tizirombo tamalungo. Izi zimachitidwa paonse omwe akuonetsa zizindikiro za matenda amalungo pathupi pawo. Pofuna kupewa majeremusi, ogwira ntchito yachipatala ovomerezedwa ndi boma, amachapa bwinobwino pamalopo pofuna kubayidwapo asanatenge magazi, ndipo chotengera magazicho chimakhala chatsopano. Ngati mwana wanu akomoka potengedwa magazi, adzalandira chithandizo choyenerera ndipo adzamuyang'anira, mpaka atapeza bwino.

KODI INU MUDZAPINDULA BWANJI POLOWA NAWO MUKAFUKUFUKU AMENEYU?

Ngati inu muvomera kutenga nawo mbali mukafukufukuyi potilora kutenga magazi anu, palibe phindu lina lililonse limene mudzapeza ndipo simupatsidwa ndalama ayi. Kafukufukuyi adzathandiza kuti tidziwe ngati pali kusiyana kwa mitundu ya malungo imene ikupezeka mu Malawi muno. Kafukufukuyi adzathandizanso kuonjezera nzeru za momwe tizirombo timalimbiranirana ndi mankhwala amalungo.

UFULU WANU

Muli ndi ufulu onse kulola kapena kukana kulowa mukafukufukuyu. Muli ndiufulu onse omuchotsa mwana wanu kapena otuluka inuyo mukafukufukuyu nthawi ina iliyonse yomwe inu mwafuna. Izi sizidzakhudza malandiridwe anu a chithandizo pachipatala chino lero kapena nthawi ina iliyonse mtsogolo muno.

ZINSINSI ZA MUKAFUKUFUKU

Magazi anu kapena a mwana wanu apatsidwa dzina lakelake monga mwa ndondomeko ya kafukufuku. Dzina lanu sililembedwa pali ponse. Pamagazi amene tatenga kuchoka kwa inu kapena mwana wanu sitidzayeza china chilli chonse koma zokhudza malungo

wokha basi monga tafotokozera m'mwambamu ndipo pamapeto pa kafukufukuyi tidzataya magaziwo.

YEMWE MUTHA KULANKHULA NAYE PA ZOKHUDZA KAFUKUFUKUYU

Ngati muli ndi mafunso kapena madandaulo ena wokhudza kafukufukuyu, mungathe kufunsa kwa Mr. George Selemani kudzera mwa Dr Fraction Dzinjalamala wogwira ntchito ku Deparment of Pharmacy, College of Medicine, University of Malawi. Mutha kuwaimbira pa nambala iyi: 265 881870475. Sukulu ya zachipatala ya College of Medicine, University of Malawi ili panseu wa Mahatma Ghandi pafupi ndi chipatala cha gulupu kapena kuti Kwinzi (Queen Elizabeth Central Hospital). Mukhoza kulandira uphungu wokhudza kafukufukuyi kuchokera ku bungwe la College of Medicine Research and Ethics Committee mutaimba pa nambala iyi: 265 1 877245/877 291.

CHILOLEZO

Ngati mwavomereza kuti mulowe m'kafukufukuyu sainani dzina lanu pansipa. Ndawerenga kapena kuwerengeredwa zones za mchikalatachi ndipo ndamvetsetsa. Ndapatsidwa nthawi yokwanira yofunsa mafunso ena alionse pazomwe sindidamvetse. Ndikumvetsetsa kuti sindili okakamizidwa kutenga nawo mbali mukafukufuku uyu ayi. Ndamvetsetsa kuti ndili ndi ufulu kukana kupitililiza kutenga nawo mbali nthawi ina iliyonse yomwe ine ndafuna.

_____

_____

Dzina lanu Nambala ya Kafukufuku

_____ _____

Saini kapena chidindo cha chala Tsiku

_____

_____

Saini ya mfufuzi Tsiku

_____

_____

Saini ya mboni Tsiku

Umboni ovomereza kutenga nawo mbali

Ine, amene ndasayinira pansipa	(maina onse)	
Chitupa changa		
kapena		
Ine mmalo mwa	(maina a oimilira)	
Amene akutenga nawo mbali	(maina onse)	
Chitupa change kapenda kudinda chala		
Tsiku		
Ndikuvomereza kutenanga nawo mbali mu kafukufukuyu		

Appendix 3- Assent Form

Title of Research Project: Genetic Diversity and Population structure of *P. falciparum* in Malawi
Principal Investigator: George Paul Selemani

Sponsor :

Study number:_____

Age of participant_____

Medication history in past 2 months_____

Introduction

Malaria is a sickness caused by a very small germ that can get into a person's body when a mosquito bites them. It can cause fevers, headaches, body aches and weakness, and if it is not treated, it can make people very sick. Malaria often affects all people can kill if not treated especially children. This consent form explains a malaria research study you are being asked to join. You have been chosen as a possible participant for this study because the doctor who has examined you thinks you have symptoms of a malaria infection. Please review this form carefully and ask any questions about the study before you agree to join.

WHAT DO WE WANT TO DO?

In the past this country was using SP (also called fansidar) and chloroquine to treat people suffering from malaria. These drugs are no longer used because they do not cure the malaria. This is because the parasites that cause malaria can no longer be killed by these drugs as they have learned to fight these drugs. At the moment this country is using a drug called LA or Lumefantrine-artemether to kill malaria parasites and that way cure people of their malaria sickness. This drug LA, now works very well. Although things are

like this now, we believe that malaria germs may soon learn to fight against LA so that it will also become a useless drug for the treatment of malaria as was the case with the drugs SP and chloroquine. Again, there is a feeling that in order to combat the disease, a vaccine for malaria need to be introduced. But as reports from other countries are to go by, vaccines for malaria which have been designed have worked in some settings and not in others. This is due to the fact that malaria parasites have been changing that the vaccine which works in one area does not work in another. We feel it is important to start right now looking for differences in the type of malaria that affect different areas and the changes they are undergoing. The aim of this research study is to find out if there are differences in the malaria parasites and how complex these differences are form one area to another. We ask you to take part in this research study to find out if there are such differences in malaria infection and how complex the situation is in Malawi.

HOW ARE WE GOING TO DO OUR WORK?

A small amount of blood (~5 drops) will be taken from your finger onto a glass slide and filter paper. Blood on the glass slide will be used to check if you have malaria parasites in your blood. This is not part of our research—it is necessary to find out if you need to be treated for malaria and how sick you are. If you choose not to take part in this study your blood will be thrown away after diagnosis. But if you agree we shall keep the drop of blood on a piece of paper so that we can learn about the parasites as said above. We shall ask you to tell us your age or when you were born, whether you took bactrium, or any other drugs used to treat malaria in the last two months.

CAN YOU BE HURT FROM TAKING PART IN THIS STUDY?

There are no foreseeable risks to participants in this study. However, fingersticks can cause a bruise, and very rarely infection or fainting. The methods used to check for the presence of malaria are standard clinical/laboratory practice in all Malawi health care centers. Thus, whether you or your child enrolls into this study or not, a fingerprick for malaria diagnosis is needed if you or child exhibits malaria-suggesting symptoms. In that case a trained and licensed healthcare provider will clean your finger with alcohol and use new lancets and needles, so that infection is very unlikely. Usually if fainting occurs, you or your child will receive medical treatment and be observed until you are feeling better according to national guidelines.

WHAT WILL YOU GAIN FROM TAKING PART IN THIS STUDY?

If you accept us to take your blood, you will not gain any money or anything like that. This study will also help us to learn what drugs are best for use against malaria in Malawi and will increase our understanding of how malaria parasites learn to resist drugs. Again it will provide information on how varied the parasites are spread in Malawi.

YOUR FREEDOM

You are free to refuse or accept participation in this study. You may decide to withdraw your child's or your consent for participation in our study any time during the procedure described above or any time during the period of the study from now until end of year 2012. This will not affect your medical treatment today or any time in the future.

KEEPING YOUR SECRETS

The blood sample collected from you or your child will be given its own study number. Your name will not be recorded. Your blood will not be subjected to any other tests apart

from those described to you here and after this study ends we shall discard and destroy your sample.

WHO CAN YOU TALK TO ABOUT THIS STUDY

Please contact Mr. George Selemani through Dr Fraction Dzinjalamala at the Blantyre Malaria Project for any questions or worries you might have about this study. You can call him at +265 881870475. The College of Medicine, University of Malawi is located on Mahatma Ghandi road close to Queen Elizabeth Central Hospital. Additional information regarding this research can be obtained from the Secretariat, College of Medicine Research and Ethics Committee if you call at 265 1 877 245/877 291.

Please sign or make your mark below if you agree that your child should take part in this study.

Print Name of Subject:_____

_____ _____

Signature or Mark of Subject or Legally Authorized Representative Date

_____ _____

Signature of Person Obtaining Consent Date

_____ _____

Signature of impartial Witness

……

…

Participant's Copy

Declaration by or on behalf of Participant

I, the participant and the undersigned	(full names)	Initial
ID number		
OR		
I, in my capacity as	(parent or guardian)	
Of the participant	(full names)	
ID number or fingerprint		
Date		
Give consent/assent to participate in the above mentioned study		

Appendix 4 Assent Form-Chichewa

KALATA YA CHILOLEZO KWA MWANA WOFUNA KULOWA MU KAFUKUFUKU

MUTU: KAFUKUFUKU WA KUPIMA KWA TIZIROMBO TAMALUNGO KU MANKHWALA A

MALUNGO

WOFUFUZA WAMKULU: George Paul Selemani

WOPEREKA CHITHANDIZO:

Nambala ya afukufuku_____

Zaka zobadwa_____

Mbiri ya mankhwala a malungo pa masiku 30

apitawa_____

MAU OYAMBA

Malungo ndi matenda omwe amayambitsidwa nditizilombo tating'onoting'ono tomwe timalowa m'thupi lamunthu akalumidwa ndi udzudzu. Matendawa amapangitsa kuti munthu adzimva kuzizira, litsipa, kuphwanya m'thupi ndi kufooka. Ngati munthu salandira chithandizo msanga, amadwalika kwambiri. Matendawa amatha kupha, makamaka ana. Chikalata chino chikufotokoza za kafukufuku yemwe tikukufunsani kuti mutengemo mbali. Inu mukhoza kutenga nawo mbali mukafukufukuyi chifukwa chakuti adokotala atatha kukuyezani akuganiza kuti muli ndi matenda amalungo. Chonde werengani chikalatachi bwino lomwe ndikufunsa mafunso aliwonse omwe mungakhale nawo musanaganize zolowa nawo mukafukufukuyi.

KODI CHOLINGA CHATHU NCHIYANI?

Mmbuyomo dziko lino limagwiritsa ntchito mankhwala a SP (amenenso amatchedwa kuti fansidar) ndi kololokwini pofuna kuchiritsa anthu odwala matenda a malungo. Mankhwala awiriwa panopa sakugwiritsidwanso ntchito pa matenda a malungo muno m'Malawi. Izi ziri chonchi chifukwa choti tizirombo toyambitsa matenda a malungo sitikufa mankhwala amenewa akagwiritsidwa ntchito. Tiziromboti tinapima kotero kuti sitingaphedwe ndi mankhwala a SP kapena kololokwini. Pakali pano dziko lino likugwiritsa ntchito mankhwala a LA (Lumefantrine -Aretemether) monga mankhwala ochizira matenda a malungo mwa anthu odwala matendawa. Pakadali pano mankhwala a LA ali ndi mphamvu ndipo akugwira ntchito bwino lomwe pa matenda a malungo. Ngakhale zinthu zili choncho panopa, tili kukhulupilira kuti tizirombo toyambitsa malungo posachedwapa tikhoza kuyamba kupima kotero kuti mankhwala a LA atha kuyamba kulephera kupha tiziromboti monga mmene zinachitikira ndi mankhwala a SP ndi kololokwini. Padakali pano pali maganizo ofuna kupeza katemera wa matenda a malungo. Koma malingana ndi kafukufuku amene anachitika ku maiko ena, katemera ameneyu amatha kugwira ntchito mu madera ena osati enanso. Tikuona kuti ndikofunka kuti tiyambiretu panopa kufufuza ngati tizirombo topima motereti tayamba kale kupezeka mwa wanthu osonyeza zizindikiro za matenda a malungo. Cholinga cha kafukufukuyi ndikufufuza kuti tipeze kuti kodi malungo akusintha bwanji mu Madera osiyanasiyana mu dziko lathu lino. Tikukufunsani inu kuti mulowe mukafukufukuyi kuti tipeze ngati malungo ali osiyanasiyana m'dziko muno.

NDONDOMEKO YA KAFUKUFUKU

Lero magazi apang'ono (madontho asanu) atengedwa kuchokera pachala chanu kuti ayezedwe ngati ali ndi tizilombo ta malungo ndinso kuti aonedwe ngati ali ndi vuto la

kuchepa kwa magazi. Zimene zachitikazi simbali imodzi ya kafukufuku wathu ayi komano izi zachitika poti nzofunikila pofuna kudziwa ngati inu muli ndi matenda a malungo kotero kuti musowa kulandira mankhwala a malungo. Ngati inu simusankha kutenga nawo mbali mukafukufukuyi, magaziwa tiwataya. Komano ngati mulora, ife tisunga magazi anu pakapepala kuti tikachotsemo tizirombo tamalungo pofuna kuphunzira za tiziromboto monga tidafotokozera mmwambamu. Tidzakufunsani kuti mutiuze zaka zanu zobadwa kapena chaka nditsiku lomwe mudabadwa. Tidzakufunsani ngati mudamwa mankhwala a bakitirimu (Bactrim) kapena mankhwala ena aliwonse amalungo mumiyezi iwiri yapitayi.

KODI PALI KUOPSYA KWANJI PA INU POLOWA NAWO MUKAFUKUFUKU AMENEYU

Palibe choopsya chenicheni chomwe tikuchiona kuti chingakuchitikireni chifukwa choti mwatenga mbali mukafukufukuyi. Kutenga magazi pa chala kumapangitsa kachilonda kakan'gono ndiponso nthawi zina koma osati kawirikawiri kukomoka kumene. Kachilondaka kamatha kulowedwa ndi tizilombo tina toyambitsa mafinya ngakhale kuti izi sizichitika kawirikawiri. Njira yomwe imagwiritsidwa ntchito kuyeza ngati munthu ali ndi malungo ndi yofanana mzipatala monse m'Malawi muno. Chotero ngakhale mutakana kapena kulora kulowa mukafukufukuyi, kubayidwa pachala kuyenera kuchitidwa monga mbali yofuna kuyeza magaziwo ndikuwaona ngati ali ndi tizirombo tamalungo. Izi zimachitidwa paonse omwe akuonetsa zizindikiro za matenda amalungo pathupi pawo. Pofuna kupewa majeremusi, ogwira ntchito yachipatala ovomerezedwa ndi boma, amachapa bwinobwino pamalopo pofuna kubayidwapo asanatenge magazi, ndipo chotengera magazicho chimakhala chatsopano. Ngati mwana wanu akomoka potengedwa

magazi, adzalandira chithandizo choyenerera ndipo adzamuyang'anira, mpaka atapeza bwino.

KODI INU MUDZAPINDULA BWANJI POLOWA NAWO MUKAFUKUFUKU AMENEYU?

Ngati inu muvomera kutenga nawo mbali mukafukufukuyi potilora kutenga magazi anu, palibe phindu lina lililonse limene mudzapeza ndipo simupatsidwa ndalama ayi. Kafukufukuyi adzathandiza kuti tidziwe ngati pali kusiyana kwa mitundu ya malungo imene ikupezeka mu Malawi muno. Kafukufukuyi adzathandizanso kuonjezera nzeru za momwe tizirombo timalimbiranirana ndi mankhwala amalungo.

UFULU WANU

Muli ndi ufulu onse kulola kapena kukana kulowa mukafukufukuyu. Muli ndiufulu onse omuchotsa mwana wanu kapena otuluka inuyo mukafukufukuyu nthawi ina iliyonse yomwe inu mwafuna. Izi sizidzakhudza malandiridwe anu a chithandizo pachipatala chino lero kapena nthawi ina iliyonse mtsogolo muno.

ZINSINSI ZA MUKAFUKUFUKU

Magazi anu kapena a mwana wanu apatsidwa dzina lakelake monga mwa ndondomeko ya kafukufuku. Dzina lanu sililembedwa pali ponse. Pamagazi amene tatenga kuchoka kwa inu kapena mwana wanu sitidzayeza china chilli chonse koma zokhudza malungo wokha basi monga tafotokozera m'mwambamu ndipo pamapeto pa kafukufukuyi tidzataya magaziwo.

YEMWE MUTHA KULANKHULA NAYE PA ZOKHUDZA KAFUKUFUKUYU

Ngati muli ndi mafunso kapena madandaulo ena wokhudza kafukufukuyu, mungathe kufunsa kwa Mr. George Selemani kudzera mwa Dr Fraction Dzinjalamala wogwira

ntchito ku Deparment of Pharmacy, College of Medicine, University of Malawi. Mutha kuwaimbira pa nambala iyi: 265 881870475. Sukulu ya zachipatala ya College of Medicine, University of Malawi ili panseu wa Mahatma Ghandi pafupi ndi chipatala cha gulupu kapena kuti Kwinzi (Queen Elizabeth Central Hospital). Mukhoza kulandira uphungu wokhudza kafukufukuyi kuchokera ku bungwe la College of Medicine Research and Ethics Committee mutaimba pa nambala iyi: 265 1 877245/877 291.

CHILOLEZO

Ngati mwavomereza kuti mwana wanu alowe m'kafukufukuyu sainani dzina lanu pansipa. Ndawerenga kapena kuwerengeredwa zones za mchikalatachi ndipo ndamvetsetsa. Ndapatsidwa nthawi yokwanira yofunsa mafunso ena alionse pazomwe sindidamvetse. Ndikumvetsetsa kuti sindili okakamizidwa kutenga nawo mbali mukafukufuku uyu ayi. Ndamvetsetsa kuti ndili ndi ufulu kukana kupitililiza kutenga nawo mbali nthawi ina iliyonse yomwe ine ndafuna.

_____ _____

Dzina la mwana Nambala ya Kafukufuku

_____ _____

Saini kapena chidindo cha chala cha oyang'anira mwana Tsiku

_____ _____

Dzina la oyang'anira mwana

_____

_____

Saini ya mfufuzi Tsiku

_____

_____

Saini ya mboni Tsiku

……

…

Umboni ovomereza

Umboni ovomereza kutenga nawo mbali

Ine, amene ndasayinira pansipa	(maina onse)	
Chitupa changa		
kapena		
Ine mmalo mwa	(maina a oimilira)	
Amene akutenga nawo mbali	(maina onse)	
Chitupa changa kapenda kudinda chala		
Tsiku		
Ndikuvomereza kutenanga nawo mbali mu kafukufukuyu		

APPENDIX 5-REC-H ETHICS APPROVAL FORM

Chairperson of the Research Ethics Committee (Human)
NMMU
Tel: +27 (0)41 504-2235

Ref: [H12-HEA-BTR-003/Approval]

Contact person: Mrs U Spies

25 July 2012

Dr N Smith
NMMU
Faculty of Health Sciences
Biomedical Technology and Radiography
F-Block - F001
North Campus

Dear Dr Smith

GENETITIC DIVERSITY AND POPULATION STRUCTURE OF PLASMODIUM FALCIPARUM IN MALAWI

PRP: Dr N Smith
PI: Mr GP Selemani

Your above-entitled application for ethics approval served at the Research Ethics Committee (Human).

We take pleasure in informing you that the application was approved by the Committee.

The ethics clearance reference number is **H12-HEA-BTR-003**, and is valid for three years. Please inform the REC-H, via your faculty representative, if any changes (particularly in the methodology) occur during this time. An annual affirmation to the effect that the protocols in use are still those for which approval was granted, will be required from you. You will be reminded timeously of this responsibility, and will receive the necessary documentation well in advance of any deadline.

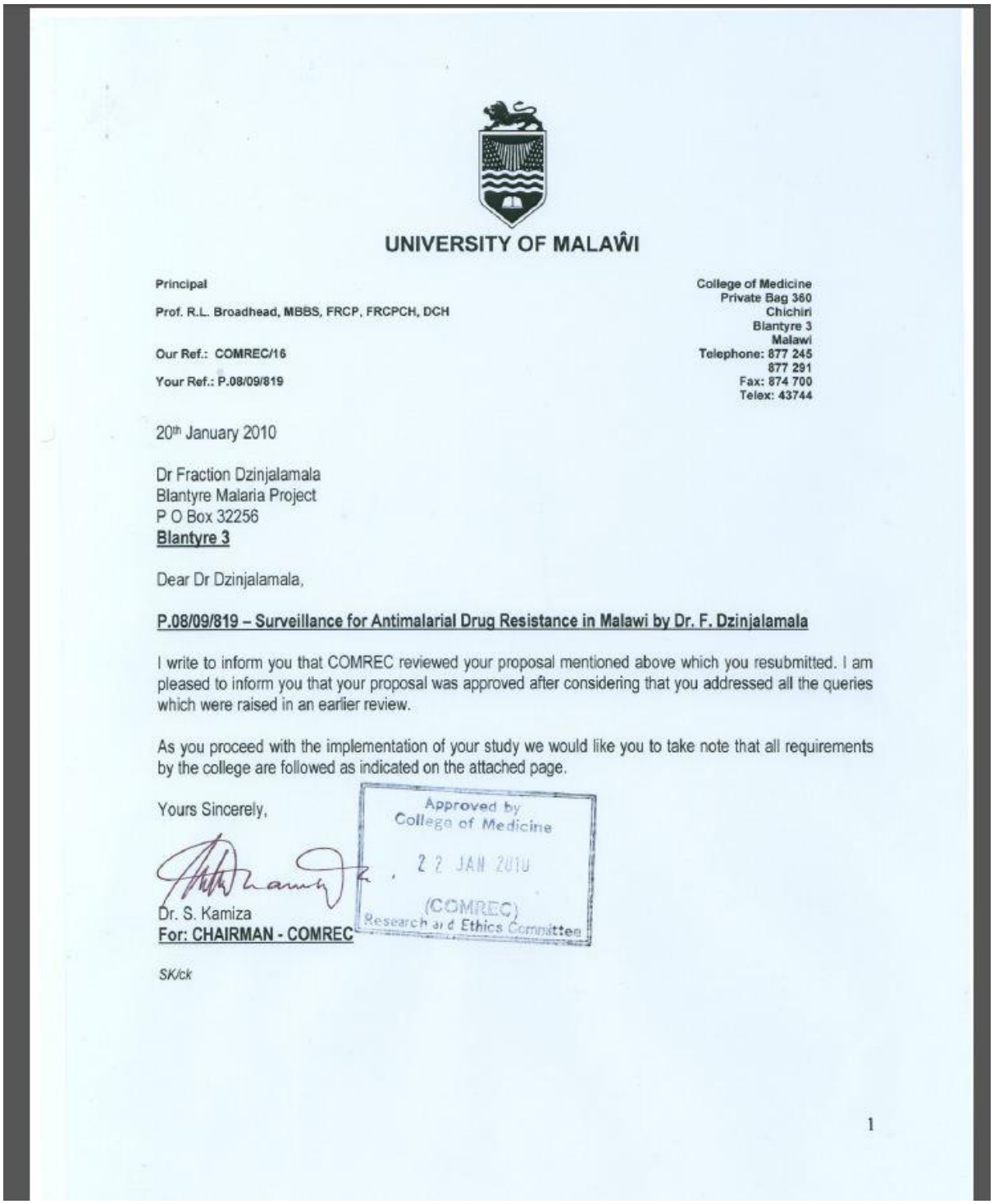
We wish you well with the project. Please inform your co-investigators of the outcome, and convey our best wishes.

Yours sincerely

Prof CB Cilliers
Chairperson: Research Ethics Committee (Human)

cc: Department of Research Capacity Development
Faculty Officer: Faculty of Health Sciences

APPENDIX 6 COMREC (MIM) STUDY APPROVAL FORM



UNIVERSITY OF MALAWI

Principal

Prof. R.L. Broadhead, MBBS, FRCP, FRCPCH, DCH

Our Ref.: COMREC/16

Your Ref.: P.08/09/819

College of Medicine
Private Bag 360
Chichiri
Blantyre 3
Malawi
Telephone: 877 245
877 291
Fax: 874 700
Telex: 43744

20th January 2010

Dr Fraction Dzinjalamala
Blantyre Malaria Project
P O Box 32256
Blantyre 3

Dear Dr Dzinjalamala,

P.08/09/819 – Surveillance for Antimalarial Drug Resistance in Malawi by Dr. F. Dzinjalamala

I write to inform you that COMREC reviewed your proposal mentioned above which you resubmitted. I am pleased to inform you that your proposal was approved after considering that you addressed all the queries which were raised in an earlier review.

As you proceed with the implementation of your study we would like you to take note that all requirements by the college are followed as indicated on the attached page.

Yours Sincerely,

Approved by
College of Medicine
22 JAN 2010
(COMREC)
Research and Ethics Committee

Dr. S. Kamiza
For: CHAIRMAN - COMREC

SK/ck

1