DETECTION AND IDENTIFICATION OF *PLASMODIUM SPECIES* CAUSING MALARIA IN MALAWI USING RAPID DIAGNOSTIC TESTS

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DETECTION AND IDENTIFICATION OF *PLASMODIUM SPECIES* CAUSING MALARIA IN MALAWI USING RAPID DIAGNOSTIC TESTS.

By

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DECLARATION

I, Gerald Loiswayo Tegha, student number 209080060, hereby declare that this dissertation for Magister Technologiae (Biomedical Technology) is my own work and that it has not previously been submitted for assessment or completion of any postgraduate qualification to another university or for another qualification.

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ABSTRACT

Malaria represents one of the oldest documented diseases among humans and even today organisms in the genus *Plasmodium* kill more people than any other infectious disease, especially in tropical and subtropical areas. The four most common species which infect humans are *Plasmodium falciparum*, *Plasmodium vivax, Plasmodium ovale* and *Plasmodium malaria*. Of these four species, *Plasmodium falciparum* and *Plasmodium vivax* account for 95% of infections globally.

Microscopy has been used since early days for the diagnosis of malaria because this method is simple, does not require highly equipped facilities, and in most cases enables differentiation among the species causing malaria in humans when performed by skilled microscopy readers. However, this method has been misleading in identifying parasite species, especially in the case of low level parasitemia, a mixed parasite infection, or modification by drug treatment as well as in placental malaria.

Malaria rapid diagnostic tests (RDT) have played a major role in malaria management; particularly in providing blood based diagnosis in remote locations where microscopy based diagnosis is unavailable. These diagnostic tests are fast and easy to perform and do not require electricity or specific equipment.

As part of strengthening malaria diagnostics in Malawi, the Ministry of Health and Population strongly recommends the use of malaria RDT's at all levels of the health care delivery system. However, malaria microscopy remains a gold standard test for malaria. All patients (regardless of age) with suspected uncomplicated malaria should have a confirmed diagnosis with malaria RDT before anti-malaria treatment is administered. Based on field performance evaluations that assessed performance, quality control and production capacities of the manufacturing companies of malaria RDT's, the Ministry of Health and Population recommended two brands of Histidine Rich Protein 2 (HRP-2), RDT's for use in Malawi. These are SD Bioline malaria Ag *Pf* and the New Paracheck malaria Ag *Pf.* All these RDT's are able to detect only *P.*

falciparum. However, other species have been reported to exist in the country and there is a need to find proper RDT's which will be able to detect all other species including *P. falciparum*.

The main aim of this study was to evaluate Paramax-3 *Pf/Pv/*Pan RDT (Zephyr Biomedicals, India), if used in Malawi, could be able to detect and identify the different species of *Plasmodium* causing malaria in Malawi. The study recruited a total of 250 adult and infants at Bwaila Hospital in Lilongwe, Malawi.

Study results showed that the overall sensitivity and specificity of the Paramax-3 RDT used in the study were 100% and 83% respectively. However, it was observed that the RDT test was not able to identify the *P. ovale*, and in some cases, the RDT test was positive for *P. falciparum* when the PCR identified the species as *P. ovale*. No *P. vivax* was detected both by RDT and PCR. This study was able to detect and identify the presence of *P. malaria* and *P. ovale* in Malawi apart from the *P. falciparum*.

There were no significant differences between microscopy results compared to both the RDT and the PCR, with 94% and 98% sensitivities of R1 and R2 compared to RDT, as well as 94% and 96% sensitivities for R1 and R2 compared to PCR respectively. Both R1 and R2 had low specificities for example, R1 had 72% and R2 had 80% compared to RDT. Comparing R1 and R2 to PCR, the sensitivities were 64.9% and 67.2% respectively. However, the readers had difficulties differentiating the different species microscopically.

The history of anti-malaria treatment had no significant effect on the outcome of the results in both the RDT and PCR.

Keywords: Histidine Rich Protein-2, Malawi, Microscopy, New Paracheck malaria Ag *Pf*, Parasitemia, Paramax-3 RDT, *Plasmodium,* PCR, SD Bioline malaria Ag *Pf*.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ACT   -   Artemisinin based Combination Therapies

AMA   -   Apical Membrane Antigen

BEC   -   Brain Endothelial Cells

CD4   -   Cluster of Differentiation 4

CD36   -   Cluster of Differentiation 36

CDC   -   Center for Disease Control

CHSU   -   Community Health Science Unit

CIDR   -   Cysteine-rich inter domain region

CR1   -   Complement Receptor 1

CSA   -   Chondroitin Surface Antigen

CSF   -   Cerebral Spinal Fluid

CSP   -   Circumsporozoite Protein

CTRP   -   Circumsporozoite TRAP related Protein

DBL   -   Duffy Binding Like domains

DBP   -   Duffy Binding Protein

DBS   -   Dried Blood Spot

DHO   -   District Health Office

dsDNA   -   double stranded Deoxyribonucleic Acid

EBA   -   Erythrocyte Binding Antigen

EDTA	-	Ethylenediaminetetraacetic Acid
EGF	-	Epidermal Growth Factor
FRTI	-	Faculty Research, Training and Innovation
HIV	-	Human Immunodeficiency Virus
HRP-2	-	Histidine Rich protein-2
HSA	-	Health Surveillance Assistant
ICAM-1	-	Intracellular Cell Adhesion Molecule
IgG	-	Immunoglobulin G
IL	-	Interleukin
IPTp	-	Intermittent Preventive Treatment in Pregnancy
ITBN	-	Insecticide Treated Bed Nets
KAHRP	-	Knob-associated Histidine Rich Protein
LA	-	Lumefantrine-Artemether
MESA	-	Mature parasite-infected Erythrocyte Surface Antigen
MRA/MR4	-	Malaria Research and Reference Reagent Resource Centre
MSP-1	-	Merozoite Surface Antigen-1
NHRC	-	National Health Science Research Committee
NIAID	-	National Institute of Allergy and Infectious Diseases
NMCP	-	National Malaria Control Program
NMMU	-	Nelson Mandela Metropolitan University
NO	-	Nitric Oxide

NSO	-	National Statistical Office
PCR	-	Polymerase Chain Reaction
PECAM-1	-	Platelet-Endothelial Cell Adhesion Molecule-1
*Pf*EMP	-	*Plasmodium falciparum* Erythrocyte Membrane Protein
*p*LDH	-	*Plasmodium* Lactate Dehydrogenase
PVM	-	Parasitophorous Vacuolar Membrane
R1	-	Microscopy Reader 1
R2	-	Microscopy Reader 2
R3	-	Microscopy Reader 3
RDT	-	Rapid Diagnostic Test
rRNA	-	Ribosomal Ribonucleic Acid
SOP	-	Standard Operation Procedure
SSP-2	-	Sporozoite Surface Protein-2
TNF	-	Tumour Necrosis Factor
TRAP	-	Thrombospondin-related Adhesion Protein
UNC	-	University of North Carolina
USA	-	United States of America
VCAM-1	-	Vascular Cell Adhesion molecule-1
VSA	-	Variant Surface Antigen
WHO	-	World Health Organisation
ZO-1	-	Zona Occludens-1

CHAPTER 1:   INTRODUCTION

1.1   INTRODUCTION

Malaria, which is caused by *Plasmodium spp.*, represents one of the oldest documented diseases of humans. The organisms in the genus *Plasmodium* are responsible for more human fatalities than any other infectious diseases, especially in tropical and subtropical areas. Globally, an estimated 350-500 million clinical malaria episodes occur annually with more than one million deaths each year. Around 60% of the clinical episodes and more than 80% of the deaths occur in young children in Africa, south of the Sahara, where malaria accounts for 25-35% of all outpatients, 20-45% of hospital admissions and 15-35% of hospital deaths (Bremen, 2001; Oduro et al. 2007; WHO Malaria Report, 2009).

Prompt and correct diagnosis of malaria is a mainstay for saving a patient's life. Therefore, one of the key principles of the Roll Back Malaria initiative of the WHO is the early detection of malaria parasites. Diagnosis based on symptoms alone, without parasitological confirmation, may lead to a lack of treatment or inappropriate treatment, the former allowing increased disease burden and continued transmission, the latter leading to under diagnosis of other treatable diseases (Metzger et al. 2007; Reyburn et al. 2007).

The WHO recently strengthened its recommendation for parasite-based diagnosis of malaria prior to treatment with anti-malaria medication. Accurate diagnosis enables targeting of anti-malaria treatment to those who will benefit and early detection of non-malaria fever requiring alternative treatment and management. Reducing medication wastage, in addition to saving money and conserving stocks of artemisinin based combination therapies (ACT) may prolong the usefulness of ACT's globally by reducing pressure towards resistance (Chandramohan, Jaffar & Greenwood, 2002; Reyburn, Mbatia, Drakeley, Carneiro & Mwakasungura, 2004; WHO Malaria Case Management Operations Manual, 2010).

The four most common species of *Plasmodium* which infect humans are *Plasmodium falciparum*, *Plasmodium vivax, Plasmodium ovale* and *Plasmodium malaria*. Of these four species, globally *Plasmodium falciparum* and *Plasmodium vivax* account for 95% of infections. Since the discovery of malaria parasites by Laveran in 1880, malaria has traditionally been diagnosed by microscopically examining Giemsa or Field Stained blood films. This method is simple as it does not require highly equipped facilities and in most cases it enables differentiation among the species causing malaria in humans when performed by a skilled microscopist. However, this method has been misleading in identifying parasite species, especially in the case of low level parasitemia, mixed parasite infection, or modification by drug treatment (Garcia, 2001; O'Meara et al. 2005; Coleman et al. 2006; Endeshaw et al. 2008; Hanscheid, Frita, Langin, Kremsner & Grobusch, 2009). In pregnancy, for example, *Plasmodium falciparum* infection of the placenta frequently occurs in the absence of parasites in the peripheral blood (Mankhambo, Kanjala, Rudman, Lema & Rogerson, 2002).

Rapid point-of-care tests are routinely used for several diseases including Human Immunodeficiency Virus (HIV), Syphilis and Hepatitis, replacing centralized laboratory testing. Over the past two decades, Malaria Rapid Diagnostic Tests (RDT's) have played a major role in malaria management; particularly in providing blood based diagnosis in remote locations where microscopy based diagnosis is unavailable. These diagnostic tests are fast and easy to perform and do not require electricity or specific equipment (Wongsrichanalai, 2001; Moody, 2002; Bell & Peeling, 2006). They are all based on the same principle and use antibodies that detect specific groups of antigens.

RDT's are built on a nitrocellulose platform and are available in dipstick or cassette format. Signals are visible as coloured lines, comprising a control line (which indicates that the test has been performed well) and one or two test lines (Gillet et al. 2009). Provision of universal access to parasite-based diagnosis for populations at risk of malaria therefore depend on the wide use of these RDT's; point of care tests first introduced in 1993 with ParaSight-F test (Thiam et al. 2011).

Automated real-time Polymerase Chain Reaction (PCR) methods are a recent development in PCR methodology. Real-time PCR uses fluorescent binding dyes, such as SYBR Green, or fluorescent probes for continuous monitoring of amplicon formation throughout the reaction. The advantages of these techniques are quantification of pathogens, reduced risk of laboratory contamination and rapidity. In malaria, most of the real-time PCR assays are performed using primers and probes of the conserved region of the *Plasmodium* 18S ribosomal Ribonucleic Acid (rRNA) of all four human malaria species (Duy Vo et al. 2007).

Available literature suggests that mixed infections detected by PCR are around 20% in Malawi. *Plasmodium falciparum* is the most frequently detected and *Plasmodium malaria* is the second most commonly detected, followed by *Plasmodium ovale.* There are no published studies indicating the prevalence of *Plasmodium vivax* in Malawi despite being listed by the WHO as one of the species causing malaria in Malawi (Bruce et al. 2008; WHO Malaria Report, 2009). With the migration of people from other malaria endemic areas into Malawi and other Southern African countries, there have been reports of an increase in cases of other species of *Plasmodium* infections detected by malaria RDT's other than the traditional *Plasmodium falciparum* widely common in this part of the world*.* However, in Malawi, most of the malaria RDT's being used in most clinics only detect and identify the *Plasmodium falciparum* (*Pf*) specific histidine rich protein-2 (*Pf* HRP-2) in human whole blood. This poses a serious problem of misdiagnosis when individuals suffering from other types of malaria, apart from falciparum malaria present themselves at these clinics.

Therefore this study aimed at detecting and identifying the diverse *Plasmodium* species in Malawi using RDT as well as PCR. A qualitative PCR was used to confirm the species identified by rapid diagnostic tests.

1.2   AIM AND OBJECTIVES OF THE STUDY

The primary aim of the study was to detect and identify the common species of *Plasmodium* causing malaria in Malawi.

1.2.1   Specific objectives of the study

The specific objectives of the study were:

- Detection of *Plasmodium* in the diagnosis of malaria using RDT.

- Identification of *Plasmodium falciparum, Plasmodium vivax* and other *Plasmodium* species causing malaria prevalent in Malawi using RDT.

- Determination of accuracy of RDT in identifying *Plasmodium* species causing malaria in Malawi using real time PCR as a confirmatory test.

CHAPTER 2:   LITERATURE REVIEW

2.1   INTRODUCTION

Traditionally, 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.

Some of the known medical writings in China, Assyria and India accurately describe the malaria-like intermittent fevers. As stated by Wiser in 2009, Hippocrates in 500 BC is generally credited with the first description of the clinical symptoms of malaria, more than 2000 years before the parasite and the lifecycle were described as shown in table 2.1 below.

Table 2.1: Historical highlights of malaria (adapted from Wiser, 2009)

YEAR	PERSON	DESCRIBED
200BC	Hippocrates	Clinical symptoms
1880	Laveran	Blood stage parasite
1898	Ross	Mosquito transmission
1948	Garnham	Liver stage of the lifecycle

Malaria parasites belong to the group of Hemosporidia, members of which as the name implies, are parasitic in the blood of vertebrates. They all use dipteran insects as their vectors. The malaria parasites of mammals all belong to the genus *Plasmodium*, which belongs to the phylum Apicomplexa. It is distinguished by the presence of an apical

complex at some stage in the lifecycle and is transmitted by female mosquitoes belonging to the genus *Anopheles* (Knell, 1991). The major transmission route is through a bite from an infective female anopheles mosquito. However, other modes of transmission (NMCP Kenya, 2009) include:

- Trophozoites introduced through blood transfusion.

- Congenital (trans-placental), however rarely.

- Sharing of needles as in drug addicts.

- Under experimental conditions through bites and by an infecting emulsion of salivary glands containing sporozoites.

Malaria has a worldwide distribution, 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. *P. falciparum* is the predominant specie in most endemic areas, with the exception being India and South America where *P. vivax* is more common. *P. ovale* is mainly found in West Africa whilst *P. malaria* is commonly found together with either *P. falciparum* or *P. vivax* (Ashley, McGready,Proux & Nosten, 2005).
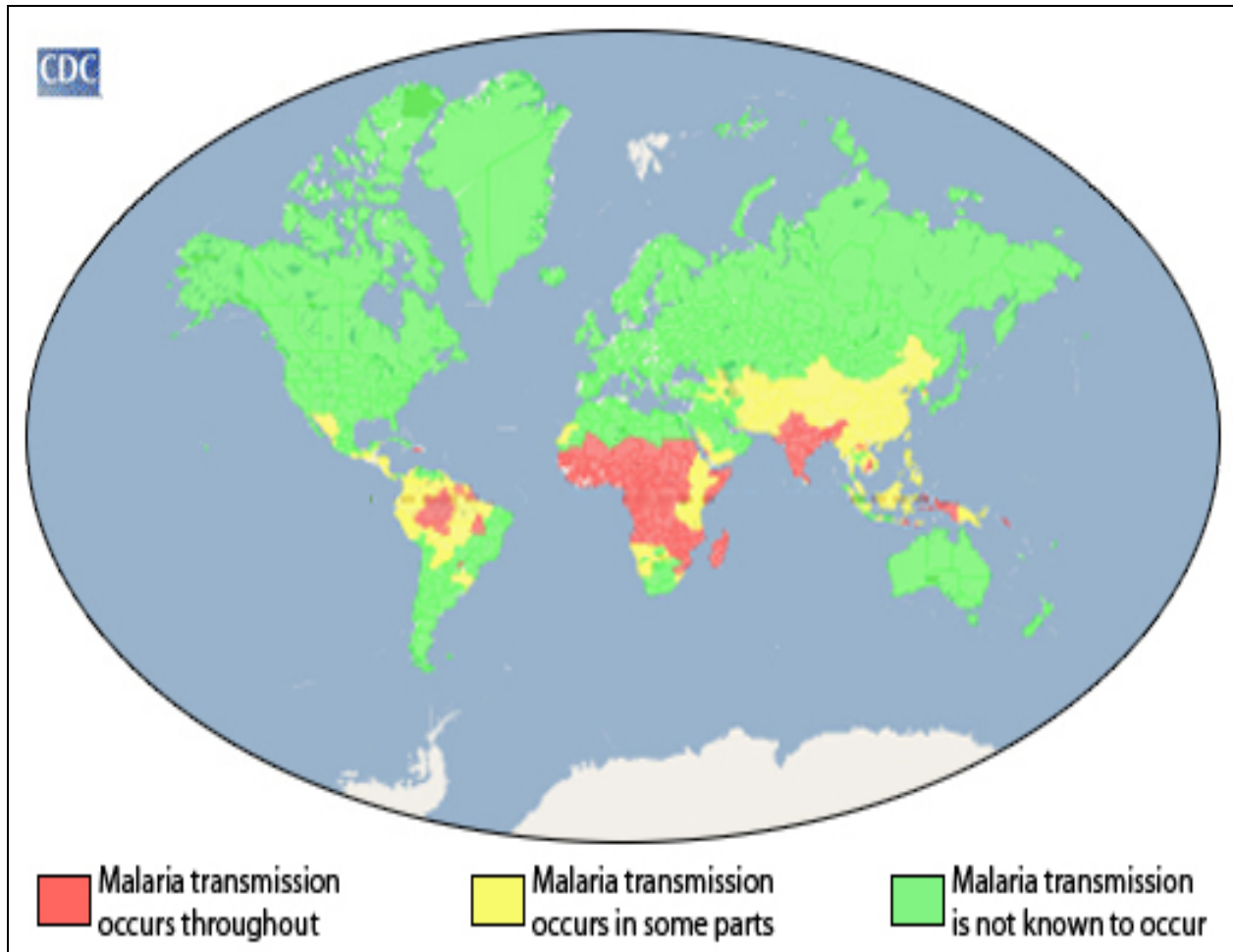
Figure 2.1: World map showing malaria endemic areas (CDC, 2010)

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 country located in the southern Africa region and has a population of approximately 13.1 million people (NSO, 2008). It is bordered by Mozambique in the east and south, Zambia in the west and Tanzania in the north (as shown in figure 2.2 and 2.3 below). These countries are situated in the malaria endemic regions as shown in the World Malaria Map (figure 2.1) above.
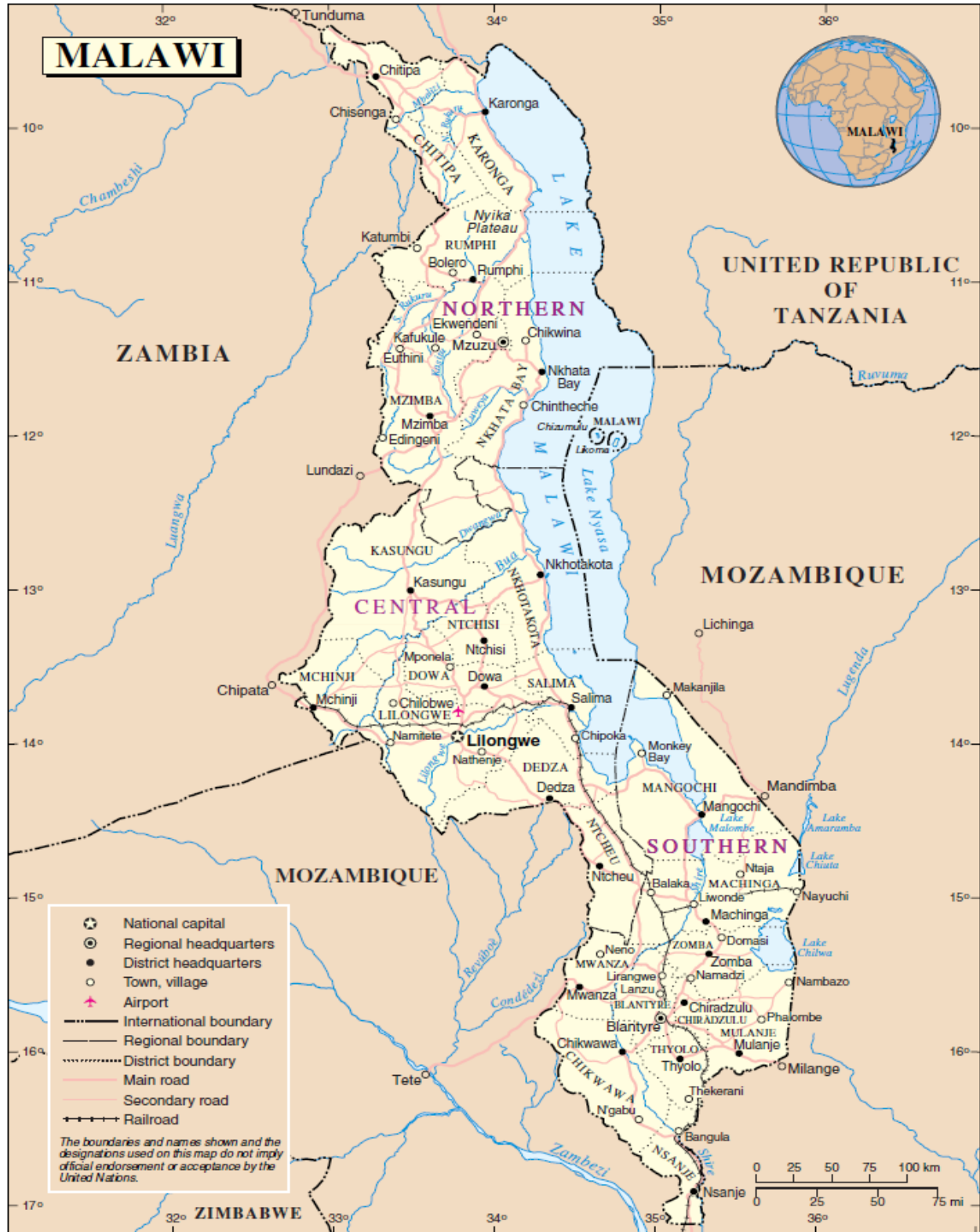
Figure 2.2: Map of Malawi showing major districts (adapted from United Nations, 2004)

Figure 2.3: Africa map showing the location of Malawi (adapted from World Atlas, 2010)

2.2 PLASMODIUM

The *Plasmodium* parasite causes malaria disease in humans. The parasite belongs to the domain Eukaryota, kingdom Chromalveolata, superphylum Alveolata, phylum Apicomplexa, class Aconoidasida, order haemosporidia, family Plasmodiidae and genus *Plasmodium* (Baun, 2006). Some forms of the *Plasmodium* parasite are shown in the blood smear in figure 2.4 below:
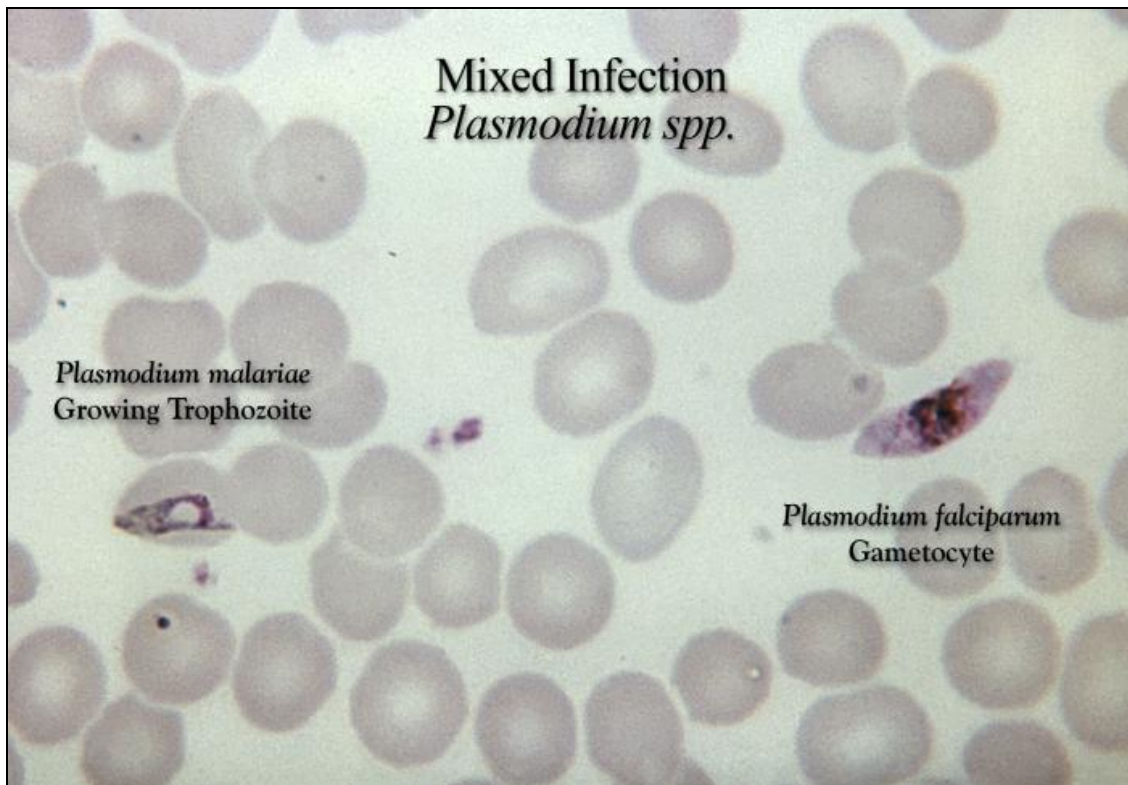
Figure 2.4: *Plasmodium* in blood (adapted from CDC, 2010)

The basic components of the malaria parasite (*Plasmodium*) inside the red blood cell of an infected host includes the chromatin, cytoplasm, vacuole and pigments as shown in figure 2.5 below.
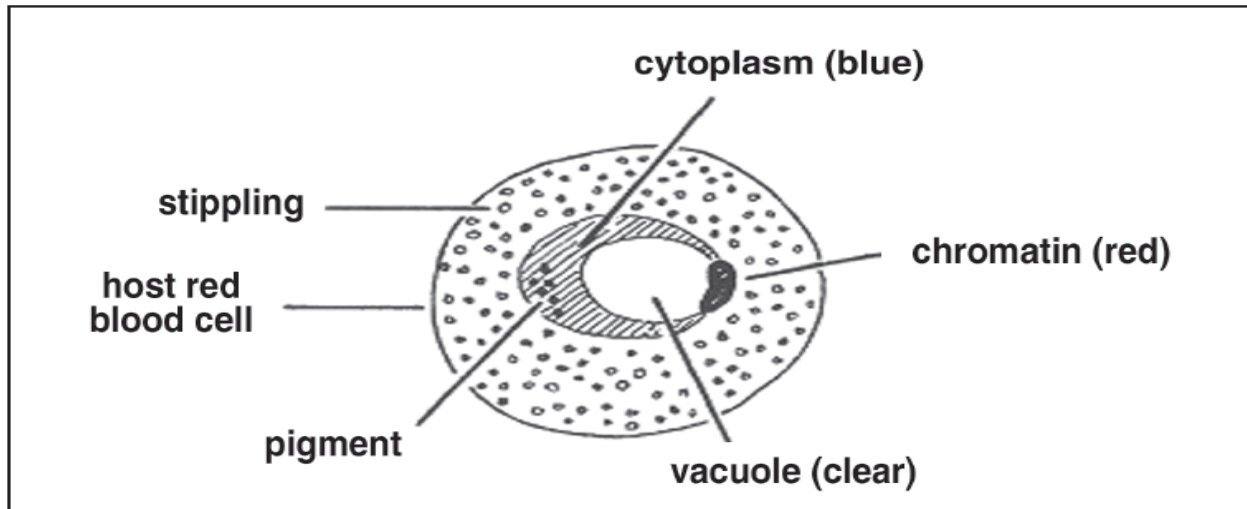
Figure 2.5: Basic components of *Plasmodium* (NMCP Kenya, 2009).

2.2.1 Lifecycle of *Plasmodium*

The infection in humans is acquired when sporozoites are injected with the saliva during a mosquito feeding. The sporozoites invade liver cells under an exoerythrocytic schizogony resulting in the production of merozoites. In *P. vivax* and *P. ovale,* some of the exoerythrocytic schizonts undergo a dormant period known as the hypnozoite stage. Merozoites are released from the infected liver cells and invade erythrocytes undergoing an erythrocytic stage schizogony thereby producing more merozoites which can re-invade new erythrocytes (NIAID, 2009).

Alternatively, some merozoites will undergo gametocytogenesis and produce either macrogametocytes or microgametocytes. The gametocytes are infective for the mosquito and when ingested will produce macrogametes and microgametes. The zygote resulting from the fusion of the gametes develops into an ookinete which will penetrate the gut epithelial cells and develop into an oocyst and later undergo sporogony. The resulting sporozoites invade the salivary glands and are infective for the human host (Wiser, 2009).

The lifecycle of the *Plasmodium*, in both human and mosquitoes, as explained by NIAID (2009), is shown in figure 2.6 below followed by a step by step description of each stage:
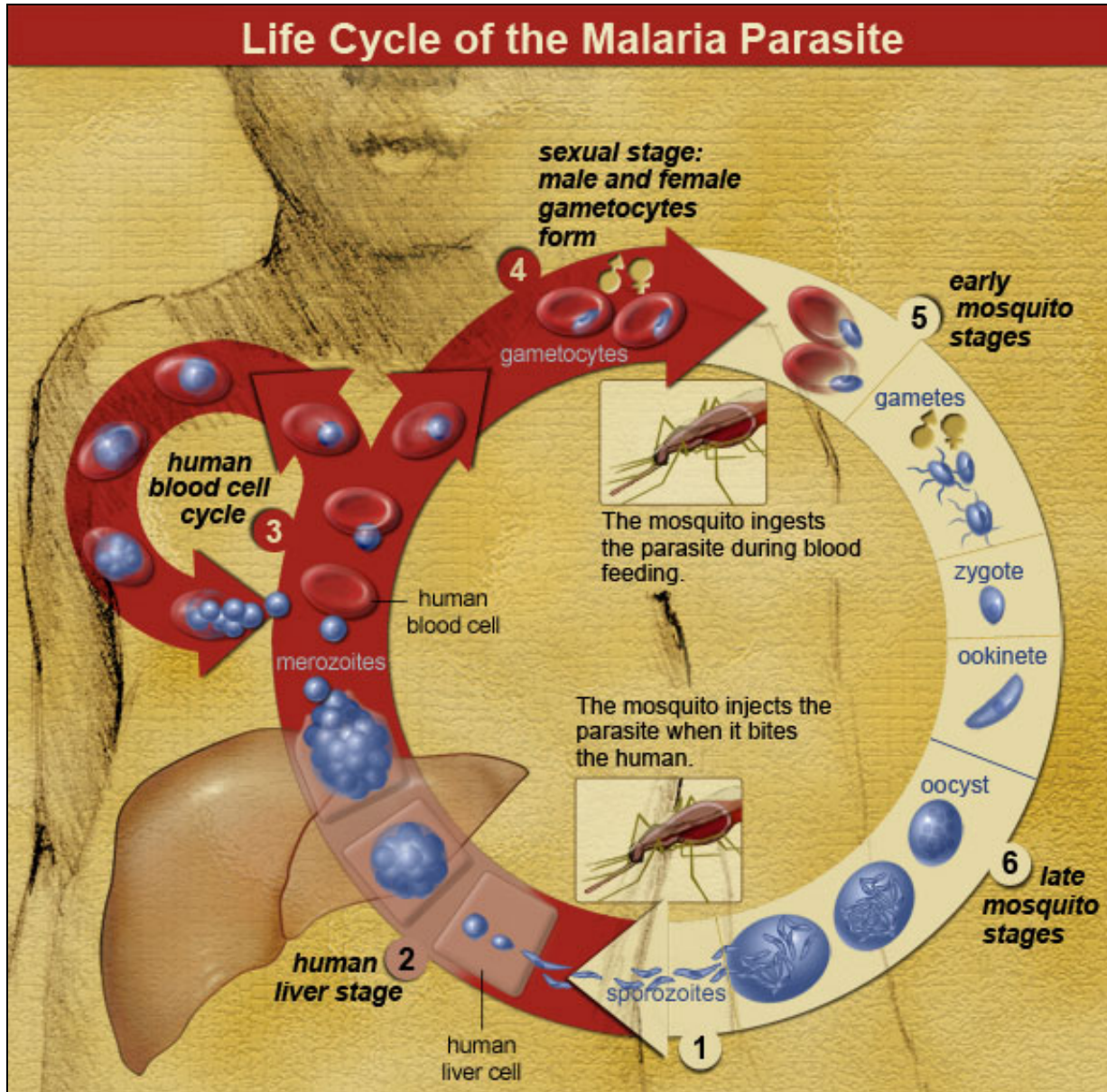
Figure 2.6: Lifecycle of *Plasmodium* in humans and mosquitoes (NIAID, 2009)

1.   A female Anopheles mosquito carrying malaria-causing parasites feeds on a human and injects the parasites in the form of sporozoites into the bloodstream. The sporozoites travel to the liver and invade liver cells.

2. During a period of five to sixteen days, the sporozoites grow, divide and produce tens of thousands of haploid forms, called merozoites per liver cell. Some malaria parasite species remain dormant for extended periods in the liver, causing relapses weeks or months later.

3. The merozoites exit the liver cells and re-enter the bloodstream, beginning a cycle of invasion of erythrocytes, asexual replication and release of newly formed merozoites from the erythrocytes repeatedly during a period of one to three days. This multiplication can result in thousands of parasite-infected cells in the host bloodstream, leading to illness and complications of malaria that can last for months if not treated.

4. Some of the merozoite-infected blood cells leave the cycle of asexual multiplication. Instead of replicating, the merozoites in these cells develop into sexual forms of the parasite, called male and female gametocytes, which circulate in the bloodstream.

5. When a mosquito bites an infected human, it ingests the gametocytes. In the mosquito gut the infected human blood cells burst, releasing the gametocytes which develop further into mature sex cells called gametes. Male and female gametes fuse to form diploid zygotes, which develop into actively moving ookinetes that burrow into the mosquito midgut wall and form oocysts.

Growth and division of each oocyst produces thousands of active haploid forms called sporozoites. After eight to fifteen days, the oocyst bursts, releasing sporozoites into the body cavity of the mosquito, from which they travel to and invade the mosquito salivary glands. The cycle of human infection begins again when the mosquito takes a blood meal, injecting the sporozoites from its salivary glands into the human bloodstream (NIAID, 2009; Wiser, 2009).

2.2.2 *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).

2.2.2.1 *Plasmodium falciparum*

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 *Plasmodium 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).

2.2.2.2 *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 *Plasmodium vivax* infection, because the Duffy blood group antigen is rare

in this population and this is the erythrocyte molecule to which the *Plasmodium vivax* merozoites bind (Markell & Voge, 1981; Knell, 1991).

2.2.2.3 *Plasmodium malaria*

Plasmodium malaria occurs primarily in those subtropical and temperate areas where other species of malaria are found and generally has a much lower incidence than *Plasmodium vivax* and *Plasmodium falciparum*. As described by Markell and Voge (1981), in the thick smear trophozoites of *Plasmodium malaria* 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.

2.2.2.4 *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 *Plasmodium 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 *Plasmodium vivax* and the nuclei in all stages are larger than corresponding stages of that species (Markell & Voge, 1981).

Tables 2.2a, b and c below show features of the malaria parasites that distinguish them in peripheral blood (Baun, 2006).

Table 2.2a: Features differentiating the four species of *Plasmodium* in blood (adapted from Baun, 2006)
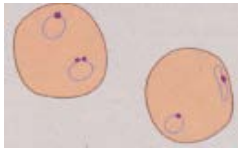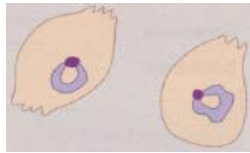
	PLASMODIUM MALARIA	*PLASMODIUM FALCIPARUM*	*PLASMODIUM OVALE*	*PLASMODIUM VIVAX*
General features and changes in erythrocytes	Cells not enlarged, sometimes contracted; staining characteristics not altered; lowest degree of parasitemia; multiple parasites per cell rare; no stippling unless over-stained; all stages usually present.	Cells not enlarged; staining characteristics usually unaltered but sometimes there are some pale cells; sometimes multiple parasites per cell and heavy parasitemia (10-40% of cells); often only ring form are present.	Red cells enlarged but not as much as *P. vivax*; cells pale and some are oval or pear-shaped; some are ragged at one or both ends; fine to coarse red stippling (Schuffner's dots); low parasitemia; often fewer than with *P. vivax*.	Cells much enlarged, irregular and pale with fine, red stippling (Schuffner's dots); usually low or moderate parasitemia; all stages of life cycle often present; sometimes multiple parasites per cell.
Early trophozoites (ring form)	Small, thick, compact rings; small chromatin dot which may be inside the ring. Double dots and accole (shoulder) forms rare.	Delicate rings, at least ¼ the diameter of the erythrocyte. Double dots and accole forms common.	Thick compact rings, at least ½ the diameter of the erythrocyte. Numerous Schuffner's dots but paler than *P. vivax.*	Thick rings, at least ½ the diameter of the erythrocyte. A few Schuffner's dots; Accole forms and double dots less common than with *P. falciparum.*

Table 2.2b: Features differentiating species of *Plasmodium* in blood (adapted from Baun, 2006)
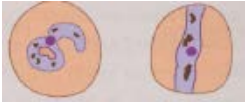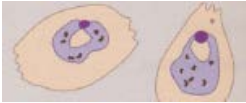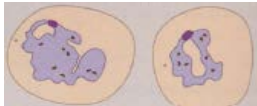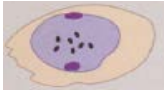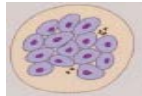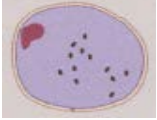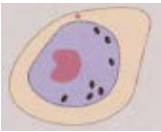
	PLASMODIUM MALARIA	*PLASMODIUM FALCIPARUM*	*PLASMODIUM OVALE*	*PLASMODIUM VIVAX*
Mature trophozoite	Ameboid form more compact than *P. vivax*; sometimes angular or band forms. Heavy, dark yellow brown pigment; no stippling unless over-stained.	Fairly delicate rings, at least ½ the diameter of the erythrocyte. Red-mauve stippling (Maurer's dots or clefts) present; mature trophozoites are less often seen in blood than ring forms.	Thick rings, less irregular than those of *P. vivax*; at least ½ the diameter of the erythrocyte. Less prominent vacuole; distinct outline; yellowish brown pigment which is coarser and darker than that of *P. vivax*; Schuffner's dots prominent.	Ameboid rings, ½-¾ the diameter of the erythrocyte; pale blue or lilac parasite with prominent central vacuole. Indistinct outline; scattered fine yellowish brown pigment granules or rods.
Early schizont	Compact, round, fills erythrocyte. Coarse dark yellow-brown pigment.	Not usually seen in blood; very small, ameboid; scattered light brown to black pigment.	Round, compact; darkish brown pigment, heavier and coarser than that of *P. vivax*, Schuffner's dots.	Rounded or irregular; ameboid; mass of fine yellow-brown pigment, Schizont almost fills cell; Schuffner's dots.
Late schizont	6-12 large merozoites, arranged in rosette or daisy head formation. Coarse dark yellowish brown pigment.	Not usually seen in blood. 8-32 very small merozoites; grouped irregularly; peripheral clump of coarse dark brown pigment.	6-12 large merozoites arranged irregularly. Central pigment; Schuffner's dots.	12-24 medium sized merozoites. Schizont almost fills the cell; Schuffner's dots.

Table 2.2c: Features differentiating the four species of *Plasmodium* in blood (adapted from Baun, 2006)
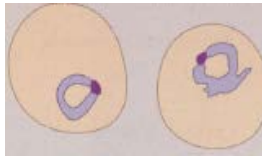
	PLASMODIUM MALARIA	*PLASMODIUM FALCIPARUM*	*PLASMODIUM OVALE*	*PLASMODIUM VIVAX*
Macrogametocyte	Similar to *P. vivax* but smaller, round or oval, almost fills cell; blue with a dark nucleus. Prominent pigment; concentrated at centre of periphery.	Sickle or crescent shaped; deforms cell which often appears empty of hemoglobin. Blue cytoplasm; compact central nucleus with pigment aggregated around it.	Similar to *P. vivax* but somewhat smaller. Pigment coarser and blacker, scattered but mainly near the periphery.	Round or ovoid, almost fills enlarged cell; blue cytoplasm. Eccentric compact red nucleus; scattered pigment.
Microgametocyte	Similar to *P. vivax* but smaller, pink or paler blue than microgametocyte with a larger paler nucleus. Prominent pigment.	Oval or crescent with blunted ends; pale blue or pink. Large pale nucleus with pigment more scattered compared to macrogametocyte.	Similar to *P. vivax* but smaller.	Round or ovoid, as large as a normal erythrocyte but does not fill the enlarged cell. Faintly staining; larger, lighter red central or eccentric nucleus; fine, scattered pigment.

2.3   PATHOGENESIS

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 malaria* 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).

Anemia 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).

Bone marrow suppression also plays an important role in the pathogenesis of malarial anemia. The normal response to hemolytic anemia 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 2.7 below is a schematic diagram showing some of the possible mediators in cerebral malaria.
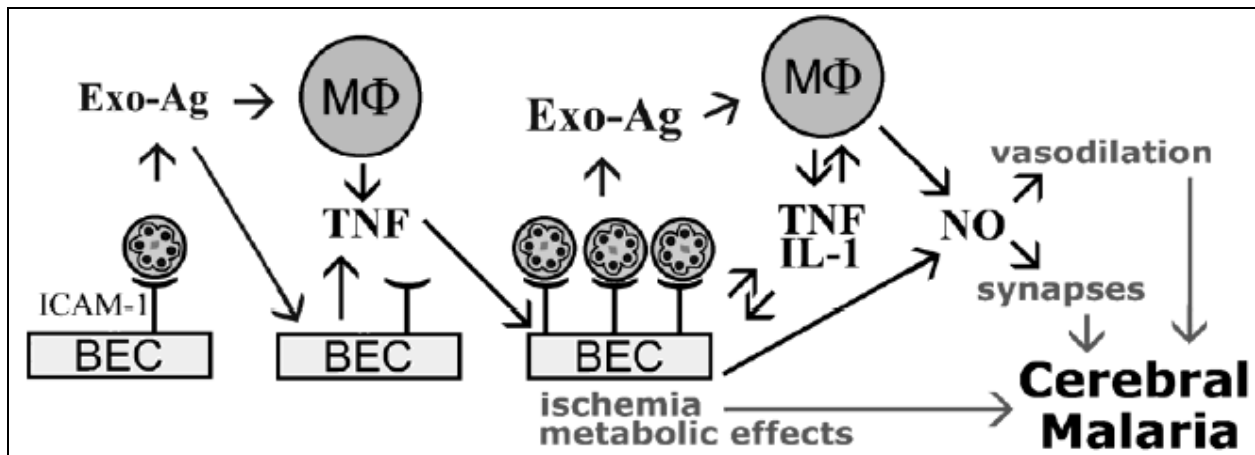
Figure 2.7: Mediators of cerebral malaria (Wiser, 2009)

The cytoadherence of infected erythrocytes to brain endothelial cells (BEC) and the release of the exo-antigens stimulate the BEC and immune effecter cells such as macrophages (Mϕ) to secret cytokines. These cytokines, such as tumor necrosis factor-α (TNF) would lead to an increased expression of possible endothelial cell receptors (e.g. ICAM-1) and promote an increase cytoadherence of infected erythrocytes. Large numbers of bound infected erythrocytes could lead to vascular blockage and hypoxia and have localized metabolic effects (e.g. hypoglycemia, lactic acidosis). TNF-α is also known to stimulate nitric oxide (NO) which can affect neuronal function by interfering with neurotransmission and causing vasodilation (Hunt et al. 2006; Wiser, 2009).

Patients who survive cerebral malaria may suffer from long term mental and psychological deficits (Boivin, 2002).

Renal complications are common and may present as acute renal failure due to the effects of sequestrated infected erythrocytes or with nephrotic syndrome, due to deposition of antigen-antibody complexes within glomeruli.

P. falciparum can also result in severe anemia, low birth weight and maternal death during pregnancy (WHO Malaria and HIV, 2005).

Figure 2.8 below gives a summary of the pathogenesis of malaria.
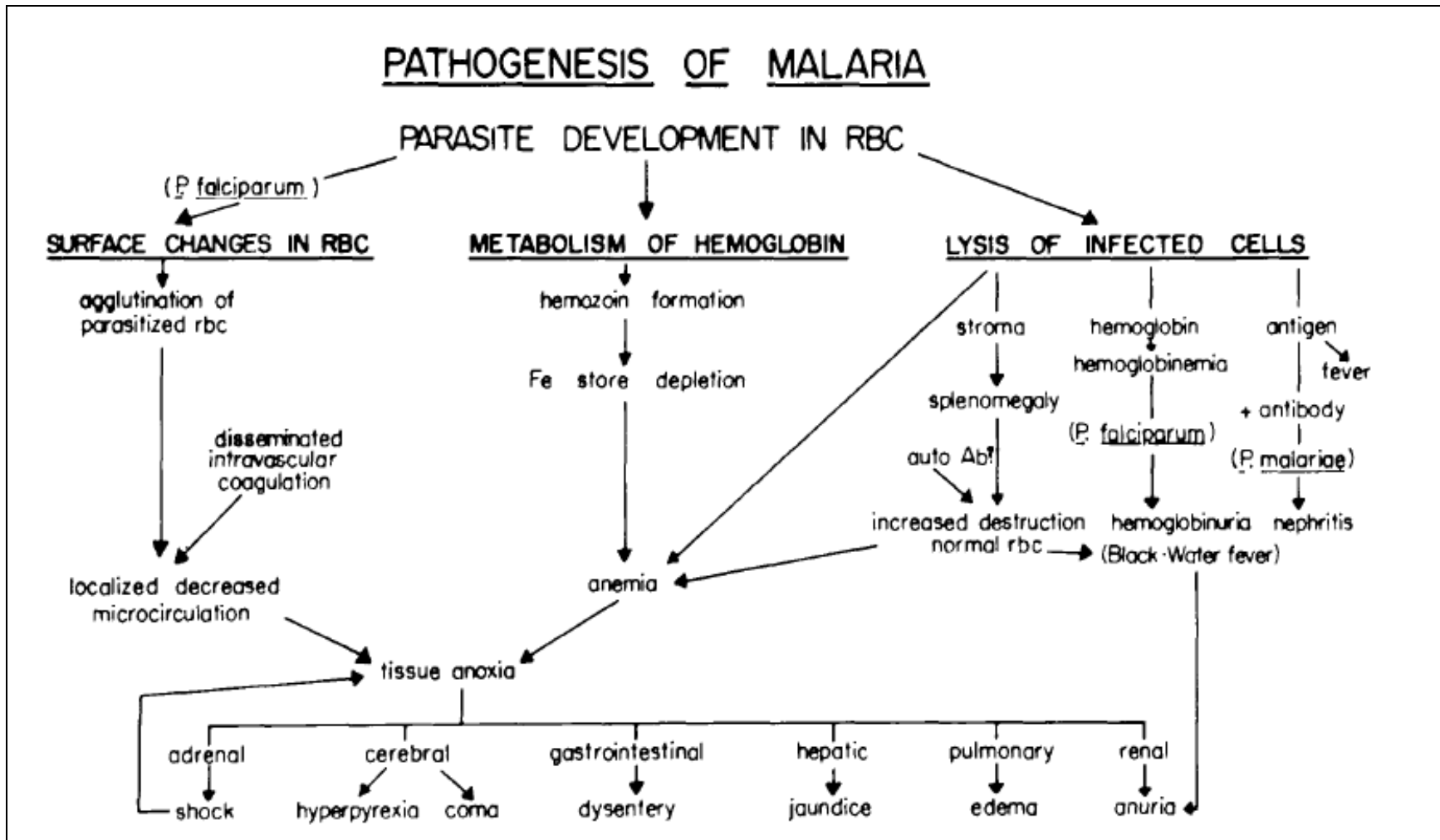
Figure 2.8: Pathogenesis of malaria (Markell & Voge, 1981)

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2.4 CELLULAR AND MOLECULAR BIOLOGY OF *PLASMODIUM*

This section discusses the detailed cellular and molecular biology of the *Plasmodium* parasite. This is important as most assays target the specific proteins or antigens present in the parasite or released by the host infected cells for its identification.

Members of the genus *Plasmodium* are eukaryotic microbes. Therefore, the cell and molecular biology of *Plasmodium* will be similar to other eukaryotes. 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).

2.4.1 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.

2.4.1.1   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-1does 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).

2.4.1.2 Reorientation 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).

2.4.1.3   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 micromenes 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 as shown in table 2.3 below

Table 2.3: Antigens involved in receptor/ligand interaction (adapted from Wiser, 2009)

Receptor/Ligand Interaction		
Species	Host Receptor	Merozoite Ligand
P. falciparum	Glycophorins (Sialic acid)	EBA-175
P. vivax	Duffy Antigen	DBP

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 2.9 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 2.9 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).
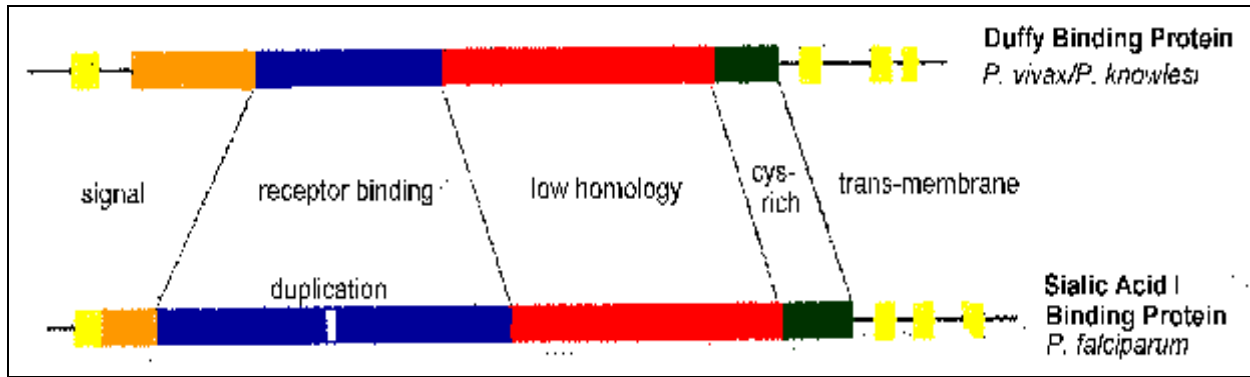
Figure 2.9: Receptor binding activity (Wiser, 2009)

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 trans-membrane domains at their C-termini (Tomley & Soldati, 2001).

In summary:

- an electron-dense junction (arrow in figure 2.10 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.
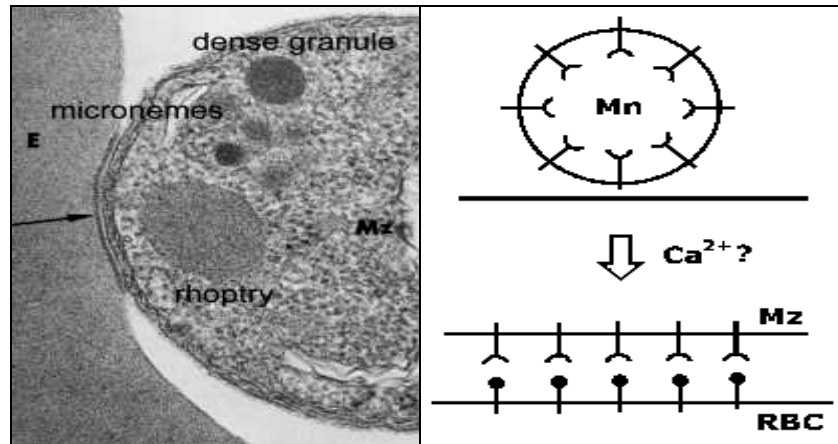
Figure 2.10: Micrograph showing binding activity

(Aikawa, Miller, Johnson & Rabbege, 1978)

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).

2.4.1.4 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 sub-membrane 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

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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).

2.4.2 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.

2.4.2.1   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 2.11 below. Their exact roles in knob formation are not known, but may involve reorganizing the sub-membrane exoskeleton
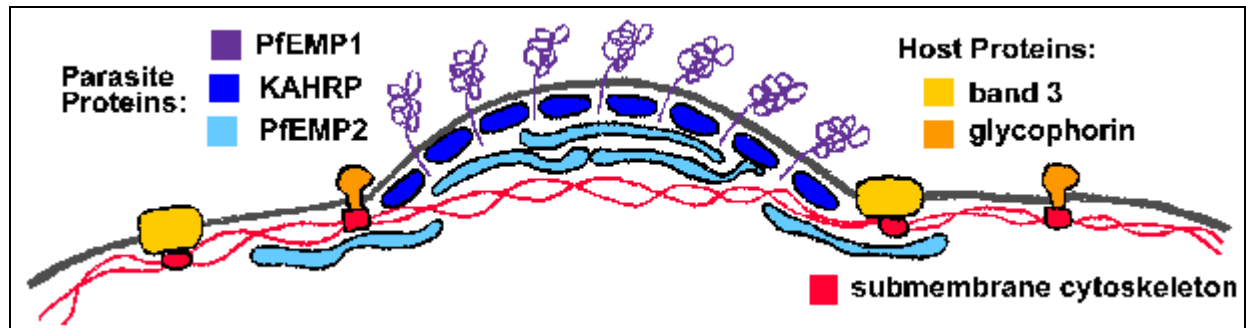
Figure 2.11: Knob formation (Wiser, 2009)

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 2.12), 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).
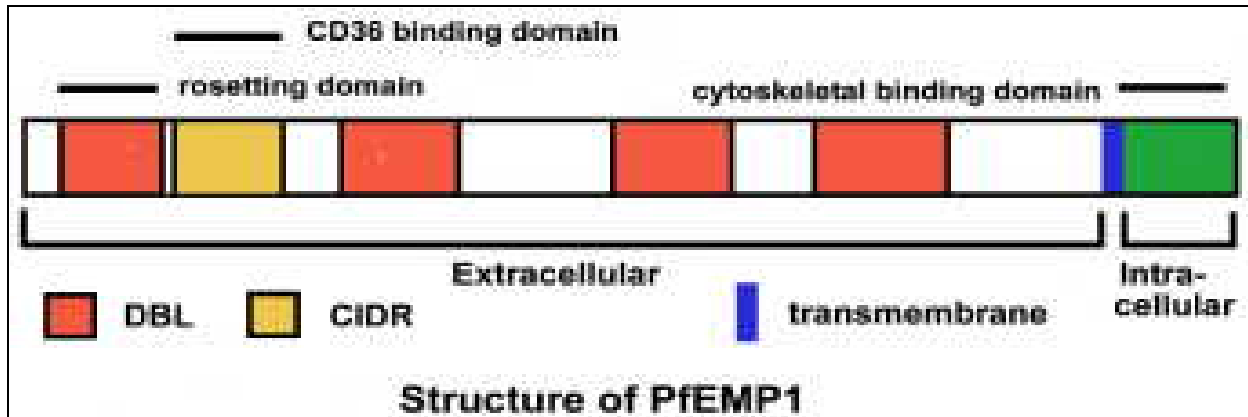
Figure 2.12: Structure of *Pf*EMP1 (Smith, Gamain, Baruch & Kyes, 2001)

*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 2.12 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).

2.4.2.2 Endothelial Cell Receptors

Several possible endothelial receptors (table 2.4 below) have been identified by testing the ability of infected erythrocytes to bind in static adherence assays (Beeson & Brown, 2002).

Table 2.4: 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 2.5 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 2.5: Binding Receptors (adapted from Craig & Scheif, 2001)

Binding Phenotypes	
Domain	**Receptor**
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).

2.4.2.3   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).

2.5   CLINICAL MANIFESTATION OF MALARIA

Symptoms of malaria usually start to appear 10-15 days after the bite of an infected mosquito. The typical pre-patent and incubation periods following sporozoite inoculation vary according to species. The pre-patent period is defined as the time between sporozoite inoculation and the appearance of parasites in the blood and represents the duration of the liver stage and the number of merozoites produced. Incubation periods tend to be a little longer and are defined as the time between sporozoite inoculation and the onset of symptoms. Sometimes the incubation periods can be prolonged for several months in *P. vivax, P. ovale* and *P. malaria*. All four species can exhibit non-specific prodromal symptoms a few days before the first febrile attack. These prodromal symptoms are generally described as 'flu-like' and include: headache, slight fever, muscle pain, anorexia, nausea and lassitude. The symptoms tend to correlate with increasing numbers of parasites (Jerrard et al. 2002).

These prodromal symptoms will be followed by febrile attacks also known as malarial paroxysms. These paroxysms will exhibit periodicities of 48 hours for *P. vivax*, *P. ovale* and *P. falciparum* and a 72-hour periodicity for *P. malaria*. Initially the periodicity of these paroxyms may be irregular as the broods of merozoites from different exoerythrocytic schizonts synchronize. This is especially true in *P. falciparum* which may not exhibit distinct paroxysms, but exhibit a continuous fever, daily attacks or irregular attacks (e.g. 36-48 hour periodicity). Patients may also exhibit splenomegaly, hepatomegaly (slight jaundice) and hemolytic anemia during the period in which the malaria paroxysms occur (Wijesekera, Carter, Rathnayake & Mendis, 1996; Karunaweera, Wijesekera, Wanasekera, Mendis & Carter, 2003).

Table 2.6: Malaria Paroxysms (adapted from Wiser, 2009)

Malaria Paroxysms		
Cold Stage	**Hot Stage**	**Sweating Stage**
Feeling intense cold	Intense heat	Profuse sweating
Vigorous shivering	Dry burning heat	Declining temperature
Lasts 15-60 minutes	Throbbing headache	Exhausted and weak – sleeping
	Lasts 2 - 6 hours	Last 2 – 4 hours

The malarial paroxysm as shown in table 2.6 above will usually last 4-8 hours and begins with a sudden onset of chills in which the patient experiences an intense feeling of cold despite having an elevated temperature. This is often referred to as the cold stage and is characterized by a vigorous shivering. Immediately following this cold stage is the hot stage. The patient feels an intense heat accompanied by severe headache. Fatigue, dizziness, anorexia, myalgia and nausea will often be associated with the hot stage. Next, a period of profuse sweating will ensue and the fever will start to decline. The patient is exhausted and weak and will usually fall asleep. Upon awakening the patient usually feels well, other than being tired and does not exhibit symptoms until the onset of the next paroxysm (Jerrard et al. 2002; Wiser, 2009).

2.6 PREVENTION AND CONTROL OF MALARIA

Strategies for preventing and controlling malaria involve three different approaches as shown in table 2.7 below. Prevention of malaria in individuals will generally involve the reduction of human-mosquito contact through the use of bed nets, repellents, etc.

Table 2.7: Malaria control and prevention strategies

CONTROL AND PREVENTION STRATEGIES		
Reduce human mosquito contact	**Reduce vector density**	**Reduce parasite reservoir**
• Impregnated bed nets • Repellant, protective clothing • Screens, house spraying	• Environmental modification • Larvicides/insecticides • Biological control	• Case detection and treatment • Chemoprophylaxis

Chemoprophylaxis, as shown in table 2.7 above, can also be used especially by travelers. However chemoprophylaxis only suppresses parasitemia and does not prevent infection (CDC, 1990).

Control activities at the community level can utilize approaches which directly reduce human-mosquito contact as well as approaches which reduce the total number of mosquitoes in an area. Such approaches include the reduction in mosquito breeding grounds (e.g. environmental modification), targeting the larva stages with chemical or biological agents and massive insecticide spraying for the adult mosquitoes. Biological control methods include the introduction of fish which eat the mosquito larvae or the introduction of bacteria (e.g. Bacillus thuringiensis) which excrete larval toxins. Case detection and treatment is another potential control method. Identifying and treating infected persons, especially asymptomatic individuals, will reduce the size of the parasite reservoir within the human population and can lower transmission rates. However, this can be a relatively expensive approach (Paul et al. 1998).

These approaches are not mutually exclusive and can be combined. Many of the successful control programs include both measures to control mosquitoes and

treatment of infected individuals. There is no standard method of malaria control that has proven universally effective. The epidemiologic, socioeconomic, cultural and infrastructural factors of a particular region will determine the most appropriate malaria control. Some of the factors which need to be considered include:

- infrastructure of existing health care services and other resources;

- intensity and periodicity, for example, seasonality of transmission;

- mosquito species (ecological requirements, behavioral characteristics, insecticide sensitivity etc.;

- parasite species and medication sensitivities; and

- cultural and social characteristics of the population.

The control of malaria in tropical Africa including Malawi has been particularly problematic because of the high transmission rates and the overall low socio-economic level. Several studies have shown that insecticide treated bed nets (ITBN) reduce the morbidity and mortality associated with malaria. In most areas the introduction of bed nets does not require large promotional programs and their use is readily accepted. This may be partly due to the reduction in mosquito nuisance biting. Some questions have been raised regarding the economic sustainability of bed net programs. It is necessary to re-treat the bed nets with insecticide periodically and the bed nets need to be repaired and replaced as they become torn and wear out. In addition, some people have raised concerns about the long-term benefits of bed nets since they reduce exposure, but do not eliminate it. This reduction in exposure may delay the acquisition of immunity and simply postpone morbidity and mortality in older age groups (Paul et al. 1998; Lengeler, 2004).

2.7 TREATMENT OF MALARIA

Several types of medication are available for the treatment of malaria. However, many factors are involved in deciding the best treatment for malaria. These factors include:

- the parasite species;

- the severity of the disease;

- the patient's age and immune status;

- the parasite's susceptibility to the medication (i.e. drug resistance); and

- the cost and availability of medication.

Therefore the exact recommendations will often vary according to geographical region (Bloland, Ettling & Meek, 2000; Bousema et al. 2003; WHO Guidelines for treatment of malaria, 2010). In addition, the various types of medication act differently on the different life cycle stages. Table 2.8 below gives a summary of some selected drugs.

Fast acting blood schizontocides, which act upon the blood stage of the parasite are used to treat acute infections and to quickly relieve the clinical symptoms. Chloroquine is generally the recommended treatment for patients with *P. vivax, P. ovale* and *P. malaria* and uncomplicated chloroquine sensitive *P. falciparum* infections. Patients infected with either *P. vivax* or *P. ovale*, and who are at high risk for re-infection, should be treated with primaquine (a tissue schizontocide). Primaquine is effective against the liver stage of the parasites, including the hypnozoites and will prevent future relapses. The combination of chloroquine and primaquine is often called 'radical cure' (Bousema et al. 2003).

Table 2.8: Selected anti-malaria drugs (adapted from Bloland, Ettling & Meek, 2000; Bousema et al. 2003; WHO Guidelines for treatment of malaria, 2010).

DRUG CLASS	EXAMPLE
Fast acting blood schizontocide	Chloroquine (plus other four aminoquinolines), quinine, quinidine, mefloquine, halofantrine, antifolates (pyrimethamine, proquanil, sulfadoxine, dapsone), artemisin derivatives
Slow acting blood schizontocide	Doxycycline (plus other tetracycline antibiotics)
Blood and mild tissue schizontocide	Proquanil, pyrimethamine, tetracycline
Tissue schizontocide (anti-relapsing)	Primaquine, tafenoquine
Gametocidal	Primaquine, artemisin derivatives, 4-aminoquinolines
Combinations	Fansidar® (pyrimethamine + sulfadoxine), Meloprim® (pyrimethamine + dapsone), Malarone® (atovaquone + proquanil), artemisin combination therapy (ACT)

The efficacy of chloroquine has been greatly diminished by the wide spread chloroquine resistance of *P. falciparum* and the emergence of chloroquine resistant *P. vivax*. If chloroquine is not effective, or if in an area with chloroquine resistant malaria, common alternative treatments include: mefloquine, quinine in combination with doxycycline or Fansidar®. Derivatives of artemisinin (dihydroartemisinin, artesunate and artemether) are now recommended as the first line treatment by the WHO. Medication used in

combination with artemisinin, include mefloquine, lumefantrine, Fansidar® and amodiaquine (Flueck et al. 2000; WHO Guidelines for treatment of malaria, 2010).

In Malawi, The Ministry of Health introduced Lumefantrine-Artemether (LA), as the first-line medication in the treatment of uncomplicated malaria, Amodiaquine-Artesunate as second-line, with Quinine for the treatment of severe malaria cases and for the management of malaria in pregnancy. The introduction of these medications was in line with the WHO recommendation to use Artemisinin combination therapies (ACT's) in order to improve malaria treatment and prolong the therapeutic life of future anti-malaria medication. Sulphadoxine-Pyrimethamine (SP) remains the recommended treatment for preventive treatment of malaria in pregnancy (IPTp) (Kalirani-Phiri, Lungu & Coghlan, 2011; NMCP Malawi, 2011).

2.8 MALARIA AND HIV

Human Immunodeficiency Virus (HIV) and malaria are major causes of morbidity and mortality in sub-Saharan Africa (Lopez, Mathers, Ezzati, Jamison & Murray, 2006). Within the region, there is widespread overlap in the distribution of the two diseases (WHO Malaria and HIV, 2004). Thus, any interaction between the two diseases may have potentially important public health implications. There is evidence that HIV infection influences susceptibility to, and the clinical course of malaria. Studies in non-pregnant (Chirenda, Siziya & Tshimanga, 2000; Francesconi et al. 2000; Whitworth et al. 2000; Cohen et al. 2005; Patnaik et al. 2005) and pregnant adults (Steketee et al. 1996; Verhoeff et al. 1999; Ladner et al. 2002) suggest that HIV infection is associated with more frequent episodes of clinical malaria and higher parasite density.

Naturally acquired immunity to malaria is dependent on exposure. Thus, in malaria endemic areas, immunity to severe disease, mild disease and parasitemia normally increases with age (Marsh & Kinyanjui, 2006). A recent report from Kilifi, Kenya suggested that HIV infection is associated with hospital admission for severe malaria among children. Importantly, those infected with HIV were older (median age, 38

months; IQR, 26–63 months) than those without HIV infection (median age, 19 months; IQR, 10– 35 months; P <0.001). HIV-infected children had higher peripheral parasite density when corrected for age. Despite the overall strong association between HIV infection and severe malaria, there was no relationship between HIV and severe malaria in infancy (Berkley et al. 2009).

Cohort studies have shown that malaria infection increases plasma viral load, even during asymptomatic parasitemia (Kublin et al. 2005). It has also been demonstrated that CD4+ T lymphocytes reduce temporarily during malarial episodes in HIV infected and uninfected patients, and repeated malaria infections are associated with a more rapid reduction in CD4+ T lymphocytes over time, suggesting that malaria may lead to faster disease progression from HIV to AIDS (Geertuyden et al. 2006; Mermin et al. 2006).

HIV co-infected women have higher placental parasite densities and higher rates of antenatal malaria transmission than HIV uninfected women. Maternal antibody surface antigens (VSA's) on malaria infected erythrocytes play a very important role in pregnancy related immunity to malaria. Blood from HIV infected mothers analysed contained fewer antibodies to VSA's in both placental and pediatric isolates of malaria than did blood from HIV uninfected mothers (Dembo et al. 2008).

Other studies showed that malaria infection during pregnancy increased the risk of mother-to-child transmission of HIV. One placental mechanism for this was evaluated in vitro, where binding of recombinant *P. falciparum* adhesin to chondroitin sulphate A on human placental cells increased HIV-1 replication in those cells, possibly via TNF-α-stimulation (Ayouba et al. 2003; Ayisi et al. 2004; Ayouba et al. 2008).

CHAPTER 3:   MANAGEMENT OF THE STUDY

3.1   RESEARCH DESIGN

Study participants were recruited from patients seeking treatment at Bwaila Hospital out-patient department and the under-five clinic situated in Lilongwe District, Malawi. Enrolment criteria included the following:

- Age limit equal or above 2 years old.

- Out-patients presenting with signs and symptoms of malaria but not very sick.

- Ability to give consent or assent.

The participants were to adhere to the routine clinical and laboratory procedures as stipulated by the Ministry of Health and Population in Malawi in the diagnosis of malaria. The procedure was as follows:

- Participants reporting at the clinic were seen by clinicians who ordered a malaria blood film for suspected malaria. These participants were then referred to the Bwaila Hospital Laboratory for malaria testing.

- Informed consent (Appendix I or Appendix II) was obtained from each participant willing to join the study after meeting the eligibility criteria.

- Venous blood (3 ml) was drawn from all participants who consented and formed part of the study. Out of this blood, a drop was used to make a thick blood smear, another drop was used for RDT and the remaining blood was used to make five dried blood spots (DBS) on protein saver filter papers and 0.5ml plasma for storage.

- The thick blood smear for microscopy was used as a laboratory result in the diagnosis of malaria for these participants by the clinician.

EDTA blood samples were collected from two hundred and fifty (250) participants and processed at Bwaila Hospital and UNC Project Research Laboratories situated in Lilongwe, Malawi. Field stain A and B (Appendix V) was used to stain the thick smear which was read by two experienced microscopists independently. In the case of discordant results, a third reader was identified to read the slide. A difference of positive and negative results was taken as a discordant, whilst a difference in grading of positive results did not require a third reader. In this case, results from the first reader were released to the participant immediately for patient care.

Malaria RDT testing was done in Bwaila Hospital Laboratory immediately after microscopy testing. However, DBS cards and plasma processing were done in UNC Project Laboratory located at Kamuzu Central Hospital. PCR testing was done at Steve Meshnick Laboratory at UNC Chapel Hill in the United States of America from the blood stored on DBS cards.

Other demographic data such as age, sex, country of origin and location of where the participant lived, were also collected. Figure 3.1 below is the flow chart outlining procedures followed during the study, from enrolment to sample processing and testing:

```
┌─────────────────────────────────────┐
│  Patients  presenting  with  signs  and │
│  symptoms of malaria visited the clinic │
└─────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────┐
│  Eligibility  checklist  verified;  Informed │
│  Consent/Assent  obtained  from  eligible │
│  participants                         │
└─────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────┐
│  Demographic  data  and  Venous  blood │
│  collection and thick blood film preparation. │
└─────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────┐
│  Staining  and  reading  of  thick  blood  film │
│  and release results immediately       │
└─────────────────────────────────────┘

┌──────────────────────────┐      ┌──────────────────────────┐
│  RDT  performed  on  venous │      │  DBS card preparation  and │
│  EDTA  blood  and  results │      │  plasma processing         │
│  recorded                  │      └──────────────────────────┘
└──────────────────────────┘

         ┌──────────────────────────────────────┐
         │  Real  time  PCR  done  in  batches  and │
         │  results recorded                      │
         └──────────────────────────────────────┘
```

Figure 3.1: Flow chart outlining study procedures

3.1.1 Ethical consideration

The study was conducted at Bwaila Hospital, which is under the Lilongwe District Health Office of the Ministry of Health and Population in Malawi. The Lilongwe District Health Officer approved the study (Appendix III).

The study proposal was also submitted to and approved by the Nelson Mandela Metropolitan University (NMMU) Health Science FRTI Committee as well as the Malawi National Health Science Research Ethics Committee, NHRC # 760, 2010 (Appendix IV). The study was also conducted in accordance with The Belmont Report (1979) following Good Clinical Practice and Good Clinical Laboratory Practice.

3.2   MATERIALS AND METHODS

Procedures followed in this study are outlined below.

3.2.1 Microscopy

Routine procedures available at the hospital laboratory for the diagnosis of malaria using microscopy were followed. Thick blood smears were used for the slides that were collected in order to aid the clinicians to accurately diagnose the participants. Field Stain A and B (Appendix V) were used to stain these slides, which were then read by experienced laboratory technicians or health surveillance assistants (HSA's) who were expertly trained by the Community Health Science Unit (CHSU) of the Ministry of Health and Population in Malawi to read malaria smears. Two independent readers read the slides. In the case of a discordant result, a third reader was identified to read the discordant slide.

3.2.1.1 Test summary

This is a useful method for rapid presumptive identification of malaria parasites. The method shows adequate staining of all stages including stippling. 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.

Field stain B is a basic stain and will stain the cytoplasm blue, whilst Field stain A is acidic and will stain the chromatin dot red if the parasite is present. The stains should be arranged so as to allow staining to start with field stain B and then A as shown in figure 3.2 below.

3.2.1.2 Test procedure

1. Prepare a blood thick smear film on a microscope slide.

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 as shown in figure 3.3, 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 (UNC Project Lab SOP 10.5.5 Rev. 2.0).

Figure 3.2: Arrangement of malaria stains

Figure 3.3: Microscopic examination of malaria thick smears

3.2.1.3   Interpretation of results

Positive: Presence of the *Plasmodium* parasite in the smear that 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).

3.2.1.4   Grading of a 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).

3.2.1.5   Quality control

Positive Control: known positive smears were used.

Negative control: known negative smears were used as negative controls (UNC Project Lab SOP 10.5.5 Rev. 2.0).

3.2.2 Rapid diagnostic test

Tests were done according to the manufacturer's instructions as stipulated by WHO Malaria Quality Assurance Manual (2008) which states that:

1. Test the RDT's as per manufacturer instructions; use a micropipette to transfer the specified blood volume to the RDT. If a micropipette is not available use the device provided in the RDT kits, but take care to transfer the exact blood volume as described in the manufacturer's instructions.

2. Use a timer to record all the steps exactly as per manufacturer instructions.

3. Read RDT results within the manufacturer's recommended time.

The *Paramax-3* (Zephyr Biomedicals, India) RDT shown in figure 3.4 below was used in this study. It is a rapid self-performing, qualitative, two sites sandwich immunoassay utilizing whole blood for the detection of *P. falciparum* specific histidine rich protein-2 (*Pf* HRP-2), *P. vivax* specific *p*LDH and pan malaria specific *p*LDH. The test can be used for the specific detection of *P. falciparum* and *P. vivax* malaria, differentiation of other malarial species and for the follow up of anti-malaria therapy.
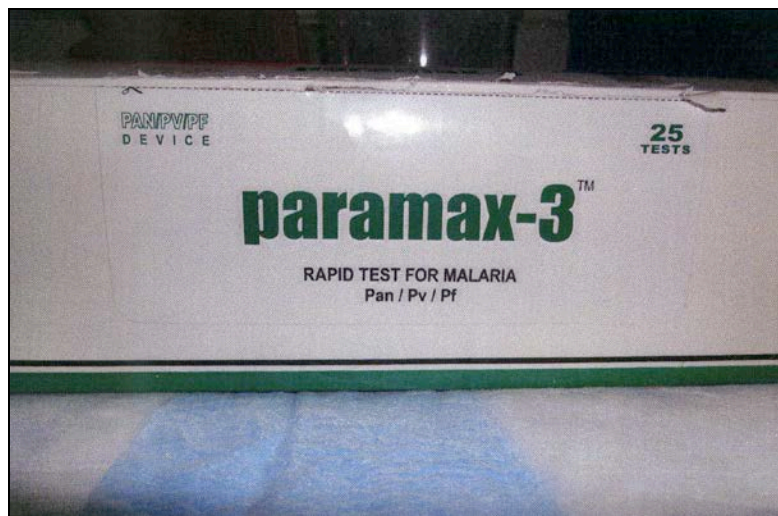
Figure 3.4: Paramax-3 Malaria RDT

3.2.2.1   Introduction

Four species of the *Plasmodium* parasites are responsible for malaria infections in human viz. *P. falciparum, P. vivax, P. ovale* and *P. malaria*. Of these, *P. falciparum* and *P. vivax* are the most prevalent. Early detection and differentiation of malaria is of utmost importance due to the incidence of cerebral malaria and drug resistance associated with falciparum malaria and the morbidity associated with the other malarial forms. As the course of treatment is dependent on the species, differentiation between *P. falciparum* and *P. vivax* is of utmost importance for better patient management and speedy recovery (Zephyr Biomedicals, 2010).

In Paramax-3 the detection system for *P. falciparum* malaria is based on the detection of *P. falciparum* specific histidine rich protein –2 (*Pf* HRP-2), which is a water-soluble protein that is released from parasitised erythrocytes of infected individuals. The detection system of *P. vivax* is based on the presence of *P. vivax* specific *p*LDH. Further detection of other *Plasmodium* species such as *P. ovale* and *P. malaria* is achieved through the pan malaria specific *p*LDH.

Since *p*LDH is a product of viable parasites, the pan band may also be used to monitor the course of effective anti-malaria therapy.

Paramax-3 detects the presence of *P. falciparum* specific *Pf* HRP-2, *P. vivax* specific *p*LDH and pan specific *p*LDH in a whole blood specimen and is a sensitive and specific test for the detection of all malaria species, differentiation for *P. falciparum and P. vivax* and the monitoring successful anti-malaria therapy (Zephyr Biomedicals, 2010).

3.2.2.2   Test principle

Paramax - 3 utilizes the principle of immunochromatography. As the test sample flows through the membrane assembly of the device after addition of the clearing buffer, the coloured colloidal gold conjugates of monoclonal anti-HRP-2 (IgG) antibody, and

monoclonal anti pan specific *p*LDH antibody complex with HRP-2/corresponding *p*LDH in the lysed sample. This complex moves further on the membrane to the test region, where it is immobilized by the monoclonal anti *Pf* HRP-2 antibody and/or monoclonal anti *P. vivax* specific *p*LDH antibody and/or monoclonal pan specific pLDH antibody coated on the membrane leading to formation of a pink/purple colored band in the respective regions which confirms a positive test result. Absence of a coloured band in the test region indicates a negative test result for the corresponding antigen. The un-reacted conjugate along with the rabbit anti-sera colloidal gold conjugate and unbound complex if any, move further on the membrane and are subsequently immobilized by anti-rabbit antibodies coated on the membrane at the control region, forming a pink/purple band. This control band serves to validate the test performance (Zephyr Biomedicals, 2010).

3.2.2.3   Specimen collection and preparation

Freshly collected EDTA anti-coagulated venous blood was used for testing.

3.2.2.4   Test procedure

1. Kit components must be brought to room temperature before testing.

2. In case the pouch has been stored at 2– 8°C, at least 30 minutes are allowed for the device to reach room temperature. The colour of the desiccant is also checked and is supposed to be blue. If it has turned colourless or faint blue, the device must be discarded and another one used.

3. Open the pouch and remove the device. Once opened, the device is supposed to be used immediately.

4. Evenly mix the anti-coagulated blood sample by gentle swirling. Dip the sample loop in to the sample.

   Blot the blood collected on to the sample pad in the sample well 'S'. (This delivers approximately 5µl of the whole blood specimen).

   Alternatively, 5µl of the anti-coagulated specimen is delivered to the sample pad in the sample well 'S' using a micropipette.

   NOTE: Ensure the blood from the sample loop has been completely taken up by the sample pad.

5. Dispense two drops of the clearing buffer into well 'R', by holding the plastic dropper bottle vertically.

6. At the end of 20 minutes, results must be read as follows:

NEGATIVE for *Plasmodium*: Only one colored band appears in the control region 'C'.
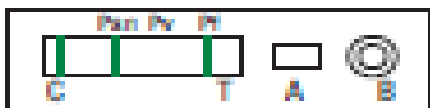
POSITIVE for *P. falciparum* malaria: In addition to the control band, a distinct coloured band also appears at the '*Pf*' and 'Pan' regions respectively.
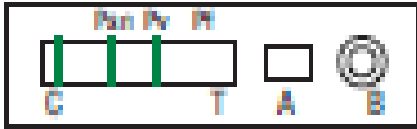
POSITIVE for *P. vivax* malaria: In addition to the control band, a distinct coloured band also appears at the '*Pv*' and 'Pan' regions respectively.
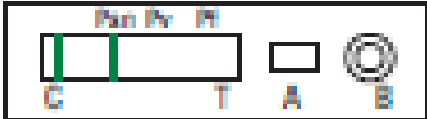
POSITIVE other species: In addition to the control band, a distinct band also appears at the 'Pan' region.
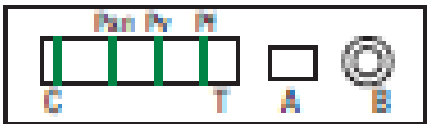
POSITIVE mixed infection: In addition to the control band, distinct coloured bands appear at '*Pf*', '*Pv*' and 'Pan' regions respectively.

INVALID RESULT: no band appears on the device. The test must be repeated with a new device ensuring that the test procedure has been followed accurately (Zephyr Biomedicals, 2010).

3.2.3 Real-time PCR test procedure

Real time PCR is a development of established methodology that uses fluorescent labels to enable the continuous monitoring of amplicon (PCR product) formation throughout the reaction. SYBR Green I (Applied Biosystems, California, USA) uses non-

specific double stranded (ds) DNA intercalators in the detection system (Bell & Ranford-Cartwright, 2007).

SYBR Green I fluoresces strongly when bound to dsDNA, but fluorescence is minimal when free in solution. The assay employs rigidly designed, highly specific primer-dimer formation and well-optimised reaction conditions. Quantification is determined during the log linear phase of amplification when the transcript number truly reflects starting copy-number. Values are recorded at the statistically calculated threshold level. This analysis is performed subsequent to amplification and monitors the precise, sequence-specific, melting temperature at which the dsDNA products formed during the reaction dissociate to ssDNA.

Various fluorescent detection systems are used in real time PCR. SYBR Green I is incorporated into dsDNA and maximum fluorescence is recorded at the end of each extension phase.

3.2.3.1 Specimen collection

Anti-coagulated blood was collected into EDTA tubes for all the 250 participants enrolled in this study. A volume of 50µl was transferred into each spot of the protein saver filter paper card until five spots were filled as shown in figure 3.5 below. These DBS cards were allowed to air dry overnight and then transferred into the -20°C freezer for temporary storage until shipping to the testing laboratory in the USA.

Figure 3.5: DBS card.

3.2.3.2 DNA isolation

Three punches using a 6mm single-hole punch from each DBS card were collected into a deep well 96-well plate. DNA was isolated using the PureLink Pro 96 Genomic DNA Kit according to the manufacturer's instructions and was eluted in 100uL elution buffer (Invitrogen, 2011).

3.2.3.3 SYBR-based real-time PCR

Pan-species primers for the 18S rRNA gene were designed as previously described (Mangold et al. 2005). Initially, all samples were run in duplicate in 25µl reactions. Each reaction contained 12.5 µl 2x iTaq Fast SYBR Green Super Mix with Rox (Bio-Rad), 3 µl 25mM $MgCl_2$, 2 µl eluted DNA, 500nM of forward and reverse primers and water to bring to volume.

Amplification was on an ABI 7300 (Applied Biosystems, 2011) with the following conditions: 50°C for 2min, 95°C for 15min, 40 repeats of 95°C for 0:15min and 60°C for 1min, with fluorescence measured at the close of each step. Amplification was

immediately followed by a dissociation program: 60°C for 1min, 95°C for 0:15min, 60°C for 0:30min, with temperature increasing until 95°C in a stepwise manner.

Samples for which fluorescence values in both values crossed the Ct threshold were deemed to be positive and dissociation values were recorded. All samples were run with 4 positive controls in duplicate, consisting of plasmids containing the 18S rRNA gene of each species at a concentration of 0.0005ng/µl (MRA-177, MRA-178, MRA-179, MRA-180, MR4, Manassas, VA) and human genomic DNA at a concentration of 5ng/ µl (ABI). Each plate also contained a non-template control (water) in duplicate. Samples for which fluorescence values in neither well crossed the Ct threshold were deemed *Plasmodium sp.* negative. Samples for which fluorescence values in just one of two wells crossed the Ct threshold were re-run in duplicate in 50µl reactions.

Each reaction contained 25µl 2x iTaq Fast SYBR Green Super Mix with Rox (Bio-Rad), 6µl 25mM MgCl², 4µl eluted DNA, 1000nM of forward and reverse primers and water to bring to volume. Again, samples were deemed positive if both wells amplified beyond the threshold as previously reported by Mangold et al. (2005).
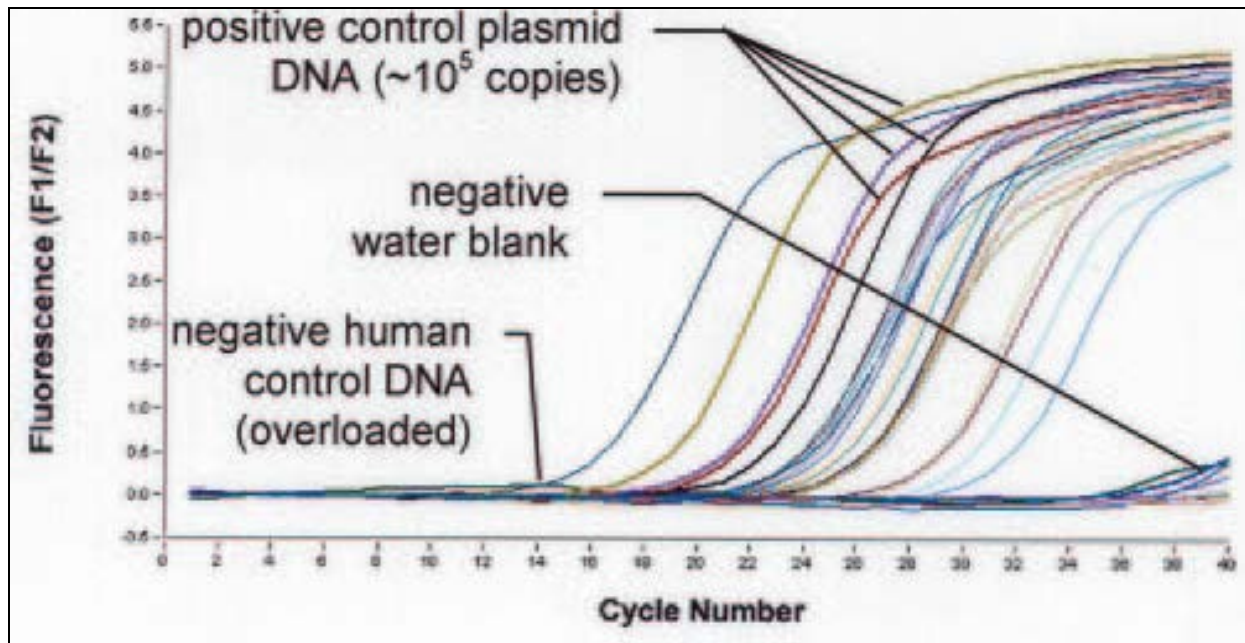
Figure 3.6: Real-time amplification with SYBR Green Fluorescence Detection (Mangold et al. 2005)

In figure 3.6 above, the plasmid controls for four species, water blank and negative human control DNA are indicated. The remaining curves are patient specimens with various parasitemia levels. The graph was generated by using the LightCycler Software v.3 (Mangold et al. 2005).

3.2.3.4 *pf*LDH Taqman Assay

All samples containing non-falciparum malaria were repeated using a Taqman assay specific to *pf*LDH. The primers, probes and conditions for this assay have been previously published (Pickard et al. 2003). The assay was run with each sample in duplicate and duplicate positive [3d7 genomic DNA at a concentration of 0.1ng/µl (MRA-102G, MR4)] and non-template controls (Applied Biosystems, 2011).

This assay was used as a check for mixed infections of *P. falciparum* and *P. malaria*, as the melting temperature of these two organisms in the SYBR-based real time PCR are very close and may overlap making mixed infections difficult to determine.

3.3   STATISTICAL ANALYSIS

Data was entered on an excel spreadsheet and analysed using StatSoft, Inc. (2011) STATISTICA (data analysis software system), version 10. The primary aim of the analysis was to determine the accuracy of RDT's to detect and differentiate different *Plasmodium* species. Sensitivity and specificity of the RDT against microscopy and PCR were also calculated with 95% confidence intervals (CI) and differences were tested for significance using the Pearson chi-square test.

CHAPTER 4:   RESULTS

4.1   INTRODUCTION

Two hundred and fifty patients suspected to be infected with the malaria parasite, *Plasmodium*, were successfully enrolled in this study. Most of these participants were between the ages of 18 and 30 years old. Of these, 142/250 (56.8%) were female and 108/250 (43.2%) were male. The ages ranged from 2 years to 63 years old, with the mean of 20.5 years and the median of 21 years with more than 82% of the participants falling below the age of 30 years. However, the study also enrolled 43 (17%) under-five infants as shown in the pie chart in figure 4.1 below:
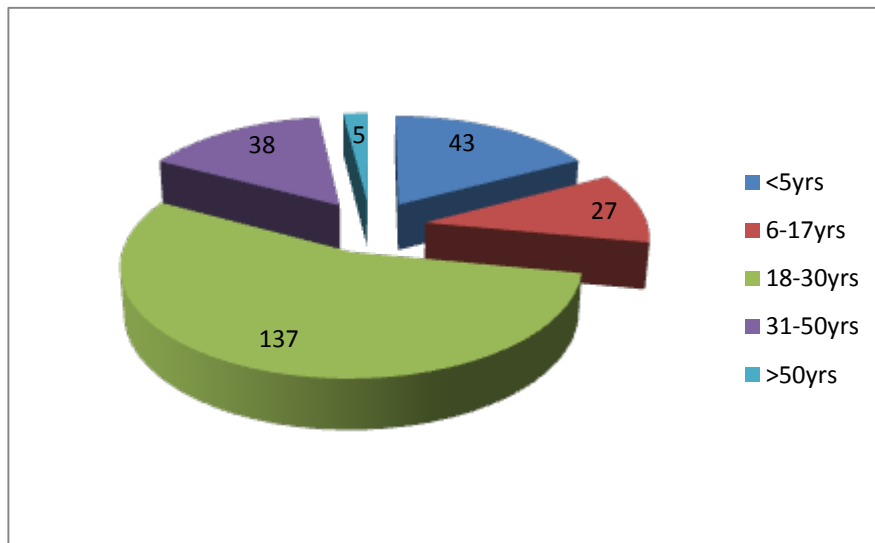
Figure 4.1: Pie chart comparing infants with other age groups

4.2   MICROSCOPY RESULTS

Microscopy results remain the gold standard for malaria diagnosis as recommended by the WHO. Two microscope readers must confirm the result before releasing it to the patient (WHO Malaria Report, 2009). However, in the setting of Bwaila Hospital Laboratory, only one reader (R1) 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 the hospital laboratory.

Therefore, for the purpose of this study, the second reading (R2) was done in the UNC Project Laboratory, and in the case of discordant results, a third reader (R3) was identified from the UNC Project Laboratory as well.

Slides were stained using Field Staining technique as described in chapter 3, and below are some examples of the positive slides taken from the microscope camera (figures 4.2 and 4.3).
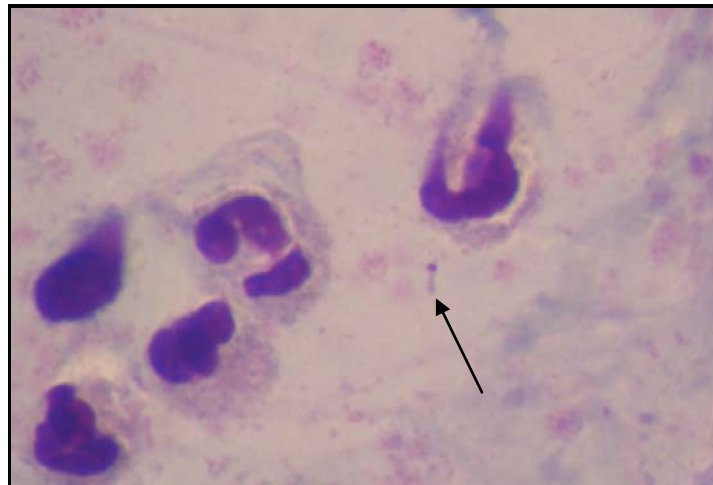
Figure 4.2: Field stained thick malaria blood smear showing low parasitemia

In the figure 4.2 above, the arrow is pointing at the malaria parasite trophozoite seen on this particular field using 100X oil objective of the light microscope.
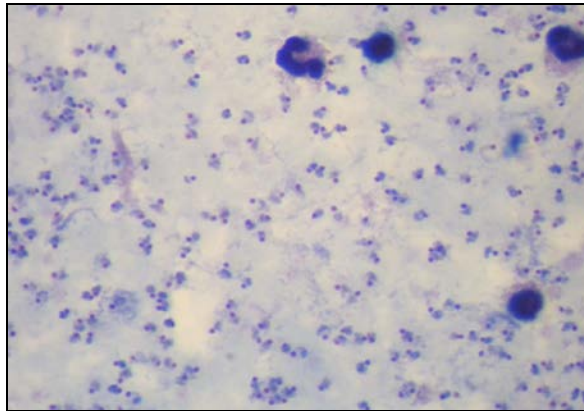
Figure 4.3: Field stained thick malaria blood smear showing high parasitemia

The 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 shown in table 4.1 below.

Table 4.1: Correlation between 'Plus System' and the parasite count per micro-litre (µl) of blood (adapted from NMCP Malawi, 2011).

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

4.2.1 Comparison of R1 and R2

A total of 250 slides were read by Reader one (R1) and Reader two (R2). Data analysis showed that 95.8% (n=185) of the slides were reported as positive by both R1 and R2. However, 4.2% (n=8) were reported by R1 as positive but R2 reported them as negative. A total of 93% (n=53) were reported as negative by both R1 and R2, whilst 7% (n=4) were reported by R1 as negative whilst R2 reported as positive. Statistically the differences are not significant (Chi-square 188.3, p=0.0000).

Table 4.2 and figure 4.4 below show the summary and graphical presentation of these results.

Table 4.2: Two-way summary comparison table for R1 and R2

	R2 Pos	R2 Neg	Total
R1 Pos	185 (95.8%)	8 (4.2%)	193
R1 Neg	4 (7.0%)	53 (93.0%)	57
Totals	189	60	250
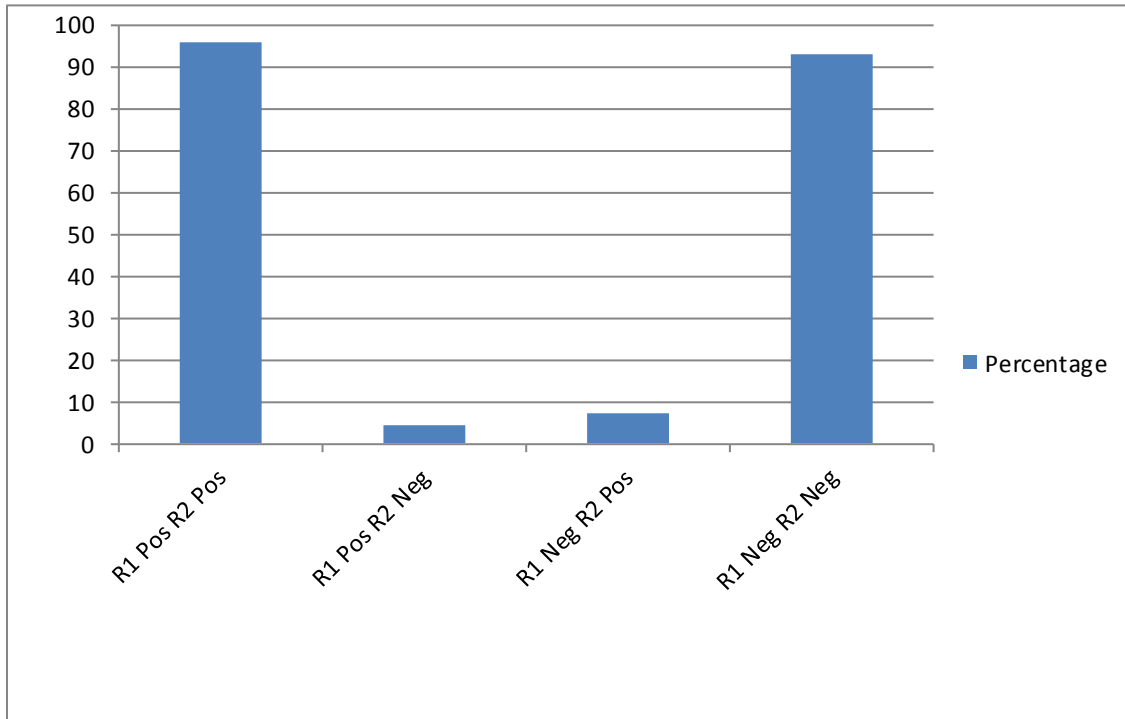
Figure 4.4: Graphical comparison of Microscopy R1 and R2

The study also compared the agreement between R1 and R2 in grading malaria densities using the plus system (Table 4.3).

Table 4.3: Comparison of malaria parasite grading between R1 and R2

	R2 (1+)	R2 (2+)	R2 (3+)	R2 (4+)	R2 (Neg)	Totals
R1 (1+)	29 (80.6%)	0	0	0	7 (19.4%)	36
R1 (2+)	1 (1.4%)	62 (86.1%)	8 (11.1%)	0	1 (1.4%)	72
R1 (3+)	0	0	23 (54.8%)	19 (45.2%)	0	42
R1 (4+)	0	0	1 (2.3%)	42 (97.7%)	0	43
R1 (Neg)	2 (3.5%)	2 (3.5%)	0	0	53 (93.0%)	57
Totals	32	64	32	61	61	250

4.3   COMPARISON OF MICROSCOPY AND RDT RESULTS

A total of 250 samples were compared between microscopy (R1) and Paramax-3 RDT results. Out of these, 5.7% (n=11) were both R1 and RDT *Pf* positive whilst 71.9% (n=41) were both R1 and RDT Negative. A total of 88.1% (n=170) were R1 positive and RDT *Pf*+Pan positive whilst 6.2% (n=12) were R1 positive but RDT negative. Another 21.1% (n=12) were R1 negative but RDT Pf positive whilst 7.0% (n=4) were R1 negative but RDT *Pf*+Pan positive. No *P. vivax* was detected by Paramax-3 from the study participants. Statistically the differences are not significant (Chi-square 142.5, p=0.0000).

 Table 4.4 and figure 4.5 illustrate the comparison between R1 and RDT results.

Table 4.4: Two-way summary comparison table for R1 and Paramax-3 RDT

	RDT Pf Pos	RDT Pf+Pan Pos	RDT Neg	Total
R1 Pos	11 (5.7%)	170 (88.1%)	12 (6.2%)	**193**
R1 Neg	12 (21.1%)	4 (7.0%)	41 (71.9%)	**57**
Totals	**23**	**174**	**53**	**250**
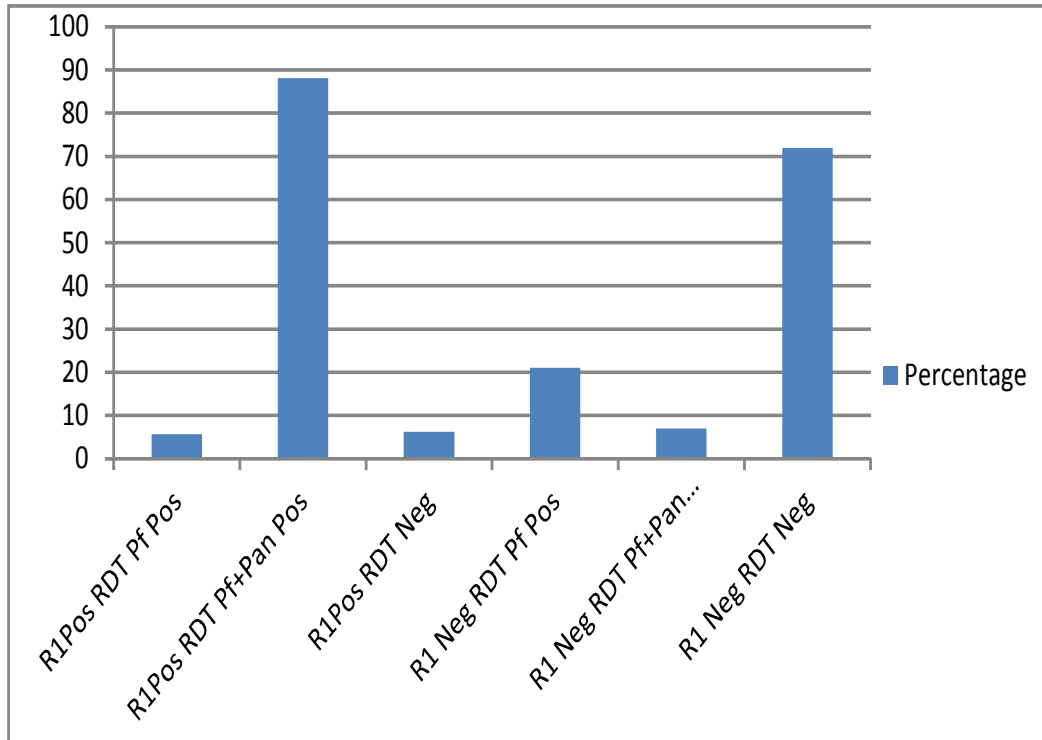
Figure 4.5: Graphical comparison of Microscopy R1 and RDT

These 250 samples were also compared between microscopy (R2) and Paramax-3 RDT. Out of these, 7.4% (n=14) were both R2 and RDT *Pf* positive, 90.5% (n=171) were R2 and RDT *Pf*+Pan positive whilst 2.1% (n=40) were R2 positive but RDT negative. Further analysis showed 80.3% (n=48) were both R2 and RDT negative, 14.8% (n=9) were R2 negative but RDT *Pf* positive, whilst 4.9% (n=3) were R2 negative but RDT *Pf*+Pan positive. The differences are not significant statistically with a Chi-square 184.3, (p=0.0000).

Table 4.5 and figure 4.6 are a summary of these results.

Table 4.5: Two-way summary comparison table for R2 and Paramax-3 RDT

	RDT Pf Pos	RDT Pf+Pan Pos	RDT Neg	Total
R2 Pos	14 (7.4%)	171 (90.5%)	4 (2.1%)	**189**
R2 Neg	9 (14.8%)	3 (4.9%)	49 (80.3%)	**61**
Totals	**23**	**174**	**52**	**250**
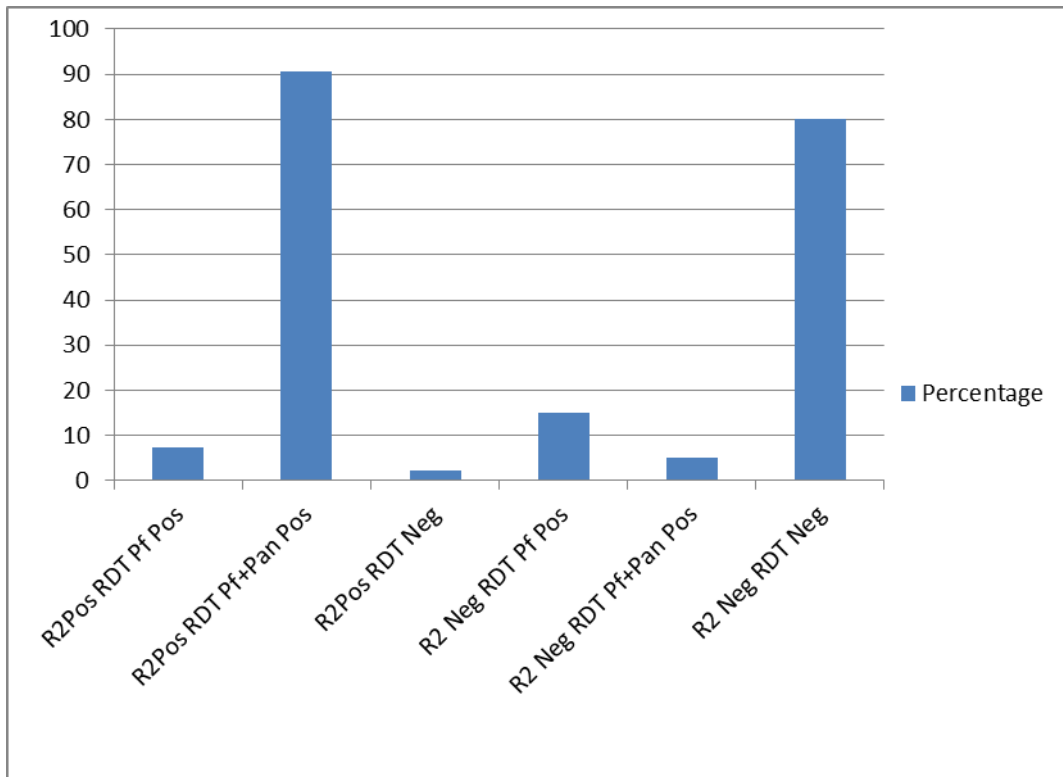
Figure 4.6: Graphical comparison of Microscopy R2 and RDT

4.4   COMPARISON OF MICROSCOPY AND PCR

PCR testing was also done on these 250 samples and results were compared between R1 and PCR. Out of these, 89.6% (n=173) were both R1 positive and PCR *Pf* positive, 1.0% (n=2) were R1 positive and PCR *Po* positive whilst 5.7% (n=11) were R1 positive and PCR mixed infection positive and 3.6% (n=7) were R1 positive and PCR negative. A total of 19 samples (33.3%) were R1 negative and PCR Pf positive, 1.8% (n=1) was R1 negative and *Po* positive whilst 64.9% (n=37) were R1 and PCR negative. Statistically the differences are not significant (Chi-square 115.5, p=0.0000).

Table 4.6 and figure 4.7 below show a summary of the comparison.

Table 4.6: Two-way summary comparison table for R1 and PCR

	PCR Pf Pos	PCR Po Pos	PCR Mixed	PCR Neg	Total
R1 Pos	173 (89.6%)	2 (1.1%)	11 (5.7%)	7 (3.6%)	**193**
R1 Neg	19 (33.3%)	1 (1.8%)	0	37 (64.9%)	**57**
Totals	**192**	**3**	**11**	**44**	**250**
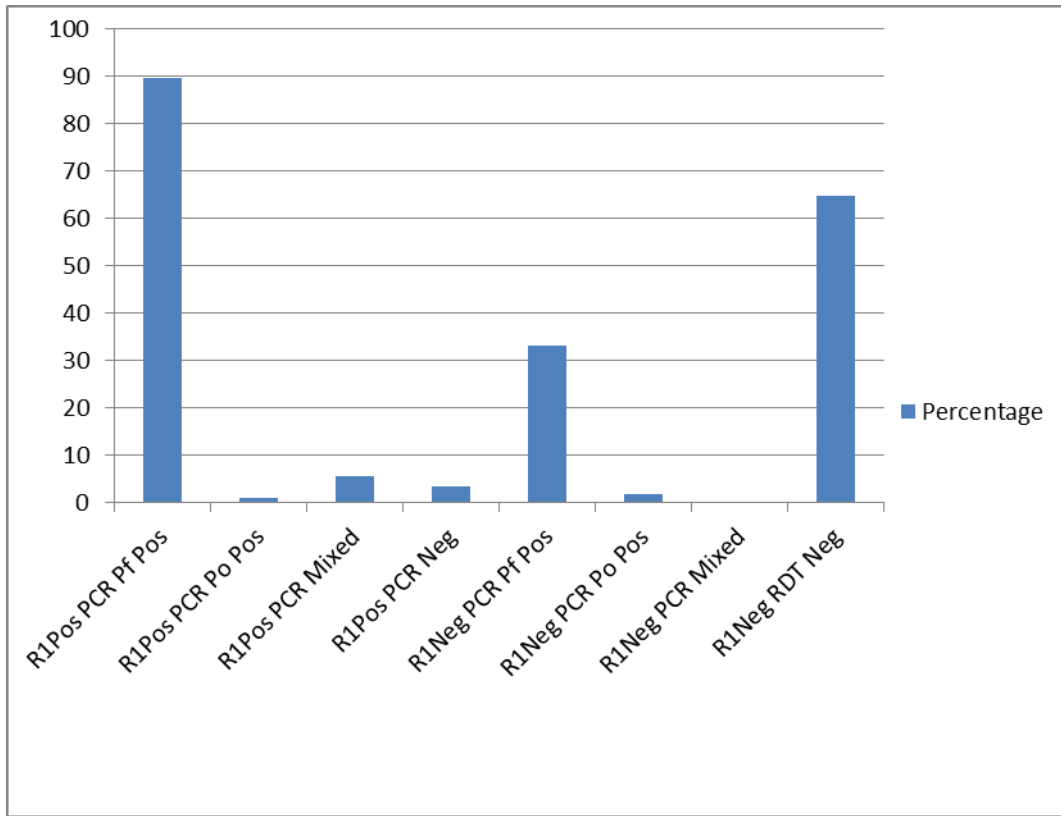
Figure 4.7: Graphical comparison of Microscopy R1 and PCR

A total of 250 samples were compared between R2 and PCR. The majority, 91.5% (n=173) were R2 positive and PCR *Pf* positive as well, 1.1% (n=2) were R2 positive and PCR *Po* positive, 5.8% (n=11) were R1 positive and PCR mixed infection positive whilst 1.6% (n=3) were R2 positive and PCR negative. Furthermore, 31.2% (n=19) were R2 negative and PCR Pf positive, 1.6% (n=1) was R2 negative and PCR Po positive whilst 67.2% (n=40) were both R2 and PCR negative as shown in table 4.7 and figure 4.8 below. Statistically the differences are not significant (Chi-square 138.4, p=0.0000).

Table 4.7: Two-way summary comparison table for R2 and PCR

	PCR Pf Pos	PCR Po Pos	PCR Mixed	PCR Neg	Total
R2 Pos	173 (91.5%)	2 (1.1%)	11 (5.8%)	3 (1.6%)	**189**
R2 Neg	19 (31.2%)	1 (1.6%)	0	41 (67.2%)	**61**
Totals	**192**	**3**	**11**	**43**	**250**
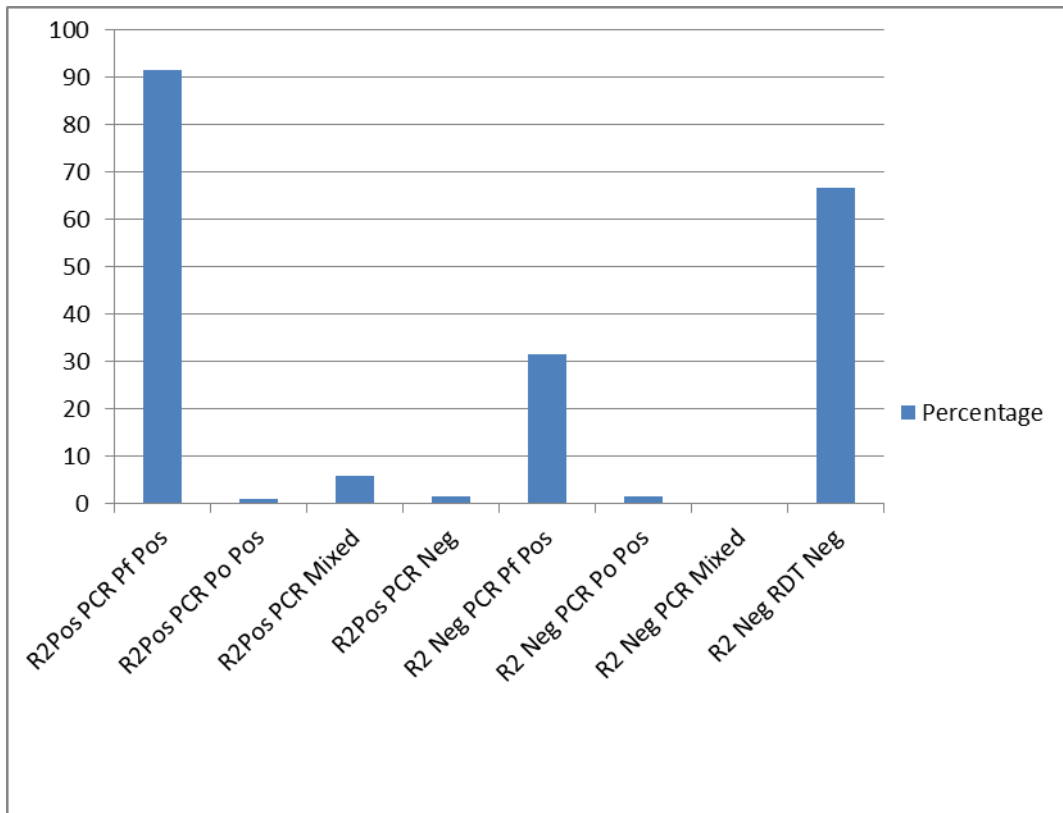
Figure 4.8: Graphical comparison of Microscopy R2 and RDT

4.5 COMPARISON OF PARAMAX-3 RDT AND PCR

A total of 250 samples were tested for RDT as well as PCR. 82.6% (n=19) tested both RDT *Pf* and PCR *Pf* positive, 13% (n=3) were RDT Pf positive and PCR Po positive, 4.4% (n=1) was RDT Pf positive and PCR mixed infection positive whilst none were RDT *Pf* positive but PCR negative. Furthermore, 94.3% (n=164) were RDT *Pf*+Pan positive and PCR *Pf* positive whilst 5.7% (n=10) were RDT *Pf*+Pan positive and PCR mixed infection positive. Another 17% (n=9) tested RDT negative but PCR *Pf* positive whilst 83% (n=44) tested both RDT and PCR negative. Statistically the differences are not significant (Chi-square 227.9, p=0.0000).

Table 4.8 and figure 4.9 give a summary of the comparison between RDT and PCR. None of the samples tested for *Pv* in both RDT and PCR.

Table 4.8: Two-way summary comparison table for RDT and PCR

	PCR Pf Pos	PCR Po Pos	PCR Mixed	PCR Neg	Total
RDT Pf Pos	19 (82.6%)	3 (13.0%)	1 (4.4%)	0	**23**
RDT Pf+Pan Pos	164 (94.3%)	0	10 (5.7%)	0	**174**
RDT Neg	9 (17.0%)	0	0	44 (83.0%)	**53**
Totals	**192**	**3**	**11**	**44**	**250**
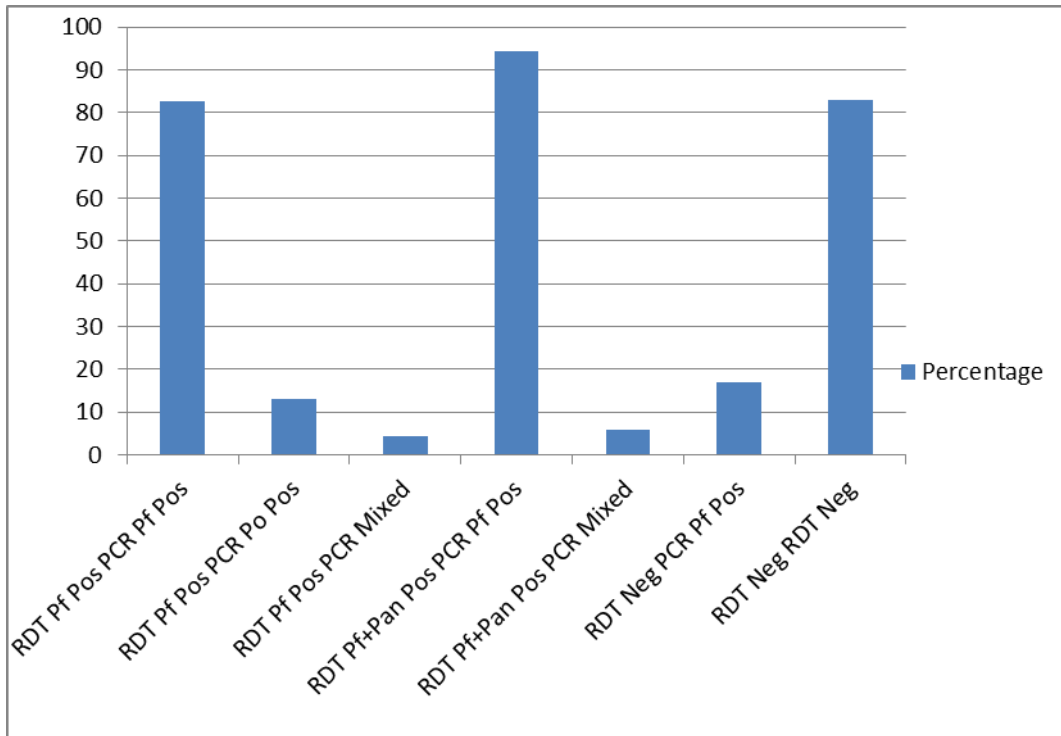
Figure 4.9: Graphical comparison of RDT and PCR

4.6   COMPARISON OF PATIENTS ON TREATMENT AND RDT TEST RESULTS

A total of 250 participants who had RDT tests done on their samples were also asked if they had taken any anti-malaria medication in the preceding four weeks. Out of these, 18.4% (n=46) had anti-malaria treatment whilst 81.6% (n=204) had not been on anti-malaria treatment in the previous four weeks. From the group who had anti-malaria treatment, 63% still were RDT positive whilst 37% were RDT negative. Figure 4.13 below gives a summary of the comparisons made.

Table 4.9: Two-way summary comparison table for anti-malaria drugs and RDT

Anti-malaria	RDT *Pf* Pos	RDT *Pf*+Pan Pos	RDT Neg	Total
Yes	3 (6.5%)	26 (56.5%)	17 (37.0%)	46
No	20 (9.8%)	148 (72.5%)	36 (17.7%)	204
Totals	23	174	53	250

CHAPTER 5:   DISCUSSION OF THE FINDINGS

5.1   INTRODUCTION

The study enrolled 250 participants seeking medical treatment at Bwaila Hospital Out-Patient Department as previously discussed in the management of the study. Out of these, 56.8% were female and 43.2% were males. These participants were recruited randomly as long as they met the eligibility criteria and were willing to participate in the study through informed consenting/assenting. The ages ranged from 2 years to 63 years old, with a mean age of 20.5 and median of 21 with more than 82% of the participant falling below the age of 30 years as shown in figure 5.1 below.
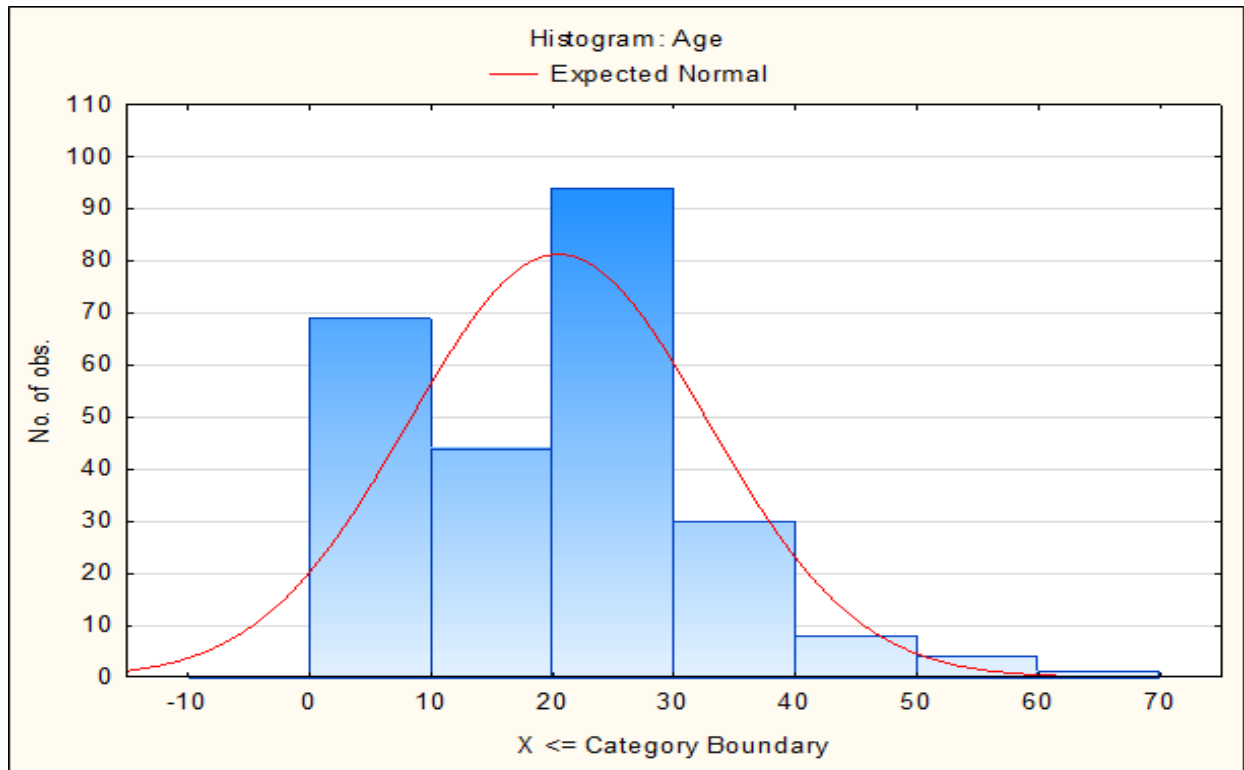
Figure 5.1: Graph showing age categories

The study participants were also examined to establish whether they had fever, which was defined as body temperatures above 37.5°C. However, only one participant did not present with fever, whilst the rest had a temperature of equal to or more than 37.5°C.

Following below are discussions pertaining to results obtained from different laboratory methods used in diagnosing malaria parasites in the study.

5.2 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. Apart from laboratory technicians, the Ministry of Health and Population in Malawi continuously train other cadres like HSA's in microscopy reading but the number of patients visiting government hospitals requiring malaria diagnostic services remains constantly high. As a result, only one reader (R1) is available at a time, therefore results that are released are only read by one microscopy reader.

However, in this study slides that were seen by this particular reader were stored and reviewed later by the second reader (R2) to confirm the malaria microscopy results. Where R1 and R2 results were discordant (R1 reporting as positive and R2 negative and vice versa), a third reader (R3) was asked to read and record his/her results. In comparing R1 and R2, there were n=8 slides which R1 reported as positive and which R2 reported as negative. Again there were n=4 slides which were reported as negative by R1 but reported positive by R2 (table 4.2). All the four smears reported negative by R1 and positive by R2 and R3 subsequently became positive in both the RDT and PCR. However, out of the eight that were reported as negative by R1 and positive by R2, R3 and RDT, only four were confirmed positive with PCR, agreeing with R1. Only one out of the four was on medication in the previous four weeks, which meant there was no

possibility of having distorted parasites as seen mostly in patients on medication (Warhurst & Williams, 1996; Srinavasan, Moody & Chiodini, 2000).

Comparing sensitivities and specificities of individual readers, R1 and R2 to PCR, results showed that both readers reported all the true positive results correctly. For instance, R1 had a sensitivity of 96.4% whilst R2's sensitivity was 98.4. However, both R1 and R2 had low specificities (64.9% and 67.2% respectively). Again, most of the samples that reduced the specificity were those with low parasitemia, which confers with available literature (Metzger et al. 2007; Mills et al. 2009).

Malaria grading comparison between R1 and R2 showed comparable results. In this study, the plus system of grading malaria was used, with the presence of malaria graded as 1+, 2+, 3+ and 4+ as described in chapter 3 of this study. Correlation of the plus system with the parasite count per microlitre is shown in table 4.1 above (NMCP Malawi, 2011).

Out of the 250 participants enrolled in the study, 80.6% (n=29) were reported by both R1 and R2 as 1+, 86.1% (n=62) were also reported by both R1 and R2 as 2+, 54.8% (n=23) were reported as 3+, 97.7% (n=42) as 4+ whilst both R1 and R2 reported 93% (n=53) negative as summarised in table 4.3 in chapter four.

There was a large disparity in the reporting of 3+ and 4+ between the two readers. Most of the slides graded as 3+ by R1 were graded as 4+ by R2. It was also observed that four participants who were reported as negative by R1 were graded as 1+ (n=2) and 2+ (n=2) respectively by R2. All of these four participants were confirmed as positive by R3 with the same grading as reported by R2. Further analysis of these samples revealed that they all tested positive for RDT and PCR, indicating they were falsely reported as negative by R1. Similarly, out of the eight which were reported as R2 negative but R1 positive, four were reported as positive by PCR whilst RDT results were negative for all eight samples.

It must be noted that these inter-microscopy-reader differences as well as differences in sensitivity of microscopy compared with RDT and PCR have been shown to occur

elsewhere as reported in other studies (Metzger et al. 2007; Ratsimbasoa, Randriamanantena, Raherinjafy, Rasoarilalao & Menard, 2007; Mills et al. 2009).

5.2 PARAMAX-3 RDT

In this study, a total of 250 samples were tested with RDT from fresh blood (within four hours of specimen collection) using the recommended method by the manufacturer. It is important to mention that the kits were bought directly from the manufacturer, Zephr Biomedicals (India), therefore chances of poor storage conditions arising were minimal. All these RDT's had one lot number (91120 expiring January 2013), manufactured in February 2011 and used in April 2011.

The main aim of this study was to determine the accuracy of this RDT method in detecting and identifying the different *Plasmodium spp.* causing malaria in Malawi. The study intended using the RDT device that was able to distinguish all four species separately, but due to its unavailability on the market at the time of the study, the RDT that was used was only able to detect, identify and differentiate the two species, *P. falciparum* and *P. vivax*. The other two remaining species, *P. malaria* and *P. ovale*, if present, were to be detected in the "Pan" section of the test device as shown in figure 5.2 below, hence the Paramax-3 RDT was not able to differentiate these two species from the others.

Figure 5.2: Paramax-3 RDT devices with results.

Microscopy using Giemsa staining remains the gold standard for malaria diagnosis as recommended by the WHO (WHO Malaria Quality Assurance Manual, 2008). However, this study used qualitative PCR as planned because Bwaila Hospital Laboratory does not use Giemsa staining, but rather Field stains. It is for this reason that the accuracy of Paramax-3 RDT was compared with SYBR Green PCR instead of the microscopy. However, microscopy results were the ones used for patient management at the hospital during the period of the study.

Despite using PCR as a gold standard, microscopy results also compared well with both the RDT and the PCR, although all the readers could not detect and differentiate *P. ovale* and *P. malaria* which were detected and differentiated by the PCR. Section 5.2.2 below gives a brief summary of the performance of the RDT compared with the PCR.

Comparing those who had anti-malaria treatment in the previous four weeks (n=46) with RDT, 63% still had malaria compared with the 69% who were positive with PCR and the 60% positive microscopically. It must be noted that the study was not able to ascertain whether these were re-infections or if they were the same existing infections.

5.3 SYBR GREEN AND TAQMAN PCR

This study used the SYBR Green real time PCR to confirm the results obtained from Paramax-3 RDT testing in order to determine its sensitivity and specificity. Samples were reported as positive when both well amplified beyond the threshold since they were run in duplicate, while those which the fluorescence values did not cross the Ct threshold were reported as negative as described before by Mangold et al. (2005) and shown in figure 5.3 below.
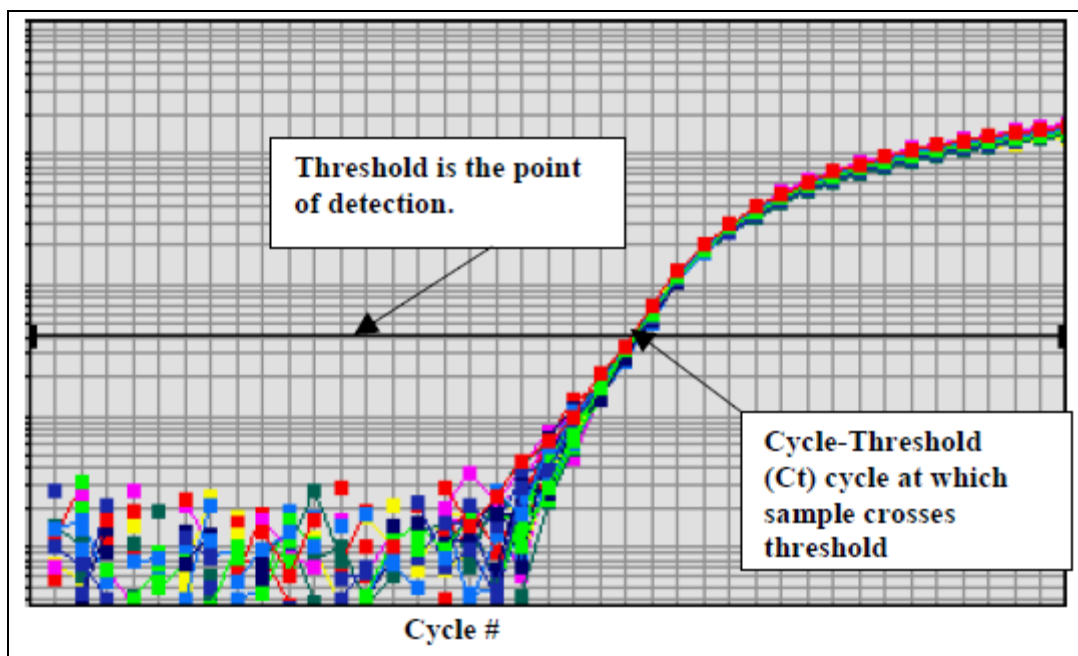
Figure 5.3: Graph showing Ct threshold (Applied Biosystems, 2011)

All samples containing non-*falciparum* malaria were repeated using a Taqman assay specific to *pf*LDH. Samples positive by the *pf*LDH assay of *P. falciparum* and positive for only non-falciparum malaria were called mixed infections. This only occurred with samples positive for *P. malaria* (n=9). No sample containing only *P. ovale* in the SYBR-based real time PCR tested positive for *P. falciparum* in the *pf*LDH assay (n=3).

There were a total of 186 positive samples detected by PCR, the majority of them being *P. falciparum*. The proportions of the figures were 91.5% (n=173) *P. falciparum*, 1.1% 9n=2) *P. ovale* and 5.8% (n=11) mixed infection. The mixed infections included n= 9 that were *P. falciparum* and *P. malaria* mixed, and n=2 that were *P. falciparum* and *P. ovale* mixed. However, there were no *P. malaria* and *P. ovale* mixed infection detected in this study.

5.4 SENSITIVITY AND SPECIFICITY OF PARAMAX-3 RDT

Using the results of PCR as gold standard, the overall sensitivity of the RDT was 100% (Chi square 227.9, p=0.0000) that is, all the samples testing positive on RDT also tested positive on PCR. However, in identifying the different species, the two results that were detected as *P. ovale* by the PCR were *P. falciparum* positive and pan negative on the RDT.

All of the nine samples that tested *P. malaria* and *P. falciparum* mixed infection on the PCR assay showed the pan band on the RDT device as compared to the two *P. ovale* and *P. falciparum* mixed infection which only one had a pan band but the other one did not. Available literature suggests that *p*LDH clears faster after treatment than the HRP-2 (Eisen & Saul, 2000). However, in this study, all except one sample that did not show the pan band where the *p*LDH reaction occurs were taken from participants who reported they did not take any anti-malaria treatment in the previous four weeks.

In terms of specificity, the overall was 83% (Chi square 227.9, p=0000), that is nine out of the 53 negative RDT results tested positive on PCR. This is far below the minimal recommended 95% specificity by the WHO (Bisoffi et al. 2010). However, it was observed in this study that all the samples that tested negative on RDT and positive on PCR had low parasitemia on microscopy, which is in congruence with many studies previously completed (Murray, Gasser, Magill & Miller, 2008; Anonymous, 2009; Willcox et al. 2009). Removing all the samples which had low parasite density of 1+, the sensitivity was higher than 83% and approached 100%.

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).

The current move to introduce ACT's into sub-Saharan Africa with the financial support provided by the Global Fund needs to be in line with confirmed diagnosis to reduce anti-malaria prescriptions on clinical grounds only, and to rationalise health budgets in view of the much higher costs of the ACT's. Rolling out these RDT's would be more feasible for health outposts, currently relying on clinical diagnosis, to incorporate RDT's into their diagnostic algorithms. The reduced expenditure on ACT's for negative patients could balance the extra costs of using RDT's (Rolland et al. 2006).

A parasitological confirmation of malaria in stable 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. 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).

In many areas in Malawi, malaria patients are treated outside of the formal health services, e.g. in the community, in the home or by private providers. Microscopy is generally not feasible in many such circumstances, but RDT's may be possible.

Although RDT's for detection of parasite antigen are generally more expensive, their deployment may be considerably more cost effective in many of these settings. The sensitivities and specificities of RDT's are variable and their vulnerability to high temperatures and humidity is an important constraint.

In the diagnosis of severe malaria cases, microscopy is a preferred option. It provides the diagnosis of malaria, as well as being useful in assessing other important parameters in a severely ill patient. In situations where an RDT has been used to confirm malaria, this allows for a rapid institution of anti-malaria treatment, however, where possible a microscopic examination is recommended to enhance the overall management of the patient (Moody, 2002; Gillet et al. 2009).

The attempt of this study to evaluate the performance of Paramax-3 *Pf/Pv/*Pan test, an alternative rapid diagnostic test that can be used in the diagnosis of malaria in endemic areas like Malawi, revealed a high level of sensitivity (100%) but lower specificity (83%). This is the first study to evaluate new generation Paramax-3 RDT tests for malaria diagnosis, performed in a Malawian setting with a predominance of *P. falciparum* infections. The study showed that this RDT proved valid, reliable and easy to use, and should be of great use in malaria-endemic countries like Malawi where microscopy is not available in all health facilities, particularly in emergency settings. For confident diagnosis of malaria in routine outpatient department conditions, a sensitivity of more than 95% is crucial (Moody, 2002; Bell & Peeling, 2006) and this was achieved by the Paramax-3 RDT.

The Paramax-3 RDT also demonstrated desirable qualities that could reduce the possibility of patients without malaria being given anti-malaria treatment and therefore could reduce medication pressure, a major concern at a time when artemisinin combination therapies (ACT's) were introduced throughout Africa including Malawi. Firstly, its specificity would reduce the number of patients with a false positive test being treated for malaria. Secondly, the ability to detect both *P. malaria* and *P. vivax* would

increase confidence in a negative test, although in the study population these species are infrequent and the study did not detect any *P. vivax*, both by RDT and PCR. The simple and easy to use nature of this test would enable any health staff to be trained to use and interpret the tests accurately. This is an advantage in countries where trained laboratory staff is scarce and cadres such as nurses are frequently in the front line for providing clinical care and diagnosis of patients in health outposts.

This study was able to confirm the availability of the three *Plasmodium* species causing malaria in Malawi using RDT and PCR, namely *P. falciparum, P. malaria* and *P. ovale*, as some studies before (Bruce et al. 2008). However, the study could not verify the availability of *P. vivax* in Malawi both by RDT and PCR, as reported by the WHO Malaria Report (2009).

A continuing area of concern with these antigen detection assays remains the occurrence of occasional false negative results (table 4.8 in chapter 4). In this study, false negative results were most common in samples with low parasitemias (reported as negative or 1+ by microscopy).

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APPENDICES

Appendix I: Informed Consent Form – Adults.

Paricipant ID ....

[stamp: This consent form should be signed only between 09-08-2010 and 08-08-2011. Approved by NHSRC. Malawi on 09-08-10]

NELSON MANDELA METROPOLITAN UNIVERSITY

PARTICIPANT INFORMATION SHEET AND INFORMED CONSENT FORM

Reference Number:

Study Title: Detection and identification of Plasmodium species causing malaria in Malawi using rapid diagnostic tests

ICF Version Number: 001

Age group: Adults

Date: 12 April 2010

What is consent?

Consent means agreeing to take part in this research study of malaria disease in your area. You have the right to decide if you want to take part in the study or not. This document is to inform you about the study. Please take time to read the following information carefully and discuss it if you wish with friends, relatives and your health workers. Ask us if there is anything that is not clear or if you would like more information.

Why is this study being done?

The research study we are asking your consent for is being carried out in order to obtain more detailed information on the malaria disease in your area. Malaria is a serious disease transmitted by the bite of a mosquito infected with malaria parasites. It can cause fever, anemia, loss of consciousness and sometimes death. Babies and young children are at greatest risk of severe illness and death due to malaria.

The information that will be collected from this study will be used to determine how reliable malaria rapid diagnostic tests are in identification of different types of malaria parasites for proper management of malaria disease in your area.

How many people are there in the study?

The study will enroll 250 people, from 2 years of age to adults.

What does this study involve?

If you agree to take part in this study, 3 ml (1 teaspoon) of blood sample will be taken in order to identify the species (type) of malaria you are infected with.

You will also be asked for details concerning

- Age

Participant ID ....

- body temperature
- location of your house.

What are the foreseeable risks for taking part in the study?

You may experience slight pain when the blood sample is taken.

Who should I contact to answer any questions on the study?

If you have any questions 1) relating to this study, 2) concerning the rights of people in research studies, you should contact Mr Gerald Tegha on 0999 200 668 or the Chairman of the committee responsible for the protection and rights of research participants, Dr. Charles Mwansambo on 0888 826 946 or write to the Chairperson, National Health Sciences Research Committee (NHSRC), P.O. Box 30377, Lilongwe 3, Malawi.

What payments will be made for the study?

You will not receive any direct payment for your participation in this study.

Are there any benefits for taking part in the study?

The information from this study will help us to better understand how to protect populations at risk against malaria which will be of great benefit in the future.

If you take part in the study and are found to be unwell due to malaria, you will be treated under the existing government services.

What will happen to blood samples taken in this study?

The blood sample collected during this study will be used to assess and identify the species (type) of malaria you have. The remaining blood sample will be stored for future studies if you agree to do so.

The blood samples will be coded with your study identification number. This means that they will not be labeled with information that directly identifies you.

Do you have to stay in the study?

You may choose to withdraw your consent from the study at any time, without giving a reason. Your decision will not affect the medical care you receive outside of the study.

Who will have access to your personal information?

If you decide to take part in the study, the study staff will collect medical and personal information about you during the study. Lectures from the university, and others like members of the ethics committee or the review board for the study will have access to this information in order to check that the study is done properly. Anyone who sees this information will keep it confidential.

Participant ID ....

What will the investigators and the collaborating partners do with the information they get?

The investigator may use the information that the study staff collects (i.e. the coded information) to:

- store and analyze the information;

- share it with government organizations (this will not include any information that directly identifies you);

- publish the results of the study (this will not include any information that directly identifies you);

- share it as part of research with other companies or universities for the purpose of further understanding malaria disease in your country (this will not include any information that directly identifies you).

- use it to plan new studies or other types of research or other medical purposes related to malaria.

Participant ID ....

SIGNATURE PAGE

If you have read and understood the written information (or have had the information explained to you), and you have voluntarily accepted to take part in the study, write your name and signature below (or thumbprint).

_____   _____

Participant Name (print)   Participant Signature and Date

_____   _____

Study Staff Conducting   Study Staff Signature and Date

Consent Discussion (print)

Participant is ☐ literate   ☐ illiterate

Witness name, signature and date are required on this form only when the consenting participant is illiterate/not able to read.

_____   _____

Participant Name if illiterate (print)   Date

Participant name and date written by: _____ On _____

(Only fill if participant is illiterate)

_____   _____

Witness Name (print)   Witness Signature and Date

(As appropriate)

Appendix II: Informed Consent Form – Infants.

NELSON MANDELA METROPOLITAN UNIVERSITY

PARTICIPANT INFORMATION SHEET AND INFORMED CONSENT FORM FOR PARENT(S)/GUARDIAN(S)

> This consent form should be signed only
> between 09 – 08 – 2010 and 0 8 – 08 – 2011
> Approved by NHSRC, Malawi on 09–08–10

Reference Number:

Study Title: Detection and identification of Plasmodium species causing malaria in Malawi using rapid diagnostic tests

ICF Version Number: 001

Age group: At least 2 years to adolescents

Date: 12 April 2010

Subject Identification: _____

What is consent?

Consent means agreeing to let your child take part in this research study of malaria disease in your area. You have the right to decide if you want your child to take part in the study or not. This document is to inform you about the study. Please take time to read the following information carefully and discuss it if you wish with friends, relatives and your health workers. Ask us if there is anything that is not clear or if you would like more information.

Why is this study being carried out?

The research study we are asking your consent for is being carried out in order to obtain more detailed information on the malaria disease in your area. Malaria is a serious disease transmitted by the bite of a mosquito infected with malaria parasites. It can cause fever, anemia, loss of consciousness and sometimes death. Babies and young children are at greatest risk of severe illness and death due to malaria.

The information that will be collected from this study will be used to determine how reliable malaria rapid diagnostic tests are in identification of different types of malaria parasites for proper management of malaria disease in your area.

How many people are there in the study?

The study will enroll 250 people, from 2 years of age to adults.

What does this study involve?

If you agree for your child to take part in this study, If you agree to take part in this study, 3 ml (1 teaspoon) of blood sample will be taken in order to identify the species (type) of malaria you are infected with.

Participant ID ....

You will also be asked for details concerning

- Age of your child

- body temperature of your child

- location of your house.

What are the foreseeable risks for taking part in the study?

Your child may experience slight pain when the blood sample is being taken.

Who should I contact to answer any questions on the study?

If you have any questions 1) relating to this study, 2) concerning the rights of people in research studies, you should contact Mr Gerald Tegha on 0999 200 668 or the Chairman of the committee responsible for the protection and rights of research participants, Dr. Charles Mwansambo on 0888 826 946 or write to the Chairperson, National Health Sciences Research Committee (NHSRC), P.O. Box 30377, Lilongwe 3, Malawi.

What payments will be made for the study?

You will not receive any direct payment for your child's participation in this study.

Are there any benefits for taking part in the study?

The information from this study will help us to better understand how to protect children against malaria which will be of great benefit to children in the future.

If your child takes part in the study and is found to be unwell due to malaria, he/she will be treated under the existing government services.

What will happen to blood samples from this study?

The blood sample collected during this study will be used to identify what type of malaria your child has. The remaining blood will be stored for future studies if you agree to do so.

The blood samples will be coded with your child's study identification number. This means that they will not be labeled with information that directly identifies your child.

Does your child have to stay in the study?

You may change your mind about your child's participation in the study at any time, without giving a reason. Your decision will not affect the medical care your child will receive outside of the study.

Participant ID ....

Who will have access to my child's personal information?

If you decide to let your child take part in the study, the study doctor and staff will collect medical and personal information about your child during the study. Lecturers from the university and others like members of the ethics committee or the review board for the study will have access to this information in order to check that the study is done properly. All staff who sees this information will keep it confidential.

What will the investigators and the collaborating partners do with the information they get?

The investigator may use the information that the study staff collects (i.e. the coded information) to:

- store and analyze the information;

- share it with government organizations (this will not include any information that directly identifies your child);

- publish the results of the study (this will not include any information that directly identifies your child);

- share it as part of research with other companies or universities for the purpose of further understanding malaria disease in your country (this will not include any information that directly identifies your child).

- use it to plan new studies or other types of research or other medical purposes related to malaria.

Participant ID ....

SIGNATURE PAGE

If you have read and understood the written information (or have had the information explained to you), and you have voluntarily accepted your child to take part in the study, write your name and signature below (or thumbprint).

_____

Name of the Participant

_____   _____

Participant's Parent/Guardian Name   Parent/Guardian's Signature and Date
        (print)

_____   _____

Study Staff Conducting   Study Staff Signature and Date

Consent Discussion (print)

Participant's Parent/Guardian is   ☐ literate   ☐ literate

Witness name, signature and date are required on this form only when the consenting participant is illiterate/not able to read.

_____   _____

Participant's Parent/Guardian's Name if illiterate   Date
        (print)

Participant's Parent/Guardian name and date written by: _____ On _____

(Only fill if participant's parent/guardian is illiterate)

_____   _____

Witness Name (print)   Witness Signature and Date

(As appropriate)

Appendix III: **Letter of Approval from the Lilongwe DHO.**

Ref. No.:
Telephone No.:   **265 727017**
Telefax No.:   **265 727817**
Telex No.:
E-Mail.   lilongwedho@malawi.net

Please address all communications to:
The District Health Officer

Lilongwe District Health Office
P.O. Box 1274
Lilongwe
Malawi

27th April, 2010

To whom it may concern,

RE: PERMISSION TO CONDUCT RESEARCH IN LILONGWE DISTRICT.

Permission has been granted to the bearer of this letter,

Mr. Gerald Loiswayo Tegha

to conduct a research in Lilongwe District on

Detection and identification of Plasmodium species causing malaria using rapid diagnostic

tests at Bwaila Hospital.

Any assistance rendered would be appreciated.

LILONGWE DISTRICT HEALRT
OFFICE

Dr. M. Mwale   2 7 APR 2010
DISTRICT HEALTH OFFICER
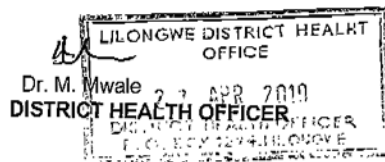
Appendix IV: Letter of Approval from Malawi NHSRC.

Telephone: + 265 789 400
Facsimile: + 265 789 431
e-mail doccentre@malawi.net
All Communications should be addressed to:
The Secretary for Health and Population

In reply please quote No. MED/4/36c

MINISTRY OF HEALTH
P.O. BOX 30377
LILONGWE 3
MALAWI

9 August 2010

Gerald Loisswayo Tegha
UNC Project

Dear Sir/Madam,

RE: Protocol # 760: Detection and identification of plasmodium species causing rapid diagnostic test, version 1.0, March 2010

Thank you for the above titled proposal that you submitted to the National Health Sciences Research Committee (NHSRC) for review. Please be advised that the NHSRC has reviewed and **approved** your application to conduct the above titled study.

- **APPROVAL NUMBER**   : NHSRC #760
  The above details should be used on all correspondence, consent forms and documents as appropriate.
- **APPROVAL DATE**   : 09/08/2010
- **EXPIRATION DATE**   :This approval expires on 08/08/2011
  After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the NHSRC secretariat should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING** :All serious problems having to do with subject safety must be reported to the National Health Sciences Research Committee within 10 working days using standard forms obtainable from the NHSRC Secretariat.
- **MODIFICATIONS:** Prior NHSRC approval using standard forms obtainable from the NHSRC Secretariat is required before implementing any changes in the Protocol (including changes in the consent documents). You may not use any other consent documents besides those approved by the NHSRC.
- **TERMINATION OF STUDY:** On termination of a study, a report has to be submitted to the NHSRC using standard forms obtainable from the NHSRC Secretariat.
- **QUESTIONS:** Please contact the NHSRC on Telephone No. (01) 789314, 08588957 or by e-mail on doccentre@malawi.net
- **Other:**
  Please be reminded to send in copies of your final research results for our records as well as for the Health Research Database.

Kind regards from the NHSRC Secretariat.

....
FOR CHAIRMAN, NATIONAL HEALTH SCIENCES RESEARCH COMMITTEE

Appendix V: Composition and Preparation of Field Stains for Malaria.

FIELD STAIN COMPOSITION AND PREPARATION

Solution A

Medicinal methylene blue - 0.8 g

Azur I - 0.5 g

Disodium hydrogen phosphate anhydrous (Na2 HPO4) - 5.0 g

Potassium dihydrogen phosphate (KH2PO4) - 6.25 g

Distilled water - 500 ml

Solution B

Eosin - 1.0 g

Disodium hydrogen phosphate anhydrous (Na2 HPO4) - 5.0 g

Potassium dihydrogen phosphate (KH2PO4) - 6.25 g

Distilled water - 500 ml

- For both solution A and B, prepare first the phosphate solutions and pour them into a hard glass bottle containing glass beads.

- Add the stains and mix accurately.

- The stock solution needs to be filtered every two weeks and to be stored in tightly stoppered bottles to avoid the growth of moulds.