Pathology of Placental Malaria in Baboons (Papio anubis) Experimentally Infected with Plasmodium knowlesi

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A Thesis submitted in partial fulfillment for the degree of Master of Science in Molecular Medicine in the Jomo Kenyatta University of Agriculture and Technology.

2009
DECLARATION

This is my original work and has not been presented for a Degree in any other University.

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DEDICATION

I dedicate this thesis to my loving husband Abraham, daughter Joy, parents Dr and Mrs. Onditi and my siblings. Thank you for your unconditional support and love during this period.
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I acknowledge with great honour the unconditional guidance of my supervisors Dr. Naomi Maina, Dr. Hastings Ozwara and Dr. Joseph Mwatha. They gave their all to ensure that this work was produced well.

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## LIST OF ABBREVIATIONS AND ACRONYMS

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<tbody>
<tr>
<td>ARD</td>
<td>Animal Resource Department</td>
</tr>
<tr>
<td>CIDR1</td>
<td>Cystein-rich Interdomain Region 1</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon Monoxide</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CS</td>
<td>Caesarean Section</td>
</tr>
<tr>
<td>CSA</td>
<td>Chondroitin Sulphate A</td>
</tr>
<tr>
<td>DBL-γ</td>
<td>Duffy Binding-like Gamma Domain</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic Acid</td>
</tr>
<tr>
<td>hCG</td>
<td>Human Chorionic Gonadotropin</td>
</tr>
<tr>
<td>HIER</td>
<td>Heat Induced Epitope Retrieval</td>
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<tr>
<td>hPL</td>
<td>Human Placental Lactogen</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IEs</td>
<td>Infected Erythrocytes</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon- Gamma</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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IRBCs  Infected Red Blood Cells
ISERC  Institution Scientific and Ethical Review Committee
IUGR  Intrauterine Growth Retardation
IVS  Intravillious space
LBW  Low Birth Weight
NK  Natural Killer
O₂  Oxygen
PAM  Pregnancy Associated Malaria
PAN  Papio Anubis
PCV  Packed cell volume
P/fEMP 1  Plasmodium falciparum erythrocyte membrane protein 1
PM  Placental malaria
RBCs  Red Blood Cells
rpm  Revolutions Per Minute
T₀  Baseline Sampling (Point of Infection)
T₁  7 Days Post Infection (Point of CS)
T₂  14 Days Post Infection
Th₁  T Helper 1
Th₂  T Helper 2
TNF-α  Tumour Necrosis Factor- Alpha
UEs  Uninfected Erythrocytes
VCAM-1  Vascular Cell Adhesion Molecule-1
<table>
<thead>
<tr>
<th>WBCs</th>
<th>White Blood Cells</th>
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ABSTRACT

Pregnant women are more susceptible to malaria than non-pregnant women. This susceptibility is highest in first and second pregnancies of continuous malaria exposure. Placental malaria leads to poor birth outcomes and poor maternal outcomes which include low birth weight (LBW), intrauterine growth retardation (IUGR), abortions, still births, anaemia and mortality. This study set out to characterize placental pathological changes in baboons infected with *Plasmodium knowlesi* parasites in order to develop a model that can be used to study placental malaria. Third trimester placentas were collected via caesarean section (CS) from four pregnant *P. knowlesi* infected and three pregnant-non infected baboons (*Papio anubis*). The placentas from all the animals were examined for gross pathology and histopathology. Findings of the study revealed that parasitaemia was six to eight folds higher in the placenta compared to peripheral blood of the same baboon. Gross pathology revealed that placentas were intact and had normal morphological features except in one baboon which showed slight fibrinoids. Histopathological findings showed that placentas from the control baboons had intact villi, good tissue perfusion and no aggregation of erythrocytes. The placentas from infected baboons on the other hand showed massive congestion of various layers, disruption of the villi, aggregation of erythrocytes, inflammatory cell infiltration, presence of infected erythrocytes and malaria pigment (haemozoin). The placental tissues from the infected baboons had significantly more pathological changes as compared to those obtained from the non infected baboons. This was evident when the median scores
were compared for each parameter using the Mann-Whitney U test. *Plasmodium knowlesi* infected placentas had significantly higher scores for damage (Med$_{1}$ = 22.5) compared to the controls (Med$_{c}$=13; p<0.05). Negative correlation was observed between placental damage and infant weight (r=-0.14, p>0.05) when tested by Spearman’s Rank correlation test. This study demonstrates that *P. knowlesi* sequesters in the placenta of baboons and damages it just like *P. falciparum* does in the placenta of humans. Consequently, the baboon model of malaria is expected to gain prominence in the study of control measures against placental malaria.

**Key Words:** *Papio anubis, Plasmodium knowlesi, placental malaria, histopathology.*
CHAPTER ONE: INTRODUCTION

1.1 Background Information

Malaria is a complex and overwhelming health problem with 300 to 500 million cases and up to 3 million deaths annually. Reports indicate that 90% of these deaths occur in Sub-Saharan Africa (World Health Organization, 2000). Most of malaria related morbidity and mortality occurs in young children (below five years) and pregnant women living in malaria endemic areas. In these endemic regions, a protective semi-immunity against *Plasmodium falciparum* is acquired during the first 10 to 15 years of life (Riley *et al.*, 1994).

In areas of stable malaria transmission where the most common species is *P. falciparum*, most infections are asymptomatic and usually persist for long periods at low densities. The clinical outcome of malaria infection depends on various factors attributed to the parasite, host, geographical area and social factors (David *et al.*, 2002).

Malaria is a disease that affects the red blood cells (RBCs) and is transmitted by the female mosquitoes of the genus *Anopheles*. In Africa, *Anopheles gambiae* and *A. funestus* are the main vectors of this disease. The protozoan parasite, *Plasmodium* is responsible for malaria. In humans, *P. falciparum, P. ovale, P. malariae, P. vivax* and *P. knowlesi* cause the disease. *Plasmodium knowlesi*, is a primate malaria
causing parasite and its infection in humans has recently been seriously witnessed in Southeast Asia (Cox-Singh et al., 2008; White, 2008).

The molecular and cellular events that occur during the life cycle of Plasmodium influence the severity of the disease. Simultaneous binding of the parasite to several receptors, to uninfected erythrocytes (rosetting), and the clumping of infected erythrocytes through platelets are associated with the pathogenesis of malaria thereby affecting many tissues and organs such as the brain, spleen, lungs, liver, heart, and placenta among others.

Various interventions have been used in the fight against malaria with the aim of controlling the impact of this disease. These interventions include: case management of infected patients through proper diagnosis and treatment; prevention of infection through vector control and disease prevention by prophylactic drugs. These interventions have had various shortcomings. The WHO recommends that persons suspected of having malaria be diagnosed and treated with effective drugs within 24 hours of the onset of symptoms. However, many patients in malaria endemic areas have no access to health care within the 24 hr period. This is mainly due to poverty, inadequate health, infrastructure and ignorance. On the other hand, vector control suffers from inadequate resources and larval resistance to larvicidal compounds. Similarly, the use of antimalarial drugs has led to drug resistance to the affordable drugs (World Health Organization, 2008).
It is estimated that at least 50 million women globally are exposed to malaria annually and approximately 3.5 million of them get infected (Brabin, 1983). With low malaria prevalence in adults, pregnant women in malaria endemic areas are highly susceptible to the disease compared to their non pregnant counterparts. This susceptibility is greatest in first and second pregnancies (Brabin, 1983).

Malaria during pregnancy leads to poor birth and maternal outcomes. Poor birth outcomes include low birth weight (LBW), intrauterine growth retardation (IUGR), abortion and still birth while poor maternal outcomes consists of anaemia and mortality (Brabin, 1983; Meeusen et al., 2001; Steketee, 2001). This is because during pregnancy the mother’s immune system is compromised by the depression of cell-mediated immunity that allows the foetus as an allograft to be retained by the mother. This however interferes with resistance to various infectious diseases which include malaria (Meeusen et al., 2001). In addition, cellular immune responses to \( P. falciparum \) antigens are depressed in pregnant women. This condition usually results in the accumulation and thriving of parasites in the intervillious space (IVS) of the placenta, leading to placental malaria (PM) (Brabin et al., 2004).

In order to effectively manage this disease, placental malaria must be ultimately understood in humans. Characterization of this disease in humans is however based on descriptive findings. Because of ethnical reasons, only a few reports have
included a histopathological examination of the placenta from infected women (Billie et al., 2000). Research on placental malaria in non-human primates has been carried out over the years (Billie et al., 2000). These studies have been based on animal species whose reproductive system is different from that of humans. There is therefore need to study placental malaria in detail using an animal model whose reproductive system and menstrual cycle is similar to humans. The olive baboon (Papio anubis) is ideal candidate for this.

In this study, the Olive Baboon (P. anubis) was used to study placental malaria. Since baboons have been used in biomedical research since 1927, there is enough baseline data which shows close parallelism to humans (Firyal, 1998). The reproductive endocrinology of the female baboon is also similar to that of female humans. Its menstrual cycle is 28-30 days and its gestation period is 27 weeks. The hormonal profile between baboons and humans is comparable (Firyal, 1998). In addition, P. anubis is fully susceptible to experimental infection by P. knowlesi leading to either severe malaria or controlled parasitaemia that results in mild infection (Ozwara et al., 2003). In order to fully take advantage of the P. knowlesi-baboon model, pathology of the infection in the placenta must be understood. This includes understanding pathology of placental malaria.
1.2 Problem Statement

Placental malaria is commonly associated with poor birth outcomes. To the foetus, it leads to LBW, IUGR, still birth, abortion, while to the mother it may cause anaemia and mortality (Menendez et al., 2000). Although placental malaria has recently attracted many research efforts, the studies have had shortcomings that result from confounding inherent variables such as mother’s health status, inaccurate estimation of gestation age, inadequate tissues for analysis, patient compliance, socio-economic conditions, moral and ethical limitations (Matteelli et al., 1997; Brabin et al., 1991).

In addition, the existing monkey models of malaria in pregnancy have not demonstrated sequestration of plasmodium infected erythrocytes in the placenta and yet that is the basis of placental pathology, which ultimately determines the clinical complications. Despite increased understanding of the pathology of placental malaria, the exact mechanisms leading to impairment of materno-fetal exchange are not well understood. There are suggestions that leucocytes, through the production of non-chemotactic cytokines, might be associated with the thickening of the trophoblastic basement membrane which might cause mechanical blockage of placental oxygen and nutrient transport (Matteelli et al., 1997).

The structural similarities between the baboon and human placenta and the cytoadherent property of P. knowlesi (Ozwara, 2005) make it likely for P. knowlesi to sequester in the placenta. Infection of pregnant baboons with P. knowlesi can
therefore be expected to produce an experimental system that will facilitate further studies of malaria in pregnancy.

1.3 Rationale

Placental histology is considered the “gold standard” of malaria determination during pregnancy. This is because it can show signs of active infection (presence of infected erythrocytes in the intervillous space), past or chronic infection (malarial pigment) or both (Shulman et al., 2003).

Because of the confounding variables that are commonly encountered in human studies, the *P. knowlesi* baboon model is a useful alternative because the reproductive anatomy and physiology of the baboons and humans are similar (Onderdonk et al., 1998) and can therefore accurately mimic the human disease state and immunological response to infection (Kennedy et al., 1997). The baboon (*P. anubis*) is also readily available in Kenya where the study was conducted.

Therefore, as an experimental system, it is possible to monitor the mother’s health status, to determine gestation age and to collect adequate samples within a specific period of time.
1.4 Justification

The ultimate goal of all biomedical research is to obtain a clear understanding of the normal physiology, biochemistry and disease processes that are usually of great relevance to humans, and malaria is not an exception. In Africa, 3.5 million women get exposed to malaria, leading to 100,000 infant deaths every year (Desai et al., 2007). Although much has been learned from studies of malaria during pregnancy in humans, progress has been limited due to lack of a suitable animal model (Billie et al., 1998). An effective disease model should be able to closely mimic the pathogenesis of malaria in pregnant women. Non-human primates are the ideal candidates for such studies. A study done by Billie et al. (2000), revealed that *P. coatneyi* did not sequester in the placenta of the rhesus monkey and as a result cannot be used to study placental malaria.

Baboons in this case are ideal because they have a similar host-pathogen interaction and a reproductive system that is physiologically similar to that of humans. They have a menstrual cycle of 28 days and a gestation period of 180 ± 7 days. These baboons are also susceptible to experimental infection by *P. knowlesi* parasites. This parasite is similar to *P. falciparum* with the exception of duration in the life cycle. The life cycle of *P. falciparum* is 48 to 72 hours while that of *P. knowlesi* is 24 hours (David et al., 2002). It is also a cytoadherent parasite (Ozware, 2005) and is likely to
sequester in the placenta. Infection of pregnant baboons with *P. knowlesi* can therefore be expected to produce an experimental system that will facilitate further pathophysiological studies of malaria in pregnancy. This will undoubtedly contribute valuable data to be used in the development of preventative, control and therapeutic measures against malaria during pregnancy.

1.5 Hypothesis

1.5.1 Null Hypothesis (H₀)
Baboon (*P. anubis*) infection with *P. knowlesi* H strain is not associated placental damage that is characterized by thickening of the trophoblastic basement membrane, trophoblastic damage, high levels of parasite and pigment (haemozoin) in the intravillious space, fibrinoid necrosis of the villi and monocyte abundance in the intravillious space.

1.5.2 Alternative Hypothesis (Hₐ)
Baboon (*P. anubis*) infection with *P. knowlesi* H strain is associated placental damage that is characterized by thickening of the trophoblastic basement membrane, trophoblastic damage, high levels of parasite and pigment (haemozoin) in the intravillious space, fibrinoid necrosis of the villi and monocyte abundance in the intravillious space.
1.6 Objectives

1.6.1 General Objective

To develop a baboon (P. anubis) model of placental malaria by characterizing the underlying pathology of placentas from P. knowlesi infected baboons.

1.6.2 Specific Objectives

The specific objectives include the following;

1) To observe parasitaemia and clinical profiles of baboons infected with P. knowlesi parasites

2) To describe the pathological changes in baboon placentas as a result of experimental P. knowlesi infection.

3) To compare the pathological changes of baboon placentas obtained from P. knowlesi infected and non-infected animals.
CHAPTER TWO: LITERATURE REVIEW

2.1 Malaria Parasites

Malaria parasites are microorganisms that belong to the genus Plasmodium. There are more than 100 species of Plasmodium that can infect various animal species ranging from reptiles, to birds, to mammals (World Health Organization, 2008). *Plasmodium gallinaceum* causes malaria in birds such as domestic fowls (chicken), *P. berghei, P. chabaudi, P. vinckei* and *P. yoelii* causes malaria in rodents while *P. knowlesi* affects non-human primates such as baboons. Humans are infected by *P. falciparum, P. vivax, P. ovale* and *P. malariae* and to a lesser extent by *P. knowlesi* (White, 2008).

Malaria distribution is based on vector distribution and climatic factors such as temperature, humidity and rainfall. Malaria is mainly transmitted in tropical and subtropical areas where *Anopheles* mosquitoes can survive and multiply (Figure 1). The highest transmission is found in Africa South of the Sahara. In cooler regions, transmission is less intense and more seasonal. In such climatic areas, *P. vivax* might be more prevalent because it is more tolerant of lower ambient temperatures (Waters *et al.*, 1991).
*Plasmodium falciparum* is found in tropical and subtropical areas (Figure 1). *Plasmodium vivax* is found mostly in Asia, Latin America, and in some parts of Africa while *P. ovale* is found mostly in Africa (especially West Africa) and the islands of the Western Pacific (Waters *et al.*, 1991).

![Figure 1: Global distribution of malaria transmission risk (Hay *et al.*, 2004)](image)

**2.2 Life Cycle of Plasmodium**

The life cycle of the malaria parasite is complex. Sporozoits (1 in Figure 2) are transmitted to the vertebrate host by the bite of an infected female mosquito of the genus *Anopheles*. Once in the blood circulation, they enter hepatocytes (liver cells)
where they develop into the preerythrocytic (exoerythrocytic) schizonts (2 to 3 in Figure 2). The period of preerythrocytic schizont development varies from one species of parasite to another. Some species like *P. vivax*, *P. ovale* and *P. cynomolgi* have a dominant stage called the hypnozoite stage where the parasite remains dormant in the liver for weeks or even years before they develop into preerythrocytic schizonts. The preerythrocytic schizonts usually contain millions of merozoites which are released into the blood circulation where they invade the red blood cells. Merozoites develop within the erythrocytes through ring, trophozoite and schizont stages (erythrocytic schizogony). Erythrocyte invasion by merozoites is dependent on the interactions of specific receptors on the erythrocyte membrane with ligands on the surface of the merozoite. A small portion of the parasites differentiates from newly invaded merozoites into sexual forms, which are macro-gametocyte (female) and micro-gametocyte (male) (4 in Figure 2). Mature macro-gametocytes, taken into the midgut of the *Anopheles* mosquito, escape from the erythrocyte to form macro-gametes while the micro-gametocytes exflagellate to form motile micro gametes after a few minutes in the mosquito midgut (5 in Figure 2). When the micro-gamete fertilizes a macro-gamete, a zygote is produced. After infection, depending on the *Plasmodium* species and ambient temperature, thousands of sporozoites are released and they invade the salivary gland epithelium (6 in Figure 2). When an infected mosquito bites a susceptible vertebrate host, the *Plasmodium* life cycle begins again.
2.3 Pathogenesis of Malaria

The molecular and cellular events during the parasite life cycle influence the severity of the disease. Disease occurs only as a result of the asexual blood stage after the parasite leaves the liver and begins to invade and grow inside RBCs. All human Plasmodium species invade by the same mechanism, but P. falciparum reaches higher parasitaemia because of greater flexibility in the receptor pathways that it can use to invade all RBCs. The RBCs infected with P. falciparum must bind to endothelium or placenta for the parasite to avoid spleen-dependent killing mechanisms (Sim et al., 1994). This binding together with the release of malaria
antigens, pigments and toxins following infection leads to a cascade of pathological events. Similarly, the process of schizont rapture results in the production of cytokines such as tumour necrosis factor (TNF) that cause fever during malaria infection. Other circulating endogenous pyrogens such as interleukin-1 (IL-1) and IL-6 are also involved in the clinical symptoms of the disease (Sim et al., 1994; Pasvol and Hogh, 1995).

The outcome of an infection and progression into pathology depends on the specific and dynamic combination of the host and the parasite properties. Parasites sequestration in various organs including bone marrow, spleen, heart, lung, brain, liver, kidney, subcutaneous tissues and placenta is through receptors. These variable and numerous amounts of receptors are expressed by the various endothelial cells in these organs and syncytiotrophoblasts in placenta (Edington, 1967; Edington 1969; Francis and Werrell 1993). The adhesion phenotype is not homogenous, and different parasites can bind to variable numbers and combinations of host receptors (Baruch 1999). This variability is believed to affect tissue distribution and pathogenesis of parasites.

In the acute malaria stage, the bone marrow vessels are full of parasitized erythrocytes, which lead to megaloblastic changes that in turn may result in folic-acid deficiency. The spleen on the other hand is characterized by intense
enlargement and blood vessels that are filled with parasitized RBCs. In the liver, the pathological changes vary according to the immunological status of the individual. The striking feature however, is due to acute malaria stage is the congestion of the sinusoids and centrilobular veins by parasitized erythrocytes. The Kupffer cells also contain parasitized and parasite free RBCs and remnants of parasites (Edington, 1967).

In the lungs, the smaller vessels are with parasitized erythrocytes while the alveolar space is thickened. The alveoli are congested with pigmented macrophages, plasma cells, neutrophils and parasitized erythrocytes. In the kidney, severe malaria is characterized by congestion of the vessels with parasitized erythrocytes. Histological changes include acute tubular necrosis due to reduced cortical perfusion, pigmentation of the vessels, intestinal tissues, and epithelial cells and within phagocytes (Gilles and Hendrickse, 1963).

Malaria deaths may occur as a result of cerebral malaria, malaria related anaemia, metabolic acidosis, placental malaria or a combination of these four. The life cycle of the Plasmodium parasite requires it to multiply within infected erythrocytes (IEs) and eventually cause them to burst. The parasite is able to infect up to 5% of the host’s red blood cells (RBCs) this being a major cause of anaemia in malaria. Recent studies have also revealed that P. falciparum infection causes modifications in both IEs and uninfected erythrocyte (UE) membranes that mimic the normal aging
process of the RBCs. As a result, the UEs that are exposed to the IEs undergo premature aging process thus contributing to anaemia (Steketee, 2001).

2.4 Malaria Control

As malaria control intensifies, it is vital to monitor malaria burden and trends, and to track the coverage and impact of interventions. The government of every country affected by malaria has a national control policy covering prevention and care management. Methods of control are mainly the use of anti-malaria treatment and vector control (WHO, 2008). The objective of anti-malarial treatment is to ensure cure of the infection, reduce morbidity and mortality of the disease, including malaria related anaemia, prevent the progression of uncomplicated malaria into severe and potentially fetal disease, reduce the impact of malaria infection of the foetus during pregnancy and prevent the spread of drug resistance. Currently, this usually is achieved by use of atemisinin-based combination therapy (ACT), (World Health Organization, 2008).

Vector control on the other hand is aimed at significantly reducing the incidence and prevalence of both parasite infection and clinical malaria. There are two main approaches to this” the use on long lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS). These can be complemented with larval control or environmental management. The insecticide treated nets are usually given free to
children and to pregnant women who are at a higher risk of getting the malaria infection (World Health Organization, 2008).

2.5 Placental Malaria

Maternal or placental malaria is a common complication in malaria endemic areas. In Africa, where the burden of maternal and foetal morbidity is high, it accounts for 100,000 infant deaths annually (Steketee, 2001; Desai et al., 2007). Indeed pregnant women in these regions have higher levels of parasitemia and parasite density compared to non-pregnant women (Nosten et al., 2004). As a result, the mother is susceptible to anaemia, cerebral malaria, pulmonary oedema and kidney failure (Shulman and Dorman, 2003; Steketee, 2001). Foetus is susceptible to abortion, stillbirth, premature delivery, low birth weight (LBW) and intrauterine growth retardation (IUGR) (Shulman and Dorman, 2003; Steketee, 2001). Low birth weight in humans is defined as birth weight that is less than 2500g, and this is usually the most important risk factor for infant mortality (McCormick, 1985; Greenwood et al., 1992).

It is thought that there is some degree of pre-existing immunity that is retained during pregnancy (Dorman and Shulman, 2000). In spite of this pre-existing immunity, these women are still susceptible to placental malaria (Menendez et al., 2000; Tako et al., 2005). Placental malaria is also higher in primigravidae and
secundigravidae than in multigravidae (Okoko et al., 2002). Several reports have shown that the risk and severity of placental malaria decreases with increase in the number of pregnancies suggesting that immune built-up is finally achieved after several pregnancies and infections (Shulman and Dorman, 2003; Steketee, 2001).

2.6 Structure of the Human Placenta

The placenta is the foetus’ extension into the mother, where it functions as the interface between the two. At term, the placenta is usually a circular-shaped structure with a diameter of about 20cm and a thickness of approximately 2.5cm. It is composed of three layers. The innermost layer surrounding the foetus is the amnion (Plate 1) while the middle layer is the allantois. Blood vessels originating from the umbilicus traverse this membrane. The outermost layer is the chorion (Plate 1) and is in contact with the endometrium. The chorion further consists of two layers of cells, the inner cytotrophoblast and the outer syncytiotrophoblast. The foetal portion of the placenta (amnion) consists of the chorionic plate, its villi and the peripheral trophoblastic shell that surrounds the intervillous space and covers the maternal tissue. The anchoring villus contacts the decidua basalis (maternal portion) that is characterized by large, polyhedral pale blue stromal (decidua) cells (Walter et al., 1982; Fried et al., 1998).
The foetal circulation enters the placenta via the umbilical arteries embedded within the umbilical cord. Once in the placenta, the foetal circulation branches into units called cotyledons (Plate 1). The finest branches of the foetal circulation are made up of capillary loops within the chorionic villi. Once nutrients have been absorbed and waste products released in the placenta, the foetal blood ultimately collects into the umbilical vein, where it returns to the foetus via the umbilical cord. The finger-like chorionic villi are the main functional units of the placenta that mediates nutrient absorption, waste elimination and generation of hormones (human chorionic gonadotropin (hCG)).

Towards the end of the first trimester there is a central mesenchymal core with embedded foetal capillaries surrounded by a layer of cytotrophoblasts and syncytiotrophoblasts. The syncytiotrophoblast layer, a flat multinucleated cell sheet for most of pregnancy, develops grape-like nucleated clusters within its cytoplasm called syncytial knots near term. Although there are fewer cytotrophoblasts visible at term, they are still present and act as precursor cells for all the other trophoblast types. Within the chorionic villi, cytotrophoblasts fuse to form the overlying syncytiotrophoblast (Peter, 2008).
Plate 1: Anatomy of a normal human placenta. The foetal surface is smooth, glistening, and covered by amnion. The maternal surface is red and is divided into many cotyledons (Peter, 2008).

Concomitant with the overall development of placental architecture is the differentiation of three distinct trophoblast types. Depending on their subsequent function in vivo, undifferentiated cytotrophoblasts can develop into either hormonally active villous syncytiotrophoblasts, or as an extra villous anchoring trophoblastic cell column, or as invasive intermediate trophoblasts. Within the villi of the human placenta there always exists a population of cytotrophoblasts (Peter, 2008).
2.7 Functions of the Placenta

2.7.1 Production and Secretion of Hormones

The syncytiotrophoblast cells of the placenta secrete four main kinds of hormones: oestrogen, progesterone, and two hormones peculiar to the placenta—human placental lactogen (hPL) and human chorionic gonadotropin (hCG). Oestrogen is one of the most important hormones during and after pregnancy. During pregnancy, oestrogen stimulates the growth of the uterus, enhances the blood flow between the uterus and the placenta, and causes the breast to enlarge as a preparation for milk production. It also stimulates contraction of the uterus muscle during baby delivery (EI-Mowafi, 1999). Progesterone maintains the inner layer of the uterus by stimulating its growth in order to provide a supportive environment for embryonic development (Martin et al., 1970). The human placental lactogen (hPL) on the other hand, has two major functions. First, it helps to regulate the mother’s metabolism, or the balancing between energy sources and wastes during pregnancy, and second, it aids in nutrient transport across the placenta ensuring that sufficient amounts of nutrients are supplied to the foetus (EI-Mowafi, 1999). The human chorionic gonadotropin (hCG) supports the production of oestrogen and progesterone in the first 10 weeks of pregnancy until the syncytiotrophoblast cells can produce these two crucial hormones by themselves.
2.7.2 Immunological Barrier

The placenta has two major roles. First, its membrane permeability permits the transport of antibodies from the mother to the foetus a phenomenon referred to as passive immunity, and second, it enables the foetus to survive in the maternal body as a genetically foreign tissue by immunosuppressing the mother’s immune system. It does this by, non-selective and specific suppression. In non-selective suppression, the placenta secretes several molecules such as progesterone that suppress the immune system. In specific suppression, the cells in the mother’s immune system that can recognize the father’s part of genetic make-up are selectively abrogated and hence blunting the immune response against the foetus (Gilbert, 1997).

2.7.3 Gas, Nutrient and Waste Exchange

The blood from the mother acts as the exchange mechanism for nutrients and waste removal. In the placenta, the blood supply comes from the arteries in the uterus and enters the blood lake. It then travels toward the chorionic plate (the baby’s side) and returns to the mother’s side, leaving through the veins in the uterus (Hamilton et al., 1962). Oxygen (O₂) and Carbon dioxide (CO₂) are the two essential gases the placenta transports. They pass across the placenta by simple diffusion due to concentration gradient. Another special property that allows efficient O₂ transport is that the baby’s RBCs tend to hold on to O₂ more tightly than a normal adult’s RBCs (El-Mowafi, 1999). Other harmful gases such as Carbon monoxide (CO) can also
pass across the placenta thereby affecting the foetus. Smaller nutrients like water, sodium, and calcium can pass to the baby by simple diffusion, whereas larger nutrients such as protein molecules pass the placenta by the cells’ packaging them into bubble sacks. Other nutrients travel across the placenta with the help of the proteins specialized in transportation.

### 2.8 Characteristics of Malaria Infected Placenta

Placental malaria is the accumulation of parasitized erythrocytes in the intervillious space, infiltration of inflammatory cells and release of pro-inflammatory mediators that cause pathologic alterations in the placenta (Walter et al., 1982; Fried et al., 1998; Ordi et al., 1998). These results to pathological characterization that is manifested by the presence of intravillous parasites and leukocytes, macrophages containing malaria pigments, fibrin deposits and trophoblasts, proliferation of cytotrophoblastic cells, and thickening of the trophoblastic basement membrane (Yamada et al., 1989; Matteelli et al., 1997). Previous studies have revealed that there are malarial brown pigments that occur in the previllious deposit of fibrinoid in macrophages and free in the intervillious space. Such studies have also revealed that placental malaria leads to excessive fibrinoid deposits that are associated with syncytiotrophoblastic necrosis or ultra structural damage (Walter, et al., 1982).
2.9 Parasite Adhesion in the Placenta

Red blood cells infected with the malaria parasites are able to sequester in the placenta. This sequestration phenomenon is key in the pathogenesis of placental malaria (Fried and Duffy, 1996). The syncytiotrophoblast cells in the placenta expresses different and variable amounts of host cell receptors onto which the parasites bind. This leads to the adherence process (Baruch et al., 1999). The adhesion phenotypes are however not homologous and as a result, different parasites can bind to various numbers and combinations of host receptors (Beeson et al., 1999). A single parasite protein- *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is usually expressed on the surface of the infected RBCs and mediates their binding to the different receptors (Baruch et al., 1999). Studies have shown that the parasitized RBCs isolated from the placenta have a unique binding property compared to parasites isolated from non pregnant individuals (Fried and Duffy, 1996; Beeson et al., 1999). As a result, these parasites are capable of binding to chondroitin sulphate A (CSA) but not CD36 which is the main host receptor for sequestration in the microvasculature. This in turn allows the parasites to sequester in the placenta and not in the endothelium. Indeed, CSA binding parasites express PfEMP1 with a duffy binding like-γ (DBL-γ) domain that binds CSA and non CD36-binding cysteine-rich interdomain region 1 (CIDR1) (Buffet *et al.*, 1999; Gamain *et al.*, 2001). On the contrary, CD36-adherent parasites express a PfEMP1 with a CD36-binding CIDR1 (Gamain *et al.*, 2001).
An additional process through which the parasite sequesters in the placenta is by binding to non-immune immunoglobulins (Ig) (Flick et al., 2001). A cloned parasite line selected from a high level of binding to Ig has been observed to bind to placental sections in vitro. Similarly, placental isolates from four infected Cameroonian women appeared to bind to IgG in vitro. This suggests that IgG could be acting as a bridge molecule between IRBCs and the Fc receptors present on the syncytiotrophoblasts, thereby mediating parasite sequestration in the placenta (Flick et al., 2001).

2.10 Immune Responses in Placental Malaria

With 50% of the genetic material derived from its father, the foetus’s susceptibility to rejection by the maternal immune system is somewhat similar to the susceptibility of a transplanted organ (Alex, 2004). Cell-mediated immunity is particularly suppressed during pregnancy such that the mother is increasingly reliant on humoral immunity for protection (Duffy and Fried, 2003). Initially, it was believed that this suppression is responsible for the high increase of malaria in pregnant women. However, it is now evident that despite the depression, the maternal immune system continues to respond to the parasite. In deed, antibodies preventing the attachment of P. falciparum to the placenta can be produced and quantified (Duffy and Fried, 2003).
During normal pregnancy, Th1 immune actors are subdued while Th2 actors are mainly relied upon so that the foetus is not rejected by the host’s immune system. However, in the case of placental malaria, an increase in Th1 cytokines in the placenta has been observed (Vasilliadis et al., 1994; Fried et al., 1998).

Observations have shown that although the peripheral circulation can be free of parasites, it is likely that these parasites tend to temporarily evade the immune system by hiding within the vascular placenta in pregnant women (Ismail et al., 2000; Menendez et al., 2000).

Primigravid women have been reported to be highly susceptible to malaria while multigravid women have a reduced risk of infection and associated complications (Brabin, 1983). This is so because when a woman is infected with the malaria parasite during her first or second pregnancy, antibodies (Abs) develop against the parasite variants that adhere to the placenta. These Abs contribute to protection from malaria in subsequent pregnancies (Beeson et al., 2002). Initial studies have shown an association between reduced placental infection and the level of antibody primed against PfEMP1 in vitro. Such antibodies were also more prevalent in multigravid women (Fried et al., 1998). A subsequent study carried out in Cameroonian women however, revealed the contrary. It showed that there was no clear association
between infection (peripheral or placental blood) and adhesion-blocking Abs (O’Niel-Dunne et al., 2001).

Examination of antibodies that bind to variant antigens (Ags) on the surface of IRBCs (studies done in Cameroon and Malawi) indicate that these Abs are significantly common in pregnant women with active infection than in uninfected pregnant women (Beeson et al., 2002).

Immunomodulation also occurs in primates challenged with malaria during pregnancy. A study conducted by Billie et al., (2005) showed that in pregnant monkeys leukocyte populations did not increase during Plasmodium infection. That study also concluded that the host immune system under the influence of pregnancy was responsible for the increased severity in clinical symptoms (Billie et al., 2005).

2.11 Pathology of Placental Malaria

Various factors may be responsible for placental pathology. Leucocytes, through the production of non-chemotactic cytokines, might be associated with the thickening of the trophoblastic basement membrane that could cause mechanical blockage of oxygen and nutrient transport across the placenta. These changes have been
associated with syncytiotrophoblastic damage and cytотrophoblastic proliferation (Galbraith et al., 1980; Walter et al., 1982; Yamada et al., 1989). It has also been suggested that these placental lesions, especially the thickening of trophoblastic basement membrane may alter the materno-fetal exchange and contribute to the deleterious effect of malaria-associated placental lesions on foetal growth (Sibley et al., 1998). In addition, *P. falciparum* infection could disturb the folate-B₁₂-metabolic pathway and there is evidence for this mechanism contributing to IUGR (Brabin et al., 2003). Larger molecules such as IgG and transferrin-bound iron are usually transferred across the trophoblast by receptor-mediated endocytosis/exocytosis mechanisms. However IUGR reduces this transfer (Sibley et al., 1998; Yang et al., 1999).

### 2.12 Use of Non-human Primate Models in Biomedical Research

The ultimate goal of all biomedical research is to get clear understanding of the normal physiology, biochemistry and disease process that are relevant to humans. Although some aspects of research can be carried out in humans directly, some experimental aspects are not ethically and practically feasible. Several animal models have so far been used (for example rats, dogs, cats, swine, sheep, goats, cattle among others) and have provided abundant data with significant correlation valuable in the field of medicine. However, because of biological differences between these animals and humans, the data has been inapplicable.
Non human primates hold a prominent key position between these animal models and humans. The most commonly used primates are the Old World species like the rhesus monkeys, the long tailed macaque and the baboon (Firyal et al., 1998). The baboon (Papio spp) has been used in medical research for a very long time (since 1927) and therefore, a lot of baseline biomedical data exists. This data shows close parallelism to humans. As a result, the baboon has been extensively used in numerous studies on cancer, cardiovascular and pulmonary diseases, and infectious diseases.

2.13 Non-human Primates as Models for Placental Malaria

Current animal models that are commonly used in the study of malaria in pregnancy are pregnant mice and rats infected with Plasmodium berghei (Tegoshi et al., 1992). However, their relevance to malaria in human pregnancy is questionable because of the many differences between rodent and human pregnancies. First, progesterone in rats is produced by the corpus luteum of the ovary, rather than the placenta as in humans and secondly because the rodent placenta is labyrinthine hemodichorial, rather than villous hemomonochorial, like the human placenta (Fischer and Wister, 1996).
An effective disease model should closely mimic the pathogenesis of malaria in pregnant women. Non-human primates are ideal candidates because they are susceptible to many species of *Plasmodium* and have humoral and cellular responses similar to those of humans (Coatney *et al.*, 1971). The macaques, great apes and baboons have discoid villous hemochorial placetas similar to those of humans (King, 1993). Macaques are readily available and have been used frequently in malaria research. Macaques used in malaria research include pig-tailed monkeys (*Macaca nemestrina*), cynomolgus or long-tailed monkeys (*M. fasicularis*), and rhesus monkeys (*M. mulatta*). Of these, according to Billie *et al.*, (1998) the rhesus monkey is the best characterized, most widely used, and most available. In addition, the rhesus placenta has been studied extensively (Billie *et al.*, 1998).

Great apes cannot be used because they are an endangered species while baboons are expensive to house because of their size, although they are used in reproductive studies. In addition, baboon malaria models are less well-developed than macaque malaria models. None the less, the reproductive endocrinology of female baboons is similar to that of human females. Its menstrual cycle being 28-30 days with gestation period of 27 weeks (shorter than that of humans but with similar hormonal profiles) makes it a unique non-human primate model for studies that involve reproductive physiology, perinatal biology and experimental embryology (Firyal *et al.*, 1998) and placental malaria.
2.14 Plasmodium knowlesi and its Definitive Hosts

Human malaria parasites are a major priority in research due to the association of malaria with mortality rates in the human population. However, because of host specificity of the malaria parasite to its human host, research on parasite biology is restricted (Graham, 1966). As a result, different experimental systems have been employed to model some of the complex interactions between parasites and their hosts. The three Plasmodium groups that are mainly used in experimental studies on host-parasite interactions are rodent, avian, and primate Plasmodia.

The rodent malaria parasite *P. berghei* is now used to study parasite biology (Cox, 1988). However, it does not effectively allow investigations of natural-host parasite interactions because of its phylogenetic distance (Ozwara *et al.*, 2003). Although the avian Plasmodia such as *P. gallinaceum* and *P. lophurae* are closely related to *P. falciparum*, differences in their life cycle, host specificity and immune system of the host limits their usefulness as models for human malaria (Coatney *et al.*, 1971). On the other hand, simian Plasmodium such as *P. knowlesi* has a comparable phylogeny and host-parasite relationships to the human malaria parasites (Coatney *et al.*, 1971). Its immune and metabolic systems are also phylogenetically similar to human Plasmodia (King *et al.*, 1988). It is also a cytoadherence parasite just like *P. falciparum*. As a result, it is capable of sequestering in the placenta (Ozwara, 2005).
In addition, it shares a lot of vaccine candidate molecules that are similar to *P. vivax* (http://www.sanger.ac.uk)

The natural host of *P. knowlesi* parasite is the *Macaca fascicularis* where it causes a chronic infection. However, it can experimentally infect and cause acute infection in other nonhuman primates such as *M. mulatta, M. radiata, M. assamensis, Presbytis entellus, Callithrix jacchus, Aotus trivigatus, Saimiri sciureus,* and baboons (Butcher, 1996; Coatney et al., 1971). In baboons, the infection has been induced in *Papio cynocephalus, P. doguera, P. jubileaus,* and *P. papio* (Coatney et al., 1971). This parasite has also been confirmed as the fifth human malaria parasite. Since 2004, it accounts for 70% of malaria cases in Southeast Asia where it is mostly found. It also has a close phylogenetic relationship with *P. vivax* (Chin et al., 1965; Yap et al., 1971; Singh et al., 2004; Vythilingam et al., 2006). To date, protocols have been developed for long term *in vitro* culturing of *P. knowlesi* (Kocken et al., 2002). This together with the availability of both natural and artificial hosts, plus primate phylogeny to humans make *P. knowlesi* infection in non human primates the ideal model to study malaria in human including placental malaria.

### 2.15 Life Cycle of *P. knowlesi*

The natural vertebrate hosts of *P. knowlesi* are *Macaca fascicularis* and *M. nemestrina* (Butcher 1996). It can also infect other monkey species such as *M. mulatta,* *M.*
radiata, M. assamensis, Presbytis entellus, Callithrix jacchus, Aotus trivigatus, Saimiri sciurens and baboons (Butcher 1996).

Malaria infection due to *P. knowlesi* is initiated by the injection of sporozoites with saliva during the feeding process of the mosquito vector (*Anopheles latens*, *A. dirus*, *A. balabacensis* and *A. hackeri*). The sporozoites then enter the circulation and are transported to the liver where they invade the hepatocytes. Here, the parasite undergoes asexual replication to form extraerythrocytic schizogony. These then culminate to produce merozoites which are released into the blood stream and on maturation the merozoites mature into schizont following the rapture of the infected erythrocytes (Shulman *et al.*, 2003). The merozoites differentiate into sexual forms called macro- or micro gametocytes (trophozoites). These are usually large parasites that fill up the erythrocytes but only contain one nucleus. In this form, the mosquito vector can ingest them and induce them to form gametes that eventually develop into oocytes that in turn enlarge to form sporozoites that lodge in the salivary gland of the mosquito. When it bites a new vertebrate host, the cycle continues (Ozwara, 2005), (Figure 3).
Figure 3: The lifecycle of the simian malaria parasite, *P. knowlesi* (Ozwara, 2005).

2.16 Importance of Histopathological Studies in Placental Malaria

Histopathology is a very sensitive method of detecting placental malaria although other methods (thin smears, thick smears and impression smears) have also been used (McGregor *et al.*, 1983). These methods have the advantages of being fast, easy to carry out, cheaper and can be used to detect parasites and pigments. In addition, they also help in the identification of different *Plasmodium* species which cannot happen with histology slides (Ismail *et al.*, 2000). Histology on the other hand, is able to detect past infection in addition to good classification of infections (Ismail *et al.*, 2000). Histological processing with tissue fixation and paraffin embedding allows tissues to be kept for a very long time (Moshi *et al.*, 1995). Walter *et al.*
(1982), reported that only 9.5% of the women studied had negative peripheral blood and positive placental parasitaemia, while 61% of the cases were positive in peripheral blood and positive in the placenta. Ismail et al. (2000), also showed in his study that more than 60% of the women with negative peripheral blood parasitaemia had some evidence of either active or past infection in their placental samples. In a study done in Kenya, histological assessment has been reported to be more sensitive than placental smear or peripheral films (Shulman et al., 2003). These studies confirm the sensitivity of histology in parasite detection.

Histological studies have shown that malaria infection of the placenta leads to: thriving and accumulation of IRBCs and haemozoin (malaria pigment) in the intravillious space; changes in the chorionic villi; monocyte abundance in the intravillious space; disruption of the syncytiotrophoblast cell membrane; chorionic plate thrombosis and thickening of the trophoblastic basement membrane (Galbarinth et al., 1980; Walter et al., 1982).

A study by Bulmer et al. (1993), showed that fibrinoid necrosis was a feature of active infection while thickening of the trophoblast membrane was a characteristic of both past and active infection. On the other hand, previllious fibrin and cytotrophoblat prominence were not in any way associated with malaria infection. Earlier studies showed that the severity of placental changes was not associated with
low birth weight (LBW). The accumulation of mononuclear cells in the intravillious space was also uncommon in both studies (Yamada et al., 1989). Another study by Leopardi et al., (1996) however, showed that active placental infection (presence of parasite and pigment) was strongly associated with LWB while past infection (presence of pigment only) was not associated with LBW (Leopardi et al., 1996).
CHAPTER THREE: METHODOLOGY

3.1 Materials and Methods

The materials used in this study included experimental animals, parasites, the culture media, study site and design. These are described in detail as follows:

3.1.1 Study Site and Design

The study was carried out under the Malaria Research Program at the Institute of Primate Research (IPR) located in Karen, Nairobi, Kenya. Seven pregnant adult baboons were acquired during third trimester pregnancy and transferred to the biocontainment facility according to IPR standard operating procedures. Four animals were infected in their third trimester (day 130) while the other three were used as non infected controls. These animals were in their first and second pregnancies (Table 1).

Table 1: Animal number, treatment group, gravidity, inoculum size and gestation days at infection and CS

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Treatment Group</th>
<th>Parasite Inoculum</th>
<th>Gestation Day at Infection</th>
<th>Gestation Day at CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAN3392</td>
<td>Infected</td>
<td>$2 \times 10^3$</td>
<td>130</td>
<td>139</td>
</tr>
<tr>
<td>PAN3314</td>
<td>Infected</td>
<td>$2 \times 10^5$</td>
<td>130</td>
<td>138</td>
</tr>
<tr>
<td>PAN3305</td>
<td>Infected</td>
<td>$2 \times 10^3$</td>
<td>130</td>
<td>136</td>
</tr>
<tr>
<td>PAN3233</td>
<td>Infected</td>
<td>$2 \times 10^3$</td>
<td>130</td>
<td>138</td>
</tr>
<tr>
<td>PAN3174</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>139</td>
</tr>
<tr>
<td>PAN3366</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>139</td>
</tr>
<tr>
<td>PAN3415</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>139</td>
</tr>
</tbody>
</table>
Placentas from all the animals were obtained via caesarean section (CS) carried out on day 9 post infection or at 5% parasitaemia for the infected animals and on day 139 for the control animals. Gross pathology was done after placental extraction and recorded on data sheets. The placental tissues were then preserved in 10% buffered formalin before histopathological analysis.

All the infected animals were treated orally with pyrimethamine at 1mg/kg body weight once for three days. Treatment commenced soon after CS.

### 3.1.2 Experimental Animals

Pregnant female baboons (*Papio anubis*, weight range 12-23 Kg) originating from Kajiado district in Kenya were used. These baboons had not been previously infected with *P. knowlesi* prior to use, shown by Giemsa staining of thin blood smears. During their quarantine period, they were tested to be free of hemoproteozoon, gastrointestinal parasites and Simian Immunodeficiency Virus (SIV) before being included in the study.

Each baboon was housed in an individual squeeze-back cage of dimensions 0.6x0.6x0.68 meters, in the bio containment facility that was well ventilated. The animals were fed on a standard non-human primate diet (UNGA, Kenya) and water provided *ad libitum* (Olobo *et al.*, 1990).
The Institutional Animal Care and Use Committee (IACUC), and the Institutions Scientific Ethical Review Committee (ISERC) of the Institute of Primate Research (IPR) approved the protocols for this study.

### 3.1.3 Parasites

*Plasmodium knowlesi* H strain, Pk1 (A+) clone (Barnwell *et al.*, 1983) blood stage parasites were used to induce malaria infection in the experimental animals. This parasite was originally isolated by Chin *et al.*, (1965). Barnwell and his team later on succeeded in cloning this parasite by micromanipulation and passaged in rhesus monkey (Barnwell *et al.*, 1983). The clone was used because it produces gametocytes and its genome has been fully sequenced (Pain *et al.*, 2008).

### 3.1.4 Culture Media

The culture media used to propagate this parasite consisted of Rowan Park Memorial Institute (RPMI) 1640 (Invitrogen, UK) and heat inactivated baboon serum. The RPMI 1640 contained Sodium hydrogen carbonate and hepes while the heat inactivated baboon serum was prepared as follows;

Blood was collected in sterile 50 ml tubes from the animal resource department (ARD) and transported in an ice box to the lab. Here, the blood was left at room
temperature for 7 hours to allow clotting to take place. It was then transferred to the fridge at 4°C overnight. On the following day, the blood was spun in the centrifuge at 2000 rpm for 10 minutes at 24°C. Clear serum was then aliquoted and heat inactivated in a water bath set at 56°C for 30 minutes. The heat inactivated serum was stored at -20°C until use in parasite propagation (Ozwara, 2005).

3.1.5 Processing Baboon Red Blood Cells for in vitro Culture of P. knowlesi

Alsever’s diluted baboon whole blood (10ml blood in equal volume of Alsever’s) was transferred from a 20ml syringe into a sterile 50ml tube and spun down at 1500 rpm for 10 minutes. The supernatant was discarded and the cells resuspended in Alsever’s solution twice the pellet’s volume. Centrifugation was then done before washing. Washing was carried out three times in twice the RBC pellet’s volume. One final washing step in RPMI 1640 was also undertaken by centrifuging as above. After this last wash, media of an equivalent volume to the pellet was added resulting in 50% baboon RBC packed cell volume (PCV) solution. The baboon erythrocytes were stored at 4°C and used within two weeks for in vitro propagation of parasites (Ozwara et al., 2003).
3.1.6 *In vitro* Propagation of *P. knowlesi*

*In vitro* cultures were initiated with cryopreserved *P. knowlesi* parasites previously isolated from *P. knowlesi* infected baboons and frozen in liquid nitrogen. For retrieval, parasite vials were removed from liquid nitrogen and quickly thawed at 37°C in a water bath and transferred into 50 ml centrifuge tubes. An equivalent volume of 3.5% NaCl (at room temperature) was then added and mixed with the parasites before centrifugation at 1200 rpm at 24°C for 10 min. The supernatant was sucked off and ½ original volumes of 3.5% NaCl added before centrifugation as before. An equivalent volume (to the first 3.5% NaCl) of media (RPMI 1640 with 10% baboon serum) was added, mixed and centrifuged again. Final washing was undertaken by adding 5X RPMI 1640 of the original volume, mixed well and centrifuged as before. The parasite pellets was then transferred into culture to a starting erythrocyte 50% PCV, 20% baboon serum, 15µg/ml gentamycin solution and RPMI 1640. For starting and daily parasitaemia, 100-200µl of culture was used for thin smear preparation. Cultures were mixed gently and transferred into sterile labelled T 25 culture flasks. New gassing needle was heat sterilized before being connected to a gas pipe fitted with a 0.2µm filter and the culture gassed with a mixture of gases (5% CO₂, 5% O₂, and 90% N₂) for 30 seconds. The flasks were tightly capped and transferred to an incubator (37°C) (Ozwara, 2005).
3.1.7 Parasitaemia Determination

Parasitaemia was evaluated every 24 hours for every culture flask. Labelled duplicate slides were prepared. Cultures were mixes gently and. 100-200µl of culture used for thin smear preparation. The volume was centrifuged for 1 minute at 3500 rpm in a micro centrifuge, supernatant removed then pellet mixed. A drop (5-10µl) was then placed on a microscope slide close to the frosted end of the slide. The blood film was touched with another slide inclined at 45° C and after the blood had spread on its edge it was moved in the forward direction to make a smear. The smear was air dried and then fixed in 100% methanol for 5 minutes. Staining was then undertaken for 15 minutes in 10% Giemsa solution. The slides were rinsed in running tap water, air dried and then observed microscopically at x 100 magnification. At least 2000 red blood cells (RBC) were counted in every parasitaemia count session. Parasitaemia was calculated as follows;

\[
\text{Total number of infected erythrocytes counted} \times 100
\]
\[
2000 \text{ erythrocytes counted}
\]

Differential parasitaemia count was also performed (for rings, trophozoites, schizonts and gametocytes). Once the parasites were established in the culture, they were maintained under similar conditions except for medium changes every 48 hours and sub culturing done when parasitaemias exceeded 5%. Parasites were cryopreserved at the young ring stage of development (Ozwara, 2005).
3.1.8 Cryopreservation of *in vitro* Cultured *P. knowlesi*

The cultures were first spun for 20 minutes at 1200 rpm and the supernatant discarded. Cryoprotectant (70 ml glycerol, added to 180ml of 4.2% sorbitol in 0.85% NaCl, filtered using 0.45μm pore size filters) was added and allowed to equilibrate for 5 to 10 min at room temperature. Aliquots 400μl volumes were then transferred into cryovials before keeping to -70°C overnight and then transferring in liquid nitrogen for long term storage (Ozwara, 2005).

3.1.9 Infection of Baboons with *P. knowlesi*

Overnight cultured *P. knowlesi* parasite was used to initiate blood stage malaria infections. Two culture flasks were pooled for parasitaemia counts before being centrifuged at 1200rpm, 24°C for 10 minutes. The parasites were then resuspended to a population of 2.0x 10⁵ parasites/ml in incomplete RPMI 1640. The baboons were sedated with ketamine-xynazimne mixture (1:20) and bled for baseline blood (T₀) samples. Inoculation was carried out via the saphenous vein with the 1ml parasite-RPMI mixture (Ozwara, 2005).

3.1.10 Sample Collection

Peripheral blood smears were taken from day 2 post inoculation until the day of CS. At CS, placental blood smear, cord blood smear and placental tissues were collected.
Blood for haematological analysis was collected in K$_2$EDTA tubes after every seven days.

### 3.1.11 Clinical Monitoring and Parasitaemia of Baboons

Daily parasitaemia was determined by thin blood smears preparations by finger prick method every 24 hours from day 2 post infection. Double smears were prepared for each thin smear preparation session. A baboon’s finger to be pricked was first cleaned by alcohol swabbing before pricking with a sterile needle. A drop of blood from the pricked finger was then transferred to the slide using a capillary tube and used to prepare thin smears as described in section 3.1.7. Determination of parasitaemia and parasite stage differential counts was also carried out on a daily basis as described before. Baboon general agility, playing habit, appetite, body temperatures and heart rates were also monitored and recorded.

### 3.1.12 Caesarean Section and Sample Collection

Caesarean section was carried out according to IPR Standard Operating Procedures at 9 days post infection or at 5% parasitaemia. Prior to this, peripheral blood samples were collected, and body temperature, body weight, heart rate, respiration, perfusion and body condition parameters taken. Cord blood was collected during surgery before the placenta delivery. The umbilical cord was clamped about two inches way from the infant’s abdomen and blood drawn from the umbilical vein (Brustoski et
al., 2005). Once the infant and the placenta had been delivered the umbilical cord was cut and its length and morphological features taken. The infant was weighed and maintained in the IPR non-human primate nursery. The placental tissues was also weighed and placed in sterile tray. The umbilical cords and chorionic membranes were then stripped off and the placenta rinsed and submerged in sterile saline buffer containing 0.1% heparin and 2% penicillin-streptomycin (Moore et al., 1999). Blood samples from the placenta were obtained by incision of placental cotyledon quadrants as described by Moore et al. (1999), and Othoro et al. (2006).

Placental blood smears and cord blood smears were collected during CS. This was carried out by pricking of placenta cotyledon quadrants on the maternal side using a scalpel and collecting of umbilical vein blood respectively (Moore et al, 1999). Parasitaemia determination was then undertaken by staining the prepared slides with 10% Giemsa solution and observing under a Zeiss® (Sigma-Aldrich, Missouri, USA) microscope’ x100 objective. Parasites enumeration was carried out as previously described in section 3.1.7. Peripheral, cord and placental blood samples were also collected in K$_2$EDTA vials for haematological analysis.

3.1.13 Gross Pathology and Sample Collection

Placental tissues were examined grossly by a trained veterinary pathologist. It was observed for any form of extensive tearing, damage, or gross abnormalities and recorded on a data sheet.
3.1.14 Processing of Placental Tissue Samples for Histopathology

Several placental biopsy specimen of approximately 2x2 cm were obtained for each placenta sample, and fixed in 10% neutral buffered formalin in separate processing jars at room temperature. These jars were then transferred to the pathology lab for processing. They were not allowed to stay in fixative for more than two weeks.

During processing, formalin fixed tissues were dehydrated in varying concentrations of ethanol. They were put in 80% ethanol for 1 hour then in two washes of 95% ethanol for 4 hours and 2 hours respectively. Alternatively they could be left in 95% ethanol solution overnight. The next three washes were carried out in absolute ethanol for 2 hours, and the rest for 1 hour respectively. Tissues were then cleared in three washes of toluene for 1 hour each followed by infiltration in paraffin bath for 2 hours. This was followed by tissue embedding process. This process provides the sectioning medium and means for correct orientation of the tissue. An appropriate size of embedding tray was selected to ensure that the tissue is placed as flat as possible at the bottom of the embedding tray. Plastic embedding molds were correctly labelled with animal number and tissue type. Immediately following paraffin infiltration, warm forceps were used to transfer the tissue from infiltration tray to a warm embedding tray ensuring that the layer of paraffin does not solidify around the tissue.
After fixation samples were embedded in wax and the sections sectioned using a microtome, processed, and stained with haematoxylin-eosin stain. This included washing sections in double distilled water, placing them in haematoxylin solution for 5 minutes then washing further in tap water. The sections were placed in 1% lithium carbonate, rinsed in tap water, put in 1% acid alcohol for a few seconds and then rinsed again in tap water before placing the sections in eosin for 5 minutes. After rinsing in tap water, sections were dehydrated in absolute alcohol and xylene before mounting on DPX mountant.

After dehydration, the rack of slides and trough of xylene was transferred to a fume cupboard. Using forceps, a glass syringe was removed from a trough of xylene kept inside the fume cupboard and filled with DPX resin. Coverslip of the appropriate size was selected, placed on a sheet of fibre-free paper and a drop of DPX applied to it. The slides were removed from the rack and excess xylene drained onto paper towel before gently lowering the slide containing the section onto the drop of DPX such that the section is sandwiched between its slide and the coverslip. The slide was then turned over to allow the DPX to spread between the section and the coverslip. Using paper tissue, excess DPX was wiped off and the slides left to air dry. After DPX had hardened sufficiently, the slides were correctly labelled with the date, animal number and either as control or infected. The labelled slides were placed on a slide tray and with the matched specimen form taken to the bench for
microscopic examination at a magnification of x40 and x100. Cells were classified either as non-infected (no parasite), or as actively infected (parasites present) or as previously infected (haemozoin pigment present with no parasites).

3.1.15 Haematological Analysis

All haematological analysis was carried out according to the IPR pathology laboratory standard operating procedures.

3.1.15.1 Determination of Haemoglobin Concentration

Two microhaematocrit (capillary) tubes were filled with well mixed EDTA whole blood by capillary action to about three quarters of each tube. One end of the capillary tube was sealed with the sealant until the plug was 4-6 mm long. Centrifugation was done for three minutes.

The packed cell volume of each tube was read using a microhematocrit reader (Howksley ®) reader. In brief, the centrifuged haematocrit tubes were first placed into the groove of the sliding capillary tube holder facing upwards. The haematocrit tube was adjusted such that the bottom line (0%) runs exactly at the inter-phase of the sealant and the packed cells. The sliding tube holder was then slid until the end of the plasma column touched the 100% mark of the PCV reader. The pointer was
slid until it was exactly at the inter-phase between the plasma and the packed cells. The packed cell volume of the sample were read and reported as a percentage. The other parameters were calculated as follows;

- Haemoglobin concentration = PCV /3 based on the formula 3Hb = PCV. The results were reported in picograms.

3.1.15.2 Total Leukocyte Count

Test tubes were labelled with animal numbers and into each tube 380µl of Turks solution dispensed. This was followed by the addition and proper mixing of 20µl volume of well mixed whole blood. The haemocytometer was assembled before remixing of the diluted blood sample. Using a capillary Pasteur pipette held at 45°, each of the grids of the chamber were filled with the diluted blood. The chambers were left undisturbed for 5 minutes for white cells to settle in a humid chamber. The underside of the chamber was then mounted onto a microscope stage. Using 10x objectives, the cells were counted in the four corner squares. The number of white cells per litre of blood was then calculated as:

- \((N \times D \times 10^6) \div V\)

(Where N was the number of cells counted, D was the dilution factor and V was the volume of the area of the chamber counted).

Results were then reported as cells per mm³. The morphology of different leucocytes, lobulation and appearance of granules was then noted.
3.2 Data Management and Analysis

Data collected was entered on datasheet both as hard copy and as soft copy in Microsoft Access software and stored for analysis. Backup was made in both hard copy and soft copy and these kept under lock and key. Data was presented on graphs, tables and pictoral description.

Geometric mean values of, body weight and temperatures of pregnant infected group of baboons were compared with the values of control group of baboons (pregnant non-infected baboons) using the student’s \( t \) test. Probability values of \( p < 0.05 \) were considered significant. Data on placental inflammatory count and placental parasite count were recorded as continuous variables and represented as medians. Statistical analysis of the resulting median scores from the evaluation of the parameters for *Plasmodium*-infected placentas (Med\(_I\)) were compared to control placentas (Med\(_C\)) using the Mann-Whitney U test.

Correlations between maternal clinical parameters, placental parameter scores and foetal outcomes were compared using the Spearman’s rank correlation test. All tests were two-tailed with the level of significance set at \( p \leq 0.05 \).
CHAPTER FOUR: RESULTS

4.1 Parasitaemia Profile

Following the infection, parasitaemia levels were assessed daily from day 2 post
infection. The infected animals developed patent parasitaemia within 4 to 7 days post
infection. They all had their peak parasitaemia between 7 and 8 days post infection
when CS was conducted. The highest parasitaemia level was manifested in
PAN3233 that had $5.38 \times 10^5$ infected red blood cells (IRBCs) per µl of blood, at the
time of caesarean section (CS). This parasitaemia level was over fifteen-times higher
than the highest parasitaemia of the other three animals. The animal with the lowest
parasitaemia was PAN3305 ($3.5 \times 10^3$ IRBCs) per µl of blood, (Figure 4).

Parasitaemia of placental blood obtained from the maternal side of the placenta was
significantly higher compared to peripheral parasitaemia (Figure 5) in all the infected
animals (Student $t$ test, $p>0.05$, $P=0.38$). This difference ranged from six times
(PAN3392) to over eight times (PAN3314). One animal (PAN3233) had a still birth
and it died 24 hours after CS due to complications related to malaria infection.
Differential parasitaemia count revealed more ring stage parasites in the peripheral
blood samples (Figure 6, Plate 5) while placental blood had high levels of
trophozoites stage followed by schizontes (Plate 2). Ring stage parasites were the
least in placental blood (Figure 5, Plate 2). The cord blood and blood obtained from
placental foetal side were all negative for parasites (Plate 3 and 4).
Figure 4: Peripheral parasitaemia in *P. knowlesi* infected baboons.
Figure 5: Comparison of peripheral versus placental (maternal side) parasitaemia in *P. knowlesi* infected baboons at the time of CS.

Figure 6: Mean differential parasitaemia count in placental and peripheral blood samples.
Plate 2: The different parasite developmental stages on a thin blood smear prepared by Giemsa staining representing (a) high infection, (b) mild infection and (c) no infection at x100 magnification.

(Key: ----> URBCs; ----> ring stage parasite; ----> trophozoite; ·····> schizont)
Plate 3: Thin blood smear from placental foetal side prepared by Giemsa staining x100.

Plate 4: Thin blood smear prepared from cord blood by Giemsa staining x100.
Plate 5: The different parasite developmental stages on a thin blood smear of peripheral blood prepared by Giemsa staining representing (a) high infection, (b) mild infection and (c) no infection at x 100 magnification.
(Key: ▸ URBCs; ➔ ring stage parasite; ➔ trophozoite; ••• schizont)
4.2 Clinical Symptoms

All the infected animals (PAN3233, PAN3392, PAN3314 and PAN3305) remained active and appeared normal from the point of parasite inoculation until four days post inoculation. Later these animals showed symptoms that were characterized by low apathy (the animals remained dull) and poor appetite. On the other hand, the control animals remained bright and active throughout the experimental period. All the experimental animals recovered fully after treatment with Pyremethamine (dose of 1mg/Kg of body weight). The treatment began soon after CS for three days. However, one animal (PAN3233) died less than 24hrs after CS. Post-mortem results revealed that the cause of death was rapid autolysis as well as degenerative state of the liver that resulted in acute liver failure due to malaria parasite infection.

The body condition of all the animals was either very good or good at the time of baseline sampling (T0). However, it changed to fair after infection (T1) and later on improved at T2. The control group had good body condition throughout the experimental period.

Weight changes were observed in all the groups. There was a general decrease throughout the experimental period. After infection (T1), the infected group showed a significantly higher decrease (Student t test, p>0.05, P=0.52) compared to their control counterparts (Figure 7a and b).
Animal number  

Body weight (Kg)  

(a)  

(b)
Figure 7: (a) Individual and (b) mean change in weight displayed of pregnant baboons at different time points.

Temperature variation was also observed in this study (Figure 8a and b). The infected animals had a higher temperature rise following infection (T1), which later on dropped after CS (T2). Their control counterparts however showed a rise in temperature at T1 and at T2. The temperature change following infection was significantly higher in the infected group (Student $t$ test, $p>0.05$, $P=0.37$) as opposed to the non infected group.
Figure 8: (a) Individual and (b) average changes in temperature displayed by both infected and non infected pregnant baboons at different time points.
4.3 Haematological Profile

Haematological data was analyzed at three time points. These included baseline samples (T0), sample following inoculation (T1) and sample after CS (T2). These samples were collected at a time interval of 7 days.

4.3.1 Haemoglobin (Hb) concentration

Apart from PAN3305 which had a slight drop from 14 to 11.3gm/dl, the other infected animals showed a rise in Hb level after infection. Their Hb levels dropped further after CS with PAN3314 showing the biggest drop (11.7 to 8.7gm/dl). Likewise, their control counterparts showed an initial increase in Hb levels followed by a slight drop after CS (T3) (Figure 9a). Similar to the RBC levels, there was a significant difference in the average change of Hb between the infected and the non-infected groups (Man-Whitney U test, p<0.05, $Z_{\text{calc}}=-1.414$, $Z_{\text{crit}}=1.96$) (Figure 9b).
Figure 9: (a) Individual and (b) average changes in Hb level displayed by both infected and non infected pregnant baboons at different time points.
4.3.2 Lymphocyte levels

There was a general decrease in the lymphocyte level for the malaria infected animals at T1, followed by a slight increase at T2. The animal with the greatest change in lymphocyte level in this case was PAN3305 that dropped from 67% to 27% one week after infection, and later on recovered after CS to a level of 63%. In contrast, the control animals had a general increase through time points T1 and T2 respectively (Figure 10a and b). The difference observed between the infected and the non infected groups was significant (Man-Whitney U test, p<0.05, $Z_{\text{calc}}=-2.141$, $Z_{\text{crit}}=1.96$).
Figure 10: (a) Individual and (b) average changes in lymphocyte level displayed by both infected and non infected pregnant baboons at different time points. T0 represents baseline sampling, T1 represents point of CS (7 days after T0) while T2 represents 7 days after CS (recovery).
4.3.3 Monocyte Levels

Following infection, the monocyte levels increased followed by a drop after CS (T2). Their non infected counterparts on the other hand, showed an initial drop at T1 and then an increase after CS (T2) (Figure 11a and b). This change was significance between the groups (Man-Whitney U test, p<0.05, $Z_{calc}=-1.468$, $U_{crit}=1.96$).
Figure 11: (a) Individual and (b) average changes in monocyte level displayed by both infected and non-infected pregnant baboons at different time points. T0 represents baseline sampling, T1 represents point of CS (7 days after T0) while T2 represents 7 days after CS (recovery).
4.4 Pathology

4.4.1 Gross Pathology

Gross pathology of placenta extracted from all the animals showed that all the placentas were intact and fully developed. They had an average weight of 167.3g and 165.9g for the infected and non infected animals respectively. All the infected animals had no fibrinoids except PAN3233 which had low levels of fibrinoids. All animals in the control group did not have fibrinoids.

Placental calcification ranged from normal to slight in the infected animals while the control had normal. Meconium staining was however observed in both the control group and the infected group ranging from chronic to acute stages. Meconium is a brown-green-colored substance that is usually passed into the amniotic fluid by a distressed foetus.

The umbilical cord was inserted to the placenta and had three vessels (Plate 7c) in all cases. The cord was centrally and eccentrically placed in both the control and infected animal samples.

The maternal surface of the placenta (Plate 7b) had cotyledons while the foetal side had blood vessels (Plate 7a) with dense blood vessel distribution for the infected samples and normal blood vessel distribution for the controls.
Plate 6. Diagram showing the anatomy of baboon placenta. The foetal side of (a) non-infected and (c) infected baboons appear smooth with well distributed blood and is covered with amnion. The maternal side of (b) non-infected and (d) infected is made up of cotyledons. The baboon placenta resembles the human placenta.
4.4.2 Histopathology

The placental tissues obtained from four infected baboons had significant higher pathological changes compared to the placental tissues obtained from three non infected baboons. Tissues were histologically evaluated using a list of parameters graded as severity scores from 0 to 4, with 0 representing “none”, 1 representing minimal and 4 the most severe. The median scores were compared for each parameter using the Mann-Whitney U test. Malaria placentas had significantly higher scores for damage and inflammation (p<0.05, Z_{calc}=-2.121, U_{crit}= 1.96).

Placental damage consisted of fibrin necrosis of the villi, chorionic plate thrombosis, syncytiotrophoblast disruption and chorionic plate syncytiotrophoblast disruption. In most of the infected placentas, the syncytiotrophoblast cell lining of the villi and of the chorionic plate was quite severe. Inflammatory scores consisted of the presence of inflammatory cells on the different layers of the placenta. The cells consisted of monocyte, lymphocytes and neutrophils. Monocytes and lymphocytes were dominant in the infected samples while neutrophils dominated in the controls (Plate 7 a, b, c, and d).
Figure 12: Comparison of placental damage severity scores of infected and non-infected placental tissues ($\text{Med}_I=22.5$, $\text{Med}_C=13$, $p<0.05$)

Figure 13: Comparison of placental inflammation severity scores of infected and non-infected placental tissues ($\text{Med}_I=14$, $\text{Med}_C=2$, $p<0.05$)
Plate 7: Inflammatory cells in the chorionic plate (a) and in the IVS (b, c, d) showing monocytes (thin arrows) and plasma cell (thick dotted arrow), lymphocytes (thick continuous arrow) and neutrophils (thin dotted arrow) at x100. (a, b & c for infected animal PAN3233, d for PAN3366 Control). These pictures are overexposed in order to visualize the specific cell types.
Total parasitaemia and pigment scores were apparent in the infected groups only in both the IVS and the chorionic plate. This difference was significant compared to the non-infected group (p<0.05, $Z_{\text{calc}}=-2.201$, $U_{\text{crit}}=1.96$).

Parasitized RBCs were observed in vessels of the basal plate (maternal side) and the IVS of the infected animals only. The most dominant parasite stages were schizonts and trophozoites (Plate 8). Haemozoin (malaria pigment) was also observed in the infected animals. Both IRBCs and haemozoin were not observed in the control placentas (Plate 8).

![Figure 14: Comparison of placental parasitaemia severity scores of infected and non infected placental tissues ($\text{Med}_I=7$, $\text{Med}_C=0$, $p>0.05$)](image_url)

Figure 14: Comparison of placental parasitaemia severity scores of infected and non infected placental tissues ($\text{Med}_I=7$, $\text{Med}_C=0$, $p>0.05$)
Correlation of placental weight with placental damage ($r= -0.9$, $p<0.05$) and pigment score ($r=-0.5$, $p<0.05$) was expressed by Spearman’s rank correlation test. As the placental damage and level of pigmentation increased, the weight of the placenta also decreased. Similarly, there was correlation between infant weight with placental damage score and total pigment scores. As the weight of the infant decreased, the damage score increased ($r= -0.14$, $p<0.05$). However the infant weight decreased with decrease in pigment scores ($r=0.24$, $p<0.05$). There was also a strong positive correlation ($r=0.927$, $p<0.05$) between placental damage score and inflammation scores.
Comparison of the slides representative of controls and those representative of infection showed distinct differences in the IVS. The IVS and chorionic plate of infected samples was thick and highly infiltrated with parasites (Plate 8 and 9a) while that of the control was clear with visible villi (Plate 9b). However, there were more parasites in the IVS compared to the chorionic plate. Fibrinoid necrosis of the villi (Plate 10) and thrombosis of the blood vessels within the chorionic plate (Plate 11) was severe in the infected animals covering almost the entire blood vessels.
Plate 8; Parasite infiltration in the (a) IVS and (b) chorionic plate. Thin arrow represents parasitized RBCs while the thick arrows represent haemozoin. Haemozoin is a brown pigment released by ruptured infected RBCs (x100). Trophozoites and schizonts are present in large amounts compared to rings.
Plate 9: Infiltration of parasitized RBCs in the IVS of (a) malaria infected pregnant baboons. (b) Non infected pregnant baboons were clear of parasites (x40). There is thickening of the IVS of the infected tissue (a) unlike in the control (b).
Plate 10: Fibrinoid necrosis of the villi in (a) malaria infected pregnant baboon and (b) control (x100). (Key: → fibrin necrosis; ←→ syncyiotrophoblast cells; ← intact villi are represented)
Plate 11 Thrombosis of blood vessels within the chorionic plate of placental tissue extracted from (a) malaria infected pregnant baboons and (b) controls (x100).
(Key: ——> thrombosis; ……> blood vessel; ——> chorionic plate).
Plate 12: Disruption of the syncytiotrophoblast cell lining of the chorionic plate was evident in the (b) infected tissue (dotted arrow) as opposed to the control (a).

(Key: •••• trophoblast cells; —— syncytiotrophoblast.)
CHAPTER FIVE: DISCUSSION AND CONCLUSION

5.1 Discussion

5.1.1 Summary of Findings

This study set out to characterize the pathological changes of malaria infected placenta in the baboon model. This included the description of pathological changes and the assessment of infiltration by inflammatory cells. Following infection, symptoms characteristic of malaria were observed. Parasitaemia levels increased progressively. Placental parasitaemia was eight-fold higher than peripheral parasitaemia. Histopathological findings indicated the presence of parasites in the chorionic plate (maternal side) and the intravillious space. No parasites were observed in the basal plate (foetal side). Results indicated that *P. knowlesi* parasites are sequestered in the baboon placenta. This sequestration is associated with fibrinoid necrosis of the villi, chorionic plate thrombosis, syncytiotrophoblast disruption and chorionic plate syncytiotrophoblast disruption in the placenta which result in placental damage. This study also revealed that placental malaria is associated with still birth. Similarly, following *P. knowlesi* infection in pregnant animals, infiltration of inflammatory cells was also observed. These findings correlated with those found in human studies (Davison *et al.*, 2000; Brabin *et al.*, 2001; Katherine and Donald, 2004).
5.1.2 Clinical Symptoms in Pregnant Baboons infected with *P. knowlesi*

Following inoculation of pregnant baboons with *P. knowlesi* parasites, this study showed that the infection was associated with clinical symptoms including apathy, inappetance and a rise in body temperature which was progressive. The manifestation of these symptoms and parasitaemia detection occurred between days 5 to day 7 post inoculation. In non-immune individuals with *P. falciparum* infection, the pre-patent period (time from sporozoite inoculation to detectable parasitemia) is from 5 to 10 days while the incubation period (time from sporozoite inoculation to development of symptoms) is between 6 to 14 days. However, the incubation period may be significantly prolonged by the level of immunity acquired through previous exposures, by antimalarial prophylaxis, or by prior partial treatment which may mitigate but not prevent the disease (Taylor and Strickland 2000). The clinical symptoms of malaria are primarily due to schizont rupture and destruction of erythrocytes. These symptoms are usually non-specific. The presentation of malaria often resembles those of common viral infections and may lead to a delay in diagnosis. The majority of patients experience fever (>92%), chills (79%), headaches (70%), and diaphoresis (64%) (Genton and Acremont, 2001). In non-immune persons, clinical examination may be completely unremarkable even without fever (Andrej et al., 2003). Observations made in this study correlates with those of human findings.
5.1.3 Haematological Findings in Baboons infected with *Plasmodium knowlesi*

Haematological findings in this study showed that there was significant change in Hb, lymphocyte and monocyte levels following *P. Knowlesi* infection of pregnant baboons. There was a decrease in the level of Hb and lymphocyte. Studies by Billie *et al.*, (2005) demonstrated that immunomodulation does occur in primates challenged with malaria during pregnancy. In this study, it was found out that there was a decrease in the level of RBC and Hb following *P. coatneyi* infection in rhesus monkeys (*Macaca mulatta*). The study also revealed that the circulating monocyte levels were lower in the infected animals. This suggests that the lower Hb levels observed in the infected animals correlated with high parasitaemia levels in these animals.

5.1.4 Parasitaemia in the Peripheral and Placental Blood

In this study, peripheral parasitaemia was eight times lower than placental parasitaemia. The ring-stage parasites were dominant in the peripheral circulation while the trophozoites and schizonts dominated in the placental blood extracted from the maternal side. It is documented that only trophozoites and schizonts have cytoadherence properties (Katherine and Donald, 2004). These parasitized RBCs adhere to the endothelium via parasite derived proteins expressed on the surface of the infected RBC. This leads to the removal of infected RBCs from the peripheral circulation (a process known as sequestration) and enables the parasite to avoid
clearance by the immune system in the spleen (Katherine and Donald, 2004). An important difference between *P. falciparum* and other human malaria parasites is the way *P. falciparum* modifies the surface of the RBC for adherence of both asexual parasites and gametocytes to the endothelium and asexual parasites within placenta. As a result, only ring forms of *P. falciparum* are found within circulation. The surface of *P. falciparum* trophozoite-and schizont-infected RBC is covered with knob-like excrescences that are the contact points with host cells. The adherence protects the parasite from destruction, as non-adherent mature parasitized RBC are rapidly cleared within the spleen (David *et al.*, 2002). This correlates with the findings in this study suggesting the cytoadherence property of *P. knowlesi* parasites in the placenta.

Malaria in pregnancy jeopardizes the outcome of pregnancy, affecting both the mother and the foetus. Studies have shown that in malaria endemic areas, the increased risk of *P. falciparum* infection during pregnancy is associated with placental parasitaemia and it contributes to maternal anaemia, low birth weight and infant mortality especially among the primigravidae (Brabin *et al.*, 2001; Guyatt and Snow, 2004;). One such study was carried out in South-eastern Nigerian hospitals. It revealed that there was a significant relationship between the prevalence of malaria in the placenta and gravidity, age and blood group (Bulmer *et al.*, 1993). This suggests that over-reliance on maternal peripheral circulation for diagnosing and excluding other possible congenital malaria infections may lead to high rates of
malaria infection because clearance of the parasites from maternal peripheral circulation does not exclude congenital malaria.

Haemozoin was also observed in the placental tissues of the infected animals. In other studies, haemozoin has also been frequently observed in placentas from malaria-infected women with and without peripheral parasitaemia (Galbraith et al., 1980; Bulmer et al., 1993; Menendez et al., 1994). A study aimed at quantitating placental haemozoin and determination of its association with the timing of infection and birth outcome revealed significant elevated haemozoin levels in women with malaria infection at delivery (active infection) unlike in women who had infections earlier in pregnancy. Although the elevated placental haemozoin was not associated with poor birth outcomes, multivariate analyses of that data supported the association between malaria parasitaemia and poor birth outcomes in this population (Sullivan et al., 1999).

5.1.5 Plasmodium knowlesi Sequesters in the Baboon Placenta

Presence of parasitized RBCs and haemozoin in the placental IVS of infected animals was evident in this study. There were more trophozoites and schizonts than were rings. The life cycle of malaria parasites involves phases through which the parasites invade the RBCs and produce proteins which attach themselves to receptors in the walls of blood vessels. This causes the blood cells to accumulate in organ capillaries, resulting in life threatening symptoms. Studies carried out over the years
have shown that *P. falciparum* erythrocyte membrane including proteins (PfEMP-1) bind to a wide range of endothelial receptors such as chondroitin sulphate A (CSA), hyaluronic acid and immunoglobulin G (Barragan *et al.*, 2000; Beeson *et al.*, 2000). Recent studies have indicated that malaria parasites bind to three different receptors in the placenta. The adherence of IRBC to the placenta is mediated by placental receptors like Chondroitin-Sulphate A (CSA) and hyaluronic acid (HA) expressed by the syncytiotrophoblast that line the placental intervillous spaces. Chondroitin sulphate A is mediated by Variant Surface antigens (VSA) expressed on the surface of the IRBC (Buffert *et al.*, 1999; Reeder *et al.*, 1999; Maubert *et al.*, 2000). Expression of this particular VSA by parasite binding to CSA in the placenta is thought to be the basis for the higher susceptibility of primigravidae who have little or no antibodies against the VSA to placental malaria. Studies by Ricke *et al.*, (2000) have shown that the multigravid pregnant women from endemic areas tend to have high levels of antibodies against the VSAs expressed by CSA-adhering parasites. Since most of the *P. falciparum* parasites could bind to three different receptors including hyaluronic acid in the placenta (Brabin, 2003).

Recent findings have reported IRBC binding to non-immune immunoglobulins (Ig) in placental malaria (Flick *et al.*, 2001). It is thought that a cloned parasite line selected for a high level of binding to Ig adheres to placental sections *in vitro* in a manner dependent on Ig, but independent of CSA and HA. In a study carried out on Cameroonian women, parasites isolated from four infected placentas appeared to
bind IgG *in vitro*. These placental isolates also bound CSA. However, it is not clear how much the binding phenotypes overlapped. On the other hand, the laboratory isolate did not bind to CSA, suggesting that parasites binding Ig in the placenta constitute a different population from those that bind CSA or HA. Flick *et al.* (2001), therefore proposed that IgG could act as a bridging molecule between IRBC and Fc receptors present on syncytiotrophoblasts, thereby mediating parasite sequestration in the placenta. In view of this, the current study was not conclusive on the exact receptors that mediated cytoadherence of IRBCs in the placenta. It would therefore be important to do further studies to conclude this.

5.1.6 The Role of Placental Malaria on Materno-foetal Exchange

One still birth was observed in this study. In addition, placental weights correlated with placental damage score and placental pigment score. Placental weight decreased with increase in both pigment and damage scores. Infant weight also decreased with increase in placental damage scores. This study suggests that placental damage due to malaria may be responsible for the impairment of maternal-foetal exchange thereby leading to low birth weight.

It is suggested that placental malaria reduces oxygen and/or glucose transport to the foetus through a local placental effect. This could be as a result of mechanical blockage arising from the thickening of the trophoblast basement membrane, by
increased nutrient use of developing and replicating parasites in the placenta or by poor oxygen and glucose transfer from the parasitized RBCs sequestered in the placenta. In addition, the immune system through cytokines has also been suggested to play an important role in placental pathophysiology and the consequential adverse pregnancy outcomes. In response to *P. falciparum*, activated placental macrophages could induce mechanical and functional placental damage through cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) (Davison *et al.*, 2000). A study done in Kenya showed that the high levels of TNF-α and IFN-γ were both significantly associated with LBW and low placental weight in primigravid women (Fried *et al.*, 1998). In Malawi, it was observed that placental production of TNF-α but not IFN-γ may be implicated in impaired foetal growth (Rogerson *et al.*, 2003).

### 5.1.7 Lymphocyte and Monocyte Levels During Placental Malaria

This study showed the presence of both lymphocytes and monocytes in the placental tissues. These cells constituted inflammatory scores which were significantly higher in malaria infected placentas compared with the controls. A study carried out by Demba *et al.* (2006), revealed that placental infection is associated with a decrease in eosinophils, monocyte, neutrophil, lymphocyte, and platelet counts. Monocyte counts were inversely related to haemoglobin levels. It is thought that the possible means of maintaining a placenta-specific memory immune response is through the lymphocyte recirculation hypothesis which involves a hypothetical antigenic
stimulus. Initial exposure to this antigen at the placental level induces recruitment and activation of maternal effector cells, namely antigen-specific lymphocytes. Unique mechanisms for cellular immune responses may be involved at this level, given that local immunomodulation is likely to be necessary to maintain the foetal allograft. As part of this primary response, some of these activated memory/effector cells circulate out of the placental intervillous space and take up residence in the decidua/endometrium. Thus, when the placenta is expelled at parturition, a pool of memory cells is retained in a placenta-associated lymphoid system and is available for subsequent pregnancies. When the mother is exposed to the same antigen in a subsequent pregnancy, recruitment of memory effector cells from the local lymphoid organs mediates a rapid and focused antigen-specific response. Recirculation of these memory cells to local sites occurs again, ensuring continued maintenance of this memory cell population (Moore et al., 2000). Nonetheless, it is likely that the nature of the immune stimulus, the type of response it elicits, and the route and mode of exposure to antigen will all play a role in the maintenance of memory lymphocytes (Ahmed et al., 1996).

Monocyte recruitment to sites of inflammation is mediated by chemoattractant proteins including chemokines, members of a super family of inducible, proinflammatory mediators that bind a large subfamily of G protein-coupled receptors (Lee and Montaner 1999; Mackey 2001). Monocyte infiltration into the IVS of malaria-infected placentas is a key risk factor for LBW. Placental monocyte
infiltration may be a crucial mediator of poor birth outcome. In earlier studies, approximately 25% of malaria-infected women have placental monocyte infiltration at delivery (Walter et al., 1982; Menendez et al., 2000). Placental monocyte density has been identified as a significant predictor of LBW (Ordí et al., 1998; Mendez et al., 2000). Placental monocytes may be the source of the placental proinflammatory cytokines that have also been associated with LBW (Fried et al., 1998; Moormann et al., 1999). In this study, the recruitment of the inflammatory cells may be responsible for placental damage.
5.2 Conclusion

i. This study has demonstrated that baboons (*P. anubis*) infected with malaria develop clinical symptoms like apathy, fever and inappetance. Similarly, the infections lead to alterations of blood cell types. The placental parasitemia was higher than the peripheral circulation, a case similar in humans.

ii. This study has also shown that baboons (*P. anubis*) are susceptible to malaria infection that is associated with clinical symptoms and chronic or acute disease courses.

iii. When baboons get infected with *P. knowlesi* parasites during pregnancy, they also develop placental malaria. This is characterized by the sequestration and thriving of parasitized red blood cells in the placental IVS via specific receptors expressed by the syncytiotrophoblast cells. This in most cases results in placental damage that in turn leads to poor birth outcomes in baboons as is the case in humans.

iv. Placental malaria results in the infiltration of inflammatory cells such as lymphocytes and monocytes in the placenta which also contributes to poor birth outcomes such as still birth, abortion, LBW and IUGR. One striking feature observed in this study is that placental malaria is associated with still birth which usually results from the impairment of the materno-foetal exchange such that the foetus is deprived of food, oxygen and nutrients.
CHAPTER FIVE: RECOMMENDATION

This study recommends the following:

i. Study of placental malaria during the different trimesters of infection.

ii. Determination of the mechanisms underlying the sequestration of *P. knowlesi* parasites in the baboon placenta.

iii. Determination of the molecules involved in the adhesion process within the baboon placenta.

iv. Animals should be challenged with secondary infection in order to assess their response during pregnancy. This would give a better picture of what happens in humans living in malaria endemic areas.

v. Studies to determine congenital infection in baboons.
REFERENCES


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http://www.niaid.nih.gov

http:// www.sanger.ac.uk


APPENDICES

Appendix I: Reagents

Alsever’s solution
Dextrose 10.25g
Nacl 2.1g
Trisodium citrate 4.0g
Double distilled water 500ml
Sterilize by filtering through 0.2µm pore size filter
Store at 4°C

Complete RPMI 1640 (pH 7.2)
RPMI 1640 445ml
FBS 50ml
L-glutamine 5ml
Gentamycin 1ml (0.5mg/ml)
Filter using 0.45µl pore size filter
Appendix II: Daily Health Record Sheet

**DAILY HEALTH REPORT SHEET**

**PLACENTAL MALARIA PROJECT, BIOCONTAINMENT FACILITY**

ANIMAL NUMBER:

<table>
<thead>
<tr>
<th>DATE</th>
<th>DEMEANOUR</th>
<th>APPETITE</th>
<th>STOOL</th>
<th>EYES</th>
<th>VET</th>
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**KEY: APPETITE CODE:** G- good, F3/4, ½ and P-poor. F= proportion of full appetite

**STOOL CODE:** N-normal, L-liquid, B-bloody, H-hard, A-absent

**EYES CODE:** N-normal, C-cloudy, O-opaque

Appendix III: Parasitaemia Data Sheet

**PATHOLOGY OF PLACENTAL MALARIA (MSc. PROJECT)**

<table>
<thead>
<tr>
<th>Animal Number: PAN</th>
</tr>
</thead>
</table>

### Sample Origin:
- [ ] Peripheral
- [ ] Cord
- [ ] Placental
- [ ] Parasite Culture
- [ ] Retrieval Vial

### Type of smear:
- [ ] Thick
- [ ] Thin

<table>
<thead>
<tr>
<th>Date</th>
<th>Time (am/pm)</th>
<th>DAI</th>
<th>R</th>
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<th>S</th>
<th>G</th>
<th>E ((10^3))</th>
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<th>Remarks</th>
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Appendix IV: Gross Pathology Data Collection Forms

PATHOLOGY OF PLACENTAL MALARIA

Sample Collection During Cesarean Section

Animal ID: __________ Date of Data Collection: ____________

Time: ____________

Number of previous births (garavidity) if known: ____________________________

Composition of anaesthetic injection solution ______________________________

1. Clinical Information:
   a) Auxillary body temperature: _________ °C
   b) Body weight: _________ Kg
   c) Heart rate: _________
   d) Feotal Heart rate: _________

2. Blood Samples
   a) Peripheral Blood Collected
      10 ml Blood in Alsever’s
      6 ml Blood in 15 ml tubes
      4 ml Blood in EDTA
      Blood Smear on 10 slides
b) Placental Blood collected
   10 ml Blood in Alsever’s
   6 ml Blood in 15 ml tubes
   4 ml Blood in EDTA
   Blood Smear on 10 slides

c) Cord Blood collected
   10 ml Blood in Alsever’s
   6 ml Blood in 15 ml tubes
   4 ml Blood in EDTA
   Blood Smear on 10 slides

3. Foetal examination
   a) Sex of the baby ________ (M/F)
   b) Birth weight ________ g
   c) Condition of the baby
   d) Abnormalities

4. Placenta Examination
   a) Weight of the placenta ________ g
   b) Colour of placenta ___________________________
   c) Cord insertion to placenta ___________________________
   d) Estimated length of cord ________ cm
   e) Number of umbilical vessels ___________________________
   f) Number of lobes ___________________________
g) Placental lobes fused Yes/No

h) Placenta diameter ______ cm

i) Tearing
   Quadrant 1: __________________________
   Quadrant 2: __________________________
   Quadrant 3: __________________________
   Quadrant 4: __________________________

j) Perfused volume ______ ml

k) Volume recovered ______ ml

l) Fibrinoids: Marked □ Normal □ None □
m) Calcification: Marked □ Normal □ None □

n) Infractions: Yes □ No □ Size ______ cm
   Superficial □ Deep □

o) Meconium staining: None □ Acute □ Chronic □
p) Placenta praevia: Complete □ Partial □ Marginal □

q) Membranes: Intact □ Marginal □ Circumvalate □ Colour

r) Retro-placental Hemorrhage: Present/Absent ____________
   (Note the age, size and extent of clotting)

s) Retro-Membranous Hemorrhage: Present/Absent ____________
   (Note the age, size and extent of clotting)

5. Foetal surface
   a) Blood vessel distribution: Sparse □ Normal □ Dense □
b) Thrombin (#) ________________

c) Subchorionic fibrosis: Marked [ ] Normal [ ] None [ ]

d) Cord length ________________

6. Cord Insertion:

   Central [ ] Eccentric ______ cm from margin

   Marginal [ ] Valamentous [ ] Furcated [ ] Vasa Previa [ ]

5. Placental Tissues
   a. Placental snap frozen tissue (store in LN2) [ ]
   
   b. Tissue in OCT medium [ ]
   
   c. Tissues in formalin [ ]
**Appendix V: Histopathology Data Collection Form**

ANIMAL NO.…………….         SLIDE NO.……………….
DATE……………………..

<table>
<thead>
<tr>
<th>CHARACTERISTIC FEATURES</th>
<th>SEVERITY SCORE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Fibrinoid necrosis of the villi (Score 0-4)</td>
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<tr>
<td>2 Infarcts (Score 0-4)</td>
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<tr>
<td>3 Chorionic plate thrombosis (CPT) (Score 0-4)</td>
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<tr>
<td>4 Syncytiotrophoblast disruption (SD) (Score 0-4)</td>
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<tr>
<td>5 Chorionic plate syncytiotrophoblast disruption (Score 0-4)</td>
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<tr>
<td>6 Total placental damage</td>
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<tr>
<td>7 Malaria pigment (hemozoin) (Score 0-4)</td>
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<tr>
<td>8 Macrophages containing malaria pigment (Score 0-4)</td>
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<tr>
<td>(a) Decidua</td>
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<td>(b) Basal plate</td>
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<td>(c) Intravillious space</td>
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<td>9 Total pigment score</td>
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<tr>
<td>10 Inflamatory cells in sections</td>
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<tr>
<td>11 Parasites in sections</td>
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<tr>
<td>12 Others</td>
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</table>
Appendix VI: Baboons;

(a) In cages

(b) Preparation during CS
Appendix VII: Caesarean Section Photos