Prevalence and Impact of Malaria Infection on Pregnancy and Prenatal Outcomes in the Blue Nile State of Sudan

A thesis submitted to Middlesex University in accordance with the requirements for the degree of Doctor of Philosophy

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I owe the spark of my life to my husband, Muzamil, to my lovely daughter, Mai, and to my lovely son, Ahmed.

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LIST OF ABBREVIATION

ABC	Avidin Biotin Complex
ACTs	Artemisinin-Based Combination Therapies
AJs	Adherent Junctions
BNS	Blue Nile State

CDPK3	Calcium Dependent Protein Kinase 3
CL4	Claudin-4
CM	Congenital Malaria
CO2	Carbon Dioxide
CRH	Corticotropin-Releasing Hormone
CSA	Chondroitin Sulfate A
CTB	Cytotrophoblast
DAB	Diaminobenzidine
DAPI	Diamidino-2-Phenylindole
DM	Desmosomes
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
GAG	Glycos Amino Glycan
GJs	Gap Junctions
H&E	Haematoxylin And Eosin Staining
HA	Hyaluronic Acid
Hb	Haemoglobin
HCG	Human Chorionic Gonadotropin
HPL	Human Placental Lactogen
HRP	Horseradish Peroxidase
IEs	Infected Erythrocytes
IgG	Immunoglobin G
IGR	Intrauterine Growth Retardation
IGU	Intrauterine Growth Retardation
IHC	Immunohistochemistry
IL	Interleukin
IL-8	Interleukin-8
IMP	Intermittent Malaria Prophylaxis
IRBCs	Infected Red Blood Cells
IVS	Intervillous Space

JAM	Junctional Adhesion Molecule
LBW	Low Birth Weight
MAGUKs	Membrane-Associated Guanylate Kinase Homologs
MDV-1	Male Development Gene-1
MIP	Malaria In Pregnancy
NMCP	National Malaria Control Programme
NSESC	Natural Ethics Sub Committee
O2	Oxygen
P. Malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
PAM	Pregnancy-Associated Malaria
PAPP	Pregnancy Associated Plasma
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
PfEMP1	*P. falciparum* Erythrocyte Membrane Protein
PM	Placental Malaria
PTD	Pre-Term Delivery
RBCs	Red Blood Cells
SAF-B	Scaffold Attachment Factor-B
SH3	S Homology3 Domain
STB	Syncytiotrophoblast
TH1	T Helper Cell
TJ	Tight Junctions
TJ	Tight Junction
TNF	Tumor Necrosis Factor
TSA	Tyramide Signal Amplification
U1-U6	Unique Variable Domains
UV	Ultraviolet

VAR2CSA	Variant Surface Antigen 2-Chondroitin Sulphate A
VSA	Variant Surface Antigen
WHO	World Health Organization
ZAK	Zo-1 Associated Kinase
ZO-1	Zonula Occludens -1
γ	Interferon

ABSTRACT

Malaria is a global public health concern. It is prevalent in the Blue Nile state of Sudan, where the incidence rate exceeds 34%, according to a 2015 survey. *P.falciparum* is the dominant infecting species in sub-Saharan Africa; it particularly affects pregnant women, for it tends to sequester in the intervillous space (IVS) of placenta, resulting in placental malaria which can subsequently

lead to congenital malaria. Congenital malaria (CM) is defined as the presence of malaria parasites in the peripheral blood of new born infants, in the first week of life. There is quite a lot of controversy associated with the frequency of occurrence of congenital malaria as the mechanism/s of how the parasites crosses the placenta barrier is not yet fully understood;

The main aim of this study was to determine the prevalence of placental malaria and congenital malaria and their effects on the pregnancy outcomes in the Blue Nile state of Sudan. The subsequent aim of this study was to explore the possible mechanism/s by which the malaria parasite crosses the placental barrier. The role of tight junction proteins in the placenta with regards to the crossing of the parasites through the placental barrier will be investigated.

A cross-sectional study has been conducted with 336 pregnant women. The mean (SD) maternal age was (25.13 ± 4.43), who have given birth in the main maternity wards at Complex Centre, Damazin, Roseris hospitals, respectively, between the years 2012–2014 in Sudan. The socio-demographic and the obstetric information of the mothers have also been collected. The peripheral blood smears of pregnant women at delivery have been used for haemoglobin (Hb gm/dl) measurement and the detection of the malaria parasite; the placental blood and tissues, cord blood, and peripheral blood smears of the new born babies have been collected and examined for malaria parasites infection by microscopic using Giemsa staining and polymerase chain reaction (PCR) techniques. The placental tissue classification of malaria infection has been done by Haematoxylin & Eosin (H&E) staining and double staining microscopically by Giemsa and Prussian blue respectively. Placental tissues have been examined for two tight junction markers' Zonulaoccludens-1 andClaudin-4 using immunohistochemistry method.

Results showed that PCR technique was more sensitive than Giemsa staining technique in detecting the presence of parasite in the blood samples tested. Results also showed that all cases of malaria infection, that has been detected in the baby's peripheral blood has been found to be positive in the corresponding cord blood.

The presence of parasites in the peripheral mother's blood is not always associated with the presence of the parasites in the placenta.

From the total 336 cases, placental tissues from only 110 cases were available to be examined and classified. Results have revealed that out of the 110 placental tissues examined, 29.09% (n=32) have shown active acute malaria infection, 28.18% (n=31) have shown active chronic infection, 26.36% (n=29) have shown past infection, while 16.4 % (n =18) of the placental tissues have been uninfected.

Results also showed that the effect of placental malaria on maternal anaemia and baby low birth weight have increased the risk of adverse infant morbidity, predominantly for primiparae, as the prevalence of low birthweight (LBW) has been at 20.83% (n=70), amongst the new born babies. The overall mean (SD) of the birth weight of the neonates was (2.5 ± 0.30) kg and the overall frequency of LBW was 29.16% (n = 98). Malaria infection was significantly associated with low birth weight (LBW). Maternal anaemia (AOR = 21.25, 95% CI 6.70; $P < 0.001$), placental malaria (AOR = 13.94, 95% CI 4.326; $P < 0.001$), were significant risk factors for low birth weight.

From the 336 cases examined in this study, it is found that malaria parasitaemia is associated with low parity and maternal age. There is a significant age effect on malaria prevalence ($p < 0.05$). The prevalence of placental malaria has decreased with age and parity significantly ($p < 0.001$). Furthermore, there was significant effect of malaria infection on Hb level in women, who were diagnosed with placental malaria ($p<0.05$). There was also a significant correlation between infant malaria prevalence (congenital malaria) and the mothers' Hb level ($p < 0.05$). To evaluate the association of tight junction markers, Claudin 4, and Zonula occludens (ZO-1) expression with both placental and congenital malaria, Spearman's correlation coefficient was used. There was no correlation between ZO-1 and Claudin 4 expressions in each category of placental malaria infection. Further analysis shows that there is no significant difference in the expression of Claudin 4 or ZO-1 and congenital malaria.

CHAPTER 1

Section -1

1. Introduction

1.1. Background

Malaria is undoubtedly one of the world's largest infectious diseases, specifically in tropical and subtropical regions of South America, Central America, Asia and Africa (World Malaria Report, 2015).

A staggering number of 106 countries are at the risk of transmitting malarial infection. The 2015 world health report estimated that~~'s~~ there have been over 216 million malaria cases; the majority in Africa (81%) then in Southeast Asia (13%) and finally in Eastern Mediterranean region (5%) (as shown in Table 1) (World Malaria Report, 2015).

Table 1: **The numbers of Malaria cases and death by geographical region**
Source: World Malaria Report, 2015 (modified).

WHO region	Malaria cases	Malaria deaths (%)
African regions	81	91
Southeast Asia	13	6
Eastern-Mediterranean regions	5	3
Others	1	<1

The cause of malarial infection is a parasite protozoan, belonging to the genus *Plasmodium.* It is spread to humans through the bite of female infected anopheles' mosquitoes. Eventually, parasite enters the blood stream and attack its red blood cells which allows them to multiply. presence of the parasite in the blood stream is a common form of malaria namely peripheral malaria (Krettli.*et al*., 2001; Herbert *et al*., 2015). There are only four types of parasites over 100 species of plasmodia that infect humans~~;~~ such as *P. malariae, P. vivax, P. falciparum,* and *P. oval.* (Krettli *et al*., 2001; Herbert *et al*., 2015).

P. falciparum causes the most severe type of malaria, which results in death in some cases. Also, it is believed that *P. falciparum* causes placental malaria in pregnant women. Placental malaria is a condition in which pregnant women become infected with the parasite and the parasite become sequestered in the placenta (Muthusamya *et al*., 2007; Herbert *et al*., 2015). This phenomenon has a very severe impact on both the mother and the baby.

In Sudan, malaria is one of the deadliest endemic diseases, and increased susceptibility of pregnant women to malaria is a long-standing public health problem (Adam I *et al*., 2005). Malaria during pregnancy is a serious public health problem in sub-Saharan Africa and about 10,000 women and 200,000 babies die annually because of malaria during pregnancy (WHO.,2015). Most of these deaths are caused by *P. falciparum*, which is found in tropical and subtropical regions (WHO.,2015). In malaria endemic areas, at least one in four pregnant

women has an evidence of peripheral or placental malaria at delivery (Steketee *et al*., 1996). Moreover, women that are pregnant for the first time (primigravidae) are highly susceptible to malaria when compared with multigravidae (Cisse *et al*.2014).

The infant's health is also at risk because of the infection in the placenta and maternal anaemia caused by malaria. Both factors contribute to LBW, which is the leading cause of perinatal and infant mortality (Mohamed.,2013). In Sudan, detailed data on the pattern and risk factors for placental malaria are rare.

Infant and child mortality are higher than in other neighbouring states. Malaria is one of the major problems that increase mortality in the state with more than 30,000 cases reported in 2010 (a prevalence of 34%). Moreover, malaria is the main cause of common morbidity and mortality in the state (Blue Nile State emerging profile, 2014). In Sudan, there is no antenatal care programme to monitor coverage of intermittent preventive treatment to all pregnant women attending antenatal clinics as recommended in areas of high malaria transmission (WHO., 2004).

1.2. Epidemiology

Malaria infection is one of the most widespread diseases the in the sub-Saharan countries. The threat of *P. falciparum* malaria infection increases every year, with 300−500 million new cases worldwide (Greenberg *et al*,.2001). It continues to be a major life-threatening problem, especially among pregnant women. Malaria is thought to be endemic throughout Sudan and all its provinces, while the hypo-endemic area being the northern part and the meso-endemic area being the central part of the country (Malik *et al*., 2004).

The rainy season is the most common period for the transmission of malaria. The duration of transmission varies from 3-6 months with an average of 4 months as Sudan's rainy season lasts for about three months (from July to September). (National malaria programme 2007-2012).

1.2.1. Prevalence of Malaria in Sudan

Over the last 20 years, as reported in many research (WHO, 2015), malaria has become one of the major diseases in Sudan. Malaria represents a real health problem, especially amongst pregnant women. It accounts for nearly 5 to 10 million cases annually and about 35,000 cases of deaths.

Malaria in pregnancy (MIP) represents a real problem which accounts fora large rate of mortality amongst pregnant women. Sudan is one of the top African and sub-Saharan countries that has about 50% of malarial infection cases, which accounts for 70%of cases leading to death (WHO, 2015). The epidemiology of the parasite infection in Sudan is unstable. On other hand, in the Blue Nile state, where this project was conducted, the incidences of infection depends on the rainy season (as shown in

Figure 1).

The National Malaria Control Programme (NMCP) in 2003 states that health personnel, up to >60%, work in these obstetric sections. In addition, another study, stated that malaria ends in death for a large number of patients which accounts for nearly 37.2% of other causes at the hospital level (Dafallah., 2003).

World health organization (WHO, 2015) states that the mortality rate among pregnant women in Sudan is 311/100,000 in 2013 (WHO, 2015). Infant and child mortality are higher in the Blue Nile state compared to other neighbouring states with more than 30,000 cases reported in 2010 (a prevalence of more than 34%). Furthermore, malaria is the main cause of common morbidity and mortality in the province (the Blue Nile State Emerging Profile, 2014). According to

Figure 1, which shows that *P. falciparum* is the dominant cause of malaria, especially amongst the pregnant women in the Blue Nile state where it constitutes about 95% of the cases. Although, a few cases of *Plasmodium malariae* and *P. vivax* have been detected where their effects on pregnant women have been rare and minor. The main vector of malaria in the Blue Nile state is *Anopheles arabiensis*. Most of the population of the state (65%) exposed to malaria transmission, with 43% being at high-risk of attaining the infection.

One of the features of unstable malaria is that the transmission may not occur during certain times of the year and may be totally absent in some years. Over the last 10 years, malaria is causing many problems in areas of unstable transmission and affecting people's lives, as shown in

Figure 1 .

The Blue Nile state lies in the tropical climate zone, between the latitudes, $9^0$30' and $12^0$30' and the longitudes, $33^0$5' and $35^0$3 east. It is characterized by high temperatures and heavy

rainfall. The average annual rainfall is around 700 mm; with the southern part of the state being the wettest. The State has an area of 45,844 km² and an estimated population of 832, 000; 75% of people reside in the rural areas and 25% in its four urban centres. Women represent 47% of the people in the state, with a maternal mortality rate of 258/100,000 people. The specific environmental, anthropological, administrative, and geographic characteristics of the Blue Nile state, which shares an international border with Ethiopia and South Sudan, uniquely impacts the epidemiology and control of malaria (BNS Emergency Profile, 2014). The malaria transmission season runs from June in the southern area of the state and July/August elsewhere to November/December

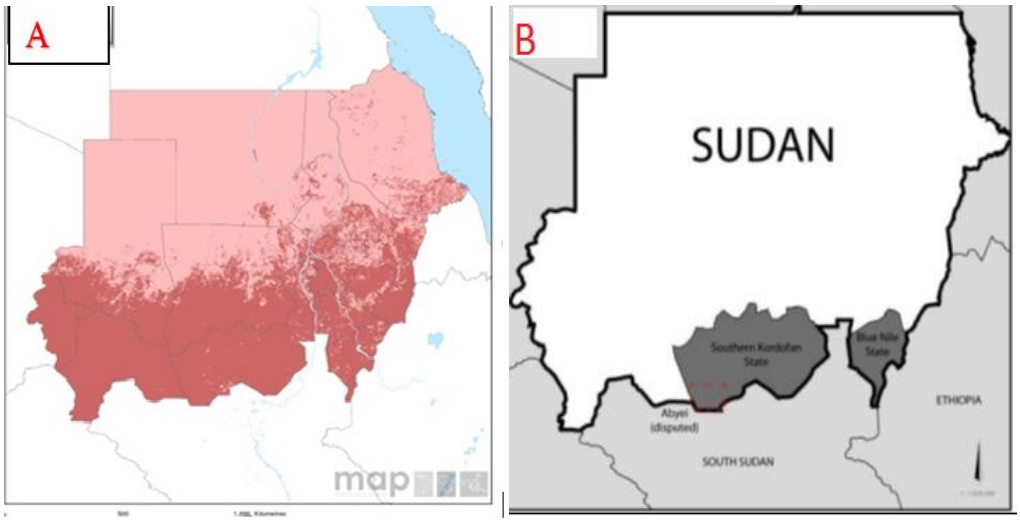


Figure 1: A) The map for the transmission of *P. falciparum* in Sudan. Grey: areas those are most likely risk-free. Light pink: areas at risk from unstable malaria transmission, with the annual case incidence recorded at <1 per 10,000. Red: areas at risk of stable malaria transmission (Malaria Atlas Project, 2010). B) Map of the Blue Nile state of Sudan where the study is conducted (Malaria Atlas Sudan, 2011).

1.3 Risk factors

Pregnant women are more vulnerable to malaria infection than their non-pregnant peers. The physiological and hormonal changes during pregnancy have an impact on increased risk of malaria during pregnancy (Lindsay *et al*., 2000).

Whilst in Africa, adult immunity to infectious transmission of falciparum malaria is commonly high and devastating effects are limited mostly to primigravidae, where a subpopulation of parasites tends to sequester inside the placenta (Fried *et al*., 1998).

In first pregnancy the immune system is defensive against these parasites; and therefore, in subsequent pregnancies the level of maternal malaria decreases due to the build -up of the mother's immunity against the parasite (Moore *et al*., 1999).

It is ambiguous what attracts mosquitos to pregnant women more than others, however it has been postulated that physiological and behavioural changes during pregnancy increases the frequency of biting by malaria- infected mosquitoes (Lindsay *et al*., 2000). This ultimately increases women's exposure to malaria parasites. The two physiological features underlying increased attractiveness during pregnancy are as follows; in the advanced pregnancy stage (meaning the gestational age of 28 weeks), they breathe out deeply more than their non-pregnant peers (Lindsay *et al*., 2000). During pregnancy, the blood level flow to the skin increases, due to dissipation of heat, mainly in the hands and feet (Lindsay *et al*., 2000). It seems that more factors leads to an increased mosquitoe bites in addition to physiological changes such as increased exposure to mosquitoes', since pregnant women leave their bed-net at night, probably to urinate, twice as frequently as non-pregnant women (Lindsay *et al*., 2000).

The infection of *P. falciparum* amongst primigravidae is common, although in multigravidae; the infection is not infrequent, as it is characterized by anaemia and low birthweight in new-borns. Few studies have approved the relation between the time of infection and the occurrence of malaria in pregnant women. Also, it is noticed that pregnant women, in general, are more susceptible to malaria infection and are extra vulnerable to infection when in their second trimester (Lindsay *et al*., 2000).

1.3.1 Vulnerability to Malaria in Pregnancy

Malaria seems to be a special case amongst pregnant women although there are other infectious diseases that can cause during pregnancy time, because pregnant women are more prone than non-pregnant women to malaria infection. Vulnerability is greatest during first and second pregnancy. In the first pregnancy, malaria is most common peaking between weeks 13–16, and decreasing towards the term (Brabin *et al*., 2001). Susceptibility to pregnancy-associated malaria (PAM) represents a combination of immunological and hormonal changes associated with pregnancy (Brabin, 1983). Age is also considered to be one of the major risk factors, as younger pregnant women are more prone to malaria infection than older ones (Saute *et al*., 2002). Parasitised cells in the placenta have specific features in which a unique variant surface antigen (VSA) is expressed predominantly. The VAR2CSA protein

and lack of immunity to these pregnancy-specific variant surface antigens, can explain some of the pregnancy-associated malaria vulnerability (Rogerson *et al*., 2007).

1.4 Life Cycle of the Malaria Parasite

Malaria infection is caused by a parasitic protozoan called *Plasmodium.* There are only four types of parasites (of the over 100 species of *Plasmodia*) that causes malaria infection to humans, namely, *Plasmodium malariae, Plasmodium vivax, P. falciparum,* and *P. ovale.* The parasite infection is spread from one person to another, by the mosquito Anopheles (Soulard *et al*., 2015).

The mosquito feeds on human blood, to produce eggs (Figure 2). These inoculated Sporozoites start to migrate and invades the liver hepatocytes by a special mechanism, which is yet to be understood (Krettli *et al*., 2001; Herbert *et al*., 2015).

1.4.1. Human Liver Stage

In the hepatocytes, the Sporozoites start to differentiate amongst themselves and divide into a massive number of liver merozoites. The Sporozoites penetrate liver cells within 30 minutes. These merozoites are then released into blood circulation and they subsequently start to invade new red blood cells (RBCs) to initiate the asexual blood-stage lifecycle of the parasites (erythrocytic cycle). In the RBCs, they mature and begin to divide within a specific period (according to the *Plasmodium* species), in three distinct stages. Then, what follows is the trophozoite phase, which is considered as a very active stage as much of the RBCs cytoplasm is consumed in this stage (Krettli *et al*., 2001) as shown in (Figure 2).

1.4.2. Human Blood Cell Cycle

In the end, the parasite undergoes around 4–5 divisions in the stage called schizont, producing a new number of merozoites that start to reinvade new RBCs and restart the cycle again (Ginsburg *et al*., 1990). This stage is called the erythrocytic cycle that is associated with clinical manifestations, such as fever and anaemia. The merozoites can also mature into the sexual forms of the parasites, giving rise to both types of gametocytes (male and female) (Figure 2). These stages are infectious for the mosquito that ingests them during its next blood meal which may continue as a cycle (Sandra *et al*., 2016).

1.4.3. Sexual Stage: Gametogenesis Formation

During the life cycle of *Plasmodium* parasite, it undergoes a stage of sexual replication. Briefly after fertilisation, the process of reproduction takes places inside the mosquito (Figure 1); however, the factors involved in the regulation of gametocytes are yet to be studied (Bennink *et al*., 2016). During a blood meal, the mosquito ingests the gametocytes, which stimulates the process of gametocyte formation known as gametogenesis, that takes place in the mosquito's midgut lumen.

The mosquito generates male and female gametes by several complex procedures; it derives from a molecule, namely xanthurenic acid that triggers male gametogenesis in addition to creating other conditions, such as shifting temperature and changing her pH, to form exflagellation (Billker *et al*., 1998; Ahmed *et al*., 2009). Specific surface proteins belonging to the family of 6-cysteine repeat proteins assist in the gametogenesis process. The proteins P48/45 are necessary for the male gametes to fertilize a female gamete and P47 is present particularly on the female's gamete surface. Moreover, a member of 6-cysteine protein family called P230 has been found on the surface of both gametes. Although it is found without a specific function, it thought to have blocking strategies (Eksi *et al*., 2002).

There are many genes implicated in the formation of gametes and they have many functions, the most essential one being macrogamete development. A recognised name of the male development gene-1 (MDV-1) is Peg 3 (KalpanaLal *et al*., 2009). Besides, there is another surface protein specifically for *P. falciparum*, a cGMP-dependent protein kinase (PKG), that is essential to produce the male gamete's flagellation, and for the activation of mediated xanthurenic acid found in the gut of mosquito (McRobert *et al*., 2008).

1.4.4. Invasion of Ookinete to Midgut Epithelium

Shortly after the development of the zygote and the completion of meiosis, the spherical zygote is transformed into an ookinete, with the co-operation of related protein kinase (Nek-4) (Reininger *et al*., 2009). The ookinete looks like an elongated motile cell, with the ability to leave the blood meal. There are essential enzymes crucial for the ookinete to cross the layer of peritrophic matrix (Reininger *et al*., 2009). Besides this, there are additional proteins, such as CDPK3 (calcium dependent protein kinase 3) that are involved in ookinete motility.

Then the ookinete break the peritrophic matrix and further makes its way into the mosquito's apical end mid-gut epithelium; (

Figure 3) with the assistance of a protein called membrane attach ookinete cross protein (MAOP) to disrupt the host T-cell membrane (Kadota K *et al.*, 2004). Then, the ookinete crosses into epithelial cells, before being present in the basal side of the epithelium through a process called traversal cell.

1.4.5. Ookinete to Oocyst transformation

The ookinete starts to inject into the mosquitoes hemocoel (Ahmed *et al*., .2009). The mid-gut is covered by laminin and collagen. The laminin plays a vital role with other host factors in the process of transformation from ookinete to a sessile oocyst (Adini *et al*., 1999; Ahmed *et al*., 2009).

The laminin-mediated transformation has major effects and is triggered by parasite ligands found on the surface of the ookinete. With the help of other surface proteins such as P25 and P28; anchored ookinete surface proteins bind and interact to assist the conversion of ookinete (Vlachou *et al*., 2004).

1.4.6. Development of oocyst and Sporozoite

Development of oocyst takes a comparatively long time, approximately between 10–12 days (Figure 1). The oocyst is considered as the only extracellular developmental stage of life cycle of malaria. Moreover, the expression of specific proteins in female gametocytes and ookinete help in the sporozoite development process (Ménard *et al.*,2013).

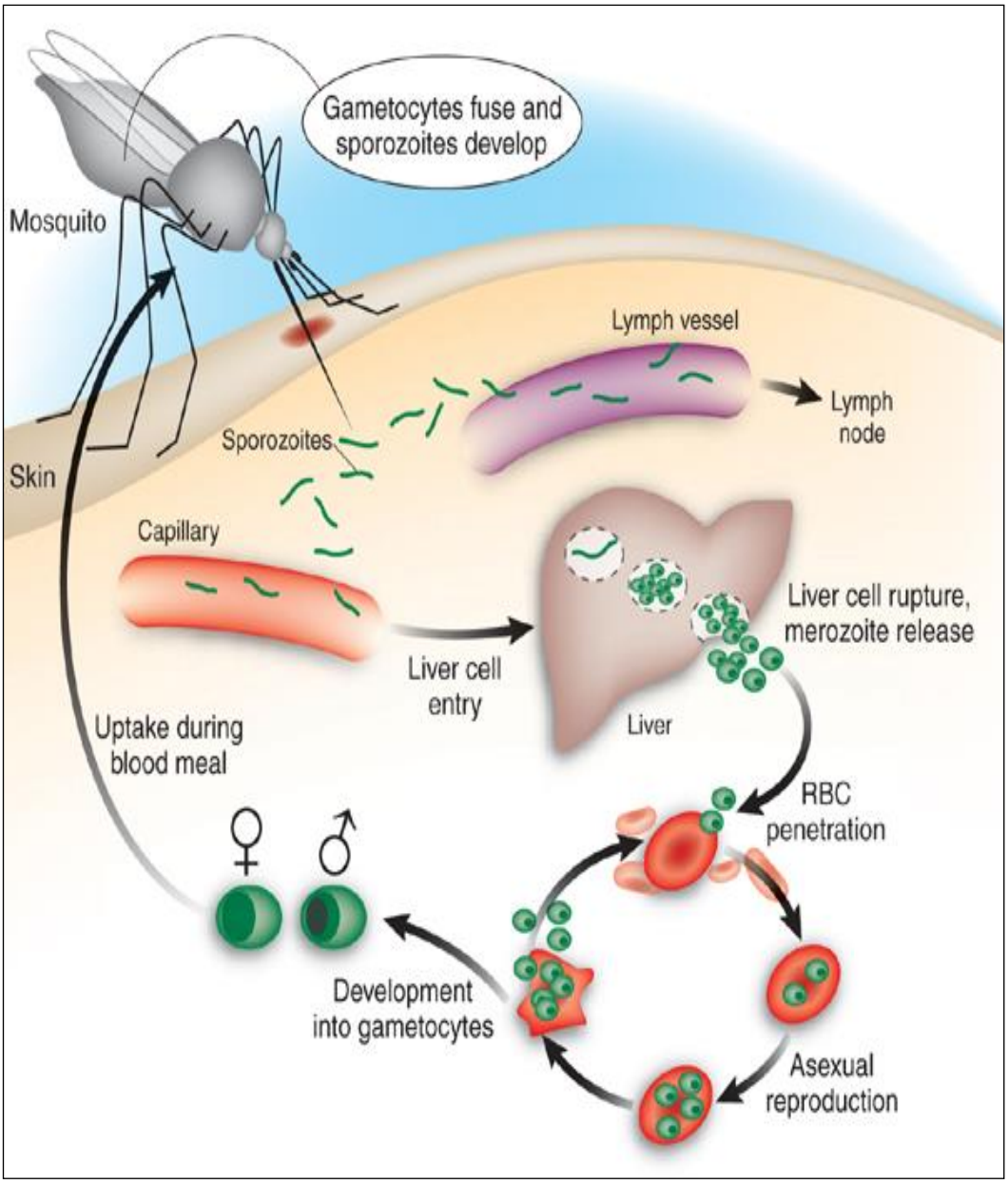


Figure 2: The life cycle of the malaria parasite, with a macroscopic view of the Malaria parasites, Source: Jones and Good, *Nat Med 12*, pp. 170–171, 2006.

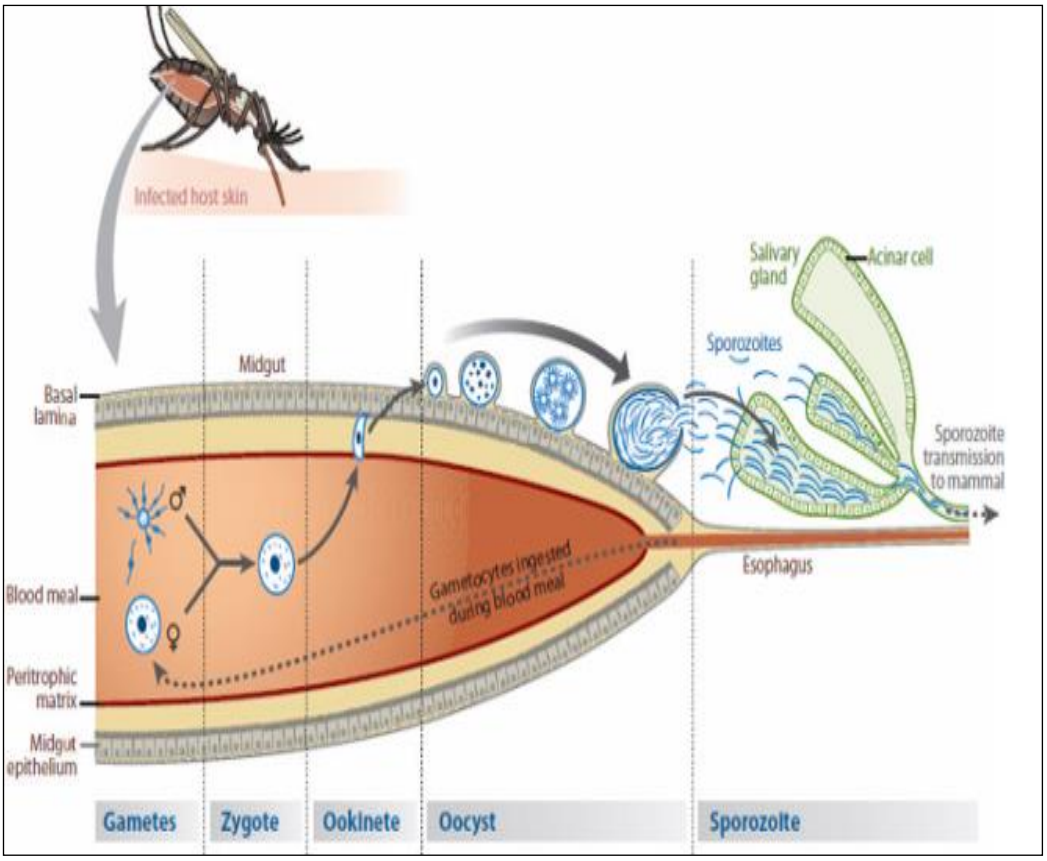


Figure 3: The various stages involved in the development of the malaria parasite Source: Christian R Engwerda & Michael F Good, Nature Medicine, 14, 912–913, 2008.

1.4.7 RBCs Invasion by Parasite

Invasion occurs in a similar way for all types of *Plasmodium*. For successful invasion to take place, the parasite first engages the receptors on RBCs for binding **(**Grau *et al*., 1989). This is followed by apical reorientation (Chitnis *et al*., 2001) and the formation of junction allowing, the parasite to starts signalling (Aikawa *et al*., 1978, Chitnis *et al*., 2001). The parasite initiates the formation of a vacuole derived from the plasma membrane of RBCs and enters the vacuole by a moving junction. There are three main parts forming the apical end of the parasite **(**micronemes, dense granules, and rhoptries) which define the phylum apicomplexa or receptors which are mainly found in micronemes, the area that studies have assessed to be the point of an invasion of RBC by merozoites and liver invasion by sporozoites (Adams al.1990, Chitnis *et al*., 2001).

The unknown answer concerns the question as to how the merozoite surface molecules recognize RBC surface and produce a signal for the invasion mechanism. Differences in

certain biological aspects, in both parasites, reflect the variety of patterns. Firstly, *P. falciparum* could invade RBCs at all stages of maturity, however, *P. vivax* can invade reticulocytes only. A recent study undertaken for the comparison of uncomplicated malaria to a severe one has suggested a similar pattern, with *P. falciparum* invading all RBCs and virulent parasites invading only a subpopulation (Chotivanich *et al*., 2000). Secondly, there is another different and redundant pathway in *P. falciparum* which is absent in *Plasmodium vivax*. *P. vivax* that invades the Duffy blood group, namely positive RBC23, that is mainly restricted to reticulocytes (Miller *et al*., 1976;). Interestingly, in areas where this blood group is absent; the *P. vivax* is also absent.

1.5. Signs and Symptoms

P. falciparum can cause many symptoms that are related to acute illness while some of these are non-specific, usually including flu-like symptoms, such as a fever, mild jaundice, headache, malaise, hyperventilation and hepatosplenomegaly (Taylor *et al*., 2000). Seizures may also occur anywhere from 7 to 30 days of the initial mosquito bite (Taylor *et al*., 2000). Febrile incidences can take from 6 to 10 hours to develop and this usually happens in three stages – the first stage is called the 'cold stage', the second stage is the 'hot stage', and the third stage is the 'sweating stage'. These stages are repeated, on-and-off, at specific times, depending on the type of malaria parasite. They repeat, at times, in 72/48/24 hours, due to the bursting of red blood cells.

Malaria symptoms start to decrease for a while, but then reappear in the above stages and can continue for a month or so, if untreated (Alessandro *et al*., 2012). Malaria presents symptoms such as thrombocytopenia, nausea, vomiting, and diarrhoea, with some cases showing signs of splenomegaly and possibly hypoglycaemia (Alessandro *et al*., 2012). Severe anaemia can be caused by both *P. falciparum* and *Plasmodium vivax*, but multiple complications such as cerebral malaria, placental malaria, metabolic acidosis, hypoglycaemia, and respiratory distress can be caused only by *P. falciparum* (

Figure 4).

In some cases of malaria, the parasites can remain in the liver inactive (in its dominant stage) from months to years. The parasite is comparatively protected from the immune system (Mueller. *et al*., 2009). These three stages (the cold stage, the hot stage, and the sweating stage) are repeated at an interval of 24, 48, and 72 hours, depending on the type of malaria.

This can be possible, according to the cyclical and coordinated bursting of red blood cells that initiate the next life stage of the malaria parasite; causing symptoms of fever amongst patients.

1.5.1. Clinical Manifestation and Clinical Outcomes

There are a few host pathologies, in which they all related to the erythrocytic stage of parasite invasion. They are very severe particularly among non-immune individuals. They appear as mild fever and some can be fatal like organ diseases (Luxemburger *et al*., 2007). People acquire partial immunity to malaria in high transmission areas specifically women at the reproductive age. Although they are at less risk of severe infections, they are still at high risk of other health disorders, such as developing anaemia and delivering babies with low birthweight (LBW) (Luxemburger *et al*., 2007).

Malaria in general is a very fatal disease but during pregnancy it has a serious impact. It causes serious consequences for pregnant women and their infants, particularly anaemia. It affects the primigravidae more than the multigravidae (Brabin *et al.*, 1990; Padhmanand,2007). It also has severe impacts on the foetus health Padhmanand, 2007). These clinical outcomes of a malaria infection are dependent on the parasite species, the host and the geographical and social factor (See

Figure 4).

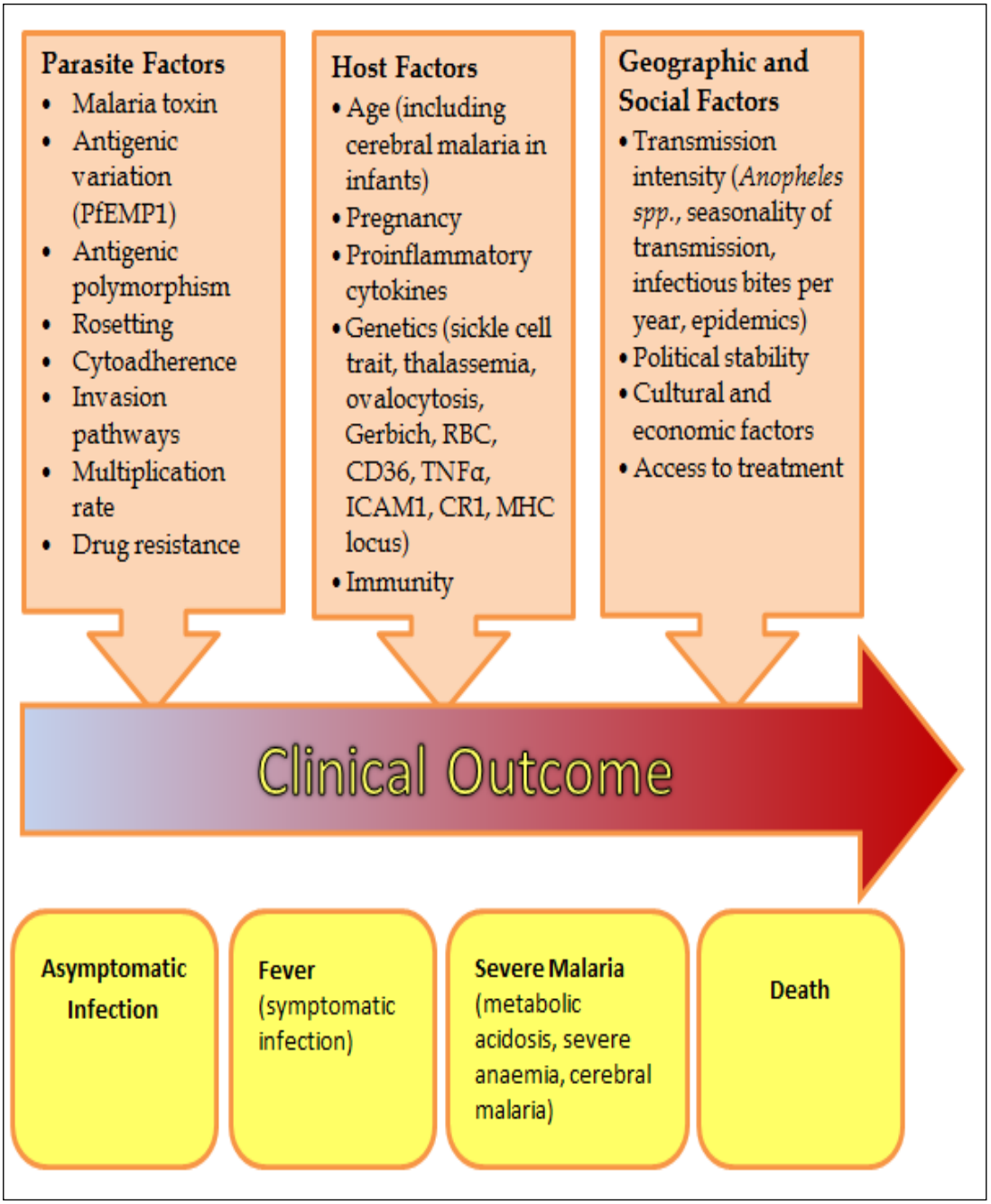


Figure 4: The clinical outcome of a malaria infection is dependent on the parasite species, the host, and the geographical and social factor (Source: Adapted from David J. Weatherall *et.al*, *Malaria and the Red Cell*, 2002).

1.6. Cellular Immunity in Placental Malaria

The immune balance of pregnant women in the placenta is interrupted by placental malaria (PM) (Qinghui,1997), which increases tumor necrosis factor (TNF), interleukin (IL) and interferon γ synthesis (Moore *et al*., 1999). LBW and anaemia have been linked with tumour necrosis factors (Fried *et al*., 1998), whereas placental malaria has been associated with the production of IFN *in vitro* by placental cells (Rogerson *et al*., 2003).

Chemokines may be important in attracting monocytes to the placenta with an increased number of neutrophils and T-cells. In malaria, the peripheral blood T-cell responses may be decreased, due to the trafficking of memory T-cells out of the circulation. Individuals develop naturally acquired immunity to both *P. falciparum* and *Plasmodium vivax,* in malaria endemic areas; which does not protect them from getting infection, but it protects them from the development of high parasitic densities and other clinical symptoms (Qinghui *et al*., 2016). Pregnant women are usually more vulnerable to *P. falciparum* and *Plasmodium vivax* irrespective of acquired protective immunity during pregnancy. The immunological changes and extensive hormonal changes that occur during pregnancy, play an essential role in this vulnerability (Beeson *et al*., 2005). Increased vulnerability has been largely pointed to the lack of immunity in pregnancy-specific isolated sequesters in the placenta, in the case of *P. falciparum* (Desai *et al*., 2007).

By the late-third month of gestation the process of sequestration starts to take place (Garnham, 1938), and by the end of the 12th week of gestation the placenta begins to develop to the point where maternal blood can begin to flow into the intervillous space (Brabin *et al*., 2004 and Wang, *et al*., 2010).

Reductions in the prevalence among multigravidae can be observed. that could be attributable to a more rapidity chronic infection clearance and help in the hypothesis that through first pregnancy particularly after the large inflammatory immunity response, the immune system is capable to support a more specific ally in subsequent pregnancies (Beeson *et al*., 2005).

Peripheral parasitaemia as shown in several studies mainly in early pregnancy at delivery, are associated with low birth weight (LBW) and anaemia and it has been pointed that infection at this point in gestation may interfere with placentation which may impair remodelling of the spiral arteries (Brabin *et al*., 2005; Rogerson *et al*., 2007).

1.7. Diagnosis

Available diagnostic assays for malaria lack either sensitivity or specificity or in some cases, it is too expensive and may need expertise; that are not usually available in the most endemic of countries. In the malaria endemic, where laboratory service is often unavailable, clinical diagnostic becomes essential, even though it is imprecise (Malaria R&D Alliance, 2005).

The use of light-microscope to examine Giemsa-stained blood smears has successfully become the golden standard for malaria diagnosis. However, it is needing a lot of effort and requires much technical skills, with a low turn-around time (the process is slow) (Ohrt *et al*., 2002). The sensitivity of the light microscopy method is reported to have a limited range, in routine labs can detect 50–100 parasites per 1μl (micro-litre).

Despite its usefulness, there are some limitations, including maintenance, skill, training, and skills of the microscopist, including the workload and preparation of slides (Durrheim *et al*., 1997 and Maguire *et al*., 2006). Even in developed countries, expert microscopists are very rare (Thomson *et al*., 2000). Besides, there are some limitations regarding the use of light microscope for example, false positive results can be reported due the poor blood film preparation, causing artefacts to misinterpret malaria parasites for cell debris, bacteria, stain precipitation, and dirt (

Figure 1 and Figure 2) (Houwen, 2002).

By using a microscope for Giemsa-stained thick blood film the detection rates around 4¬20 parasites/dL. Moreover, scientists have introduced new methods for malaria detection, such as the immunochromatographic assay which forms the basis of commercial malaria RDTs (Rapid Diagnostic Test) (Moody *et al*., 2002). Thus, allows much easier and simpler ways to interpret the methods, with a higher turn-around time.

Molecular methods such as polymerase chain reaction (PCR) have been introduced in the 1980s–1990s; that play a basic role in malaria detection (Wongsrichanalai *et al*., 2007). Moreover, another method that has emerged to detect malaria parasite is fluorescent staining (Hanscheid, 1999; Levine and Wongsrichanalai *et al*., 2007).

In some cases of examination, normal blood components like platelets can also cause confound in diagnosis. Some results might come as falsely negative, as the chances increase according to the parasite's decreasing densities. Other errors can be identified as result of species identification (Wongsrichanalai *et al*., 2007). To reduce false positive and false

negative results numbers of necessities are needed such as experienced microscopists, increased examination time, improvement in training, and a higher quality of smear preparation and staining (Wongs richanalai *et al*., 2007).

1.8. Treatment

Malaria can be prevented and cured. The basic objective of the treatment is to eradicate the parasite completely from the body system, to avoid any further progression of parasite which can lead to complications, such as anaemia (WHO, 2015). Treatment for uncomplicated malaria includes artemisinin-based combination therapies (ACTs). This is given as a combination of two active medical drugs.

ACTs are considered as one of the most effective antimalarial drugs. WHO has recommended regular monitoring for checking the efficiency of antimalarial drugs (Stephen, 2007) to avoid artemisinin resistance, artemisinin and its derivatives must not be taken as oral monotherapy (WHO, 2015).

Furthermore, there is a type of fixed-dose formulations, which combines two different active products in one tablet. To enable adherence to treatment and reduce the potential use of the individual components of co-blistered medicines as monotherapy it is preferred strongly and recommended over co- packed, co-blistered, or loose combination tablets (WHO, 2015).

In cases of severe malaria, injection of artesunate, either intramuscular or intravenous, is a suitable treatment, followed by a complete course of ACT.

In case of very sever malaria; the patients must take pre-referral treatment with intra-rectal artesunate, after which they can be referred to suitable facility to avail complete parenteral treatment (WHO, 2015).

1.9. Special Care and Treatment for Pregnant Women

World health organization (WHO) states that all pregnant women in their second or third trimester of pregnancy who have uncomplicated *P. falciparum* malaria, should be treated with artemisinin-based combination therapy (WHO, 2015).

Potent artemisinin component with its short acting (artemether, artesunate, or dihydro artemisinin) could reduce the number of parasites substantially during the first 3 days of treatment. Other group includes Lumefantrine, piperaquine, amodiaquine, or mefloquine with its longer acting partner drug, its role is to eliminate the remaining parasites, by preventing

malaria recurrence. The post-treatment prophylactic effect and prevention of new infections could be controlled under the effect of the longer-acting partner drug (WHO, 2015). The post-treatment prophylactic effect is a consequence of the potency and elimination of the drug half-life. An intermittent preventive treatment by repeated anti malaria curative is also used to eliminate potential asymptomatic infections and prevent the susceptibility to new infections. WHO states that malaria-endemic areas' women in Africa must have intermittent preventive treatment, with sulfadoxine pyrimethamine as part of their antenatal care (WHO, 2015)

Safety and side-effect profiles have recorded for the combination of dihydro artemisinin piperaquine and artemether lumefantrine. The placental malaria infection rates are similar among all the treatment groups and 15% of their babies have a low birthweight while prophylactic effect post-treatment is noticed in the artemether lumefantrine group (Ashley *et al*., 2014).

The best acceptable efficacy and safety side effects seen amongst the four studied drug combinations are dihydro artemisinin piperaquine due to its suitability support as a chemoprophylaxis or chemoprevention mediator (Tarning *et al*., 2012). The prevalence of parasitemia and symptomatic incidence in pregnant women is substantially noticed in high level among sulfadoxine users. On other hand Artemisinin-based combination therapy is essential for uncomplicated *P. falciparum* malaria among pregnant women whereas dihydro artemisinin piperaquine is used for malaria prevention (Tarning *et al*., 2012).

New anti-malarial drugs with reduced risk and resistance have emerged that may increase the therapeutic life span. However, it is several years away from clinical use that might be useful in some areas such as South East Asia due to low acquired immunity and artemisinin resistance (Amaratunga *et al*., 2016).

1.10. The Placenta

Placenta is a specialised organ formed during pregnancy, beside the membranes and the amniotic fluid that protect the foetus. The process of placenta formation is highly coordinated, involving interactions of the cells from both mother and embryo (Loke and King, 2000). The placenta is like a haemochorial, villous structure that acts as a barrier between the foetus and the mother. This organ plays basic roles, such as enabling the process of oxygen exchange, delivering nutrients to the foetus, and taking away the waste via the mother's blood (Schneider *et al*., 1990). There is difference between human placenta and other mammalian placenta

where; in human, the maternal blood runs into contact with foetal-derived tissue placenta. The role of placenta is to retain the foetus's blood supply separated from the mother's blood supply, though still providing an attachment between both enabling the performance of the foetus' normal bodily functions in the womb. The placenta is described as a 'heamochorial' structure in which maternal blood comes close into contact with the placental trophoblastic cells (Jane and Melvyn,2011). It also functions as important exchange of maternal immunoglobin G (IgG) transferred from mother to foetus through 14 weeks' time, over this route the foetus gains passive immunity against all infectious diseases that the mother immunised against before, including malarial infection (Palmeira *et al*., 2012)

Placenta starts by blastocyst after the fertilization step; the blastocyst must find its own place to persist and become the womb and so the blastocyst invades itself into the endometrium to find its security in growth and survival. This is aided by multifactor, which allows the endometrium receptivity process to allow the grown foetus to be implanted in the future; these multifactors are divided into maternal signals, such as estradiol, progesterone, CRH, and relaxin and foetal signalling like HCG and EFG (Ahokas *et al*., 2008).

Throughout this time, the conceptus needs nutrients to facilitate its growth; this is attained by utilizing the maternal debris and using extra cellular material from the endometrium and the uterine gland that accumulates into an area between the maternal and the foetal tissues. This phase is known as the *histiotrophic phase* (Martin, 2013**)** and this phase lasts until the end of the first trimester, as the foetus needs to grow more, and these nutrients are not enough for the embryo and therefore other sources are needed to be developed in the maternal endometrial tissue that results in more specialized and vascularized area. This area is known as the *haemotrophic placenta* where the exchange of blood-borne materials between the mother the foetus takes place (Martin, 2013).

1.10.1. The Mature Placental Development

The mature placenta steps start at the time of 8 weeks and continue until 10 weeks of gestation, by the following succeeding steps (Wang yuping, 2017):

- The CTB breaks through the trophoblast shell;
- The CTB, as it breaks the shell, reaches the maternal decidua where the invasion of the decidua occurs; and
- As the invasion goes on, the CTB reaches the maternal spiral arteries where remodelling occurs (widening of the spiral arteries); this helps to facilitate more blood flow between the mother and the foetus and allows the survival and growth of the foetus.

Therefore, the villous trophoblast is the barrier between the foetus and the mother and its formation is provoked by the oxygen gradient differences between the mother and the foetus (Wang yuping, 2017).

As a result, placental maturation occurs as pregnancy advances and the relative trophoblast T-cell numbers are increased as the exchange between the mother and the foetus increases to dominate the placental secretory function (Red-Horse *et al*,.2004). Placental development is largely regulated by a series of hormones and factors.

1.10.2. Histology of Placenta

Placenta has three main parts (see Figure 5). The amnion which is known as well as the water bag, the chorionic section which is the heaviest of the placenta. The chorion is composed of villi and has capillary-like structures. In early pregnancy, during the first trimester, the placenta does not have any internal blood vessels which is called as the immature placenta and as pregnancy advances, that is in the second and the third trimesters, the placental villi contain a large network of internal blood supply which is termed as the mature placenta. The chorion has a layer of epithelial cells facing the mother's side known as the Syncytiotrophoblast (STB) layer; beneath this layer, there are cells which looks like a connective tissue called cytotrophoblast (CTB). Furthermore, there are decidual' cells which are differentiated maternal endometrium cells and these decidual cells are under the influence of the progesterone hormone. They are known from their stroma, which looks like connective tissues between the endometrial glands and the umbilical cord that contain two umbilical arteries.

The placenta plays an essential role in hormone synthesis during the process of oxidation, reduction; hydrolysis, and conjunction (Sastry, 1996). Placenta secretes large amounts of hormones such as, human Chorionic Gonadotropin (HCG), steroid, oestrogen, progesterone, human placental lactogen (HPL), and human chorionic somato mammotropin.

The placenta also has a sustaining role in controlling the foetus osmotic balance and blood pressure. The placenta is formed from foetal origin cells and maternal origin cells namely trophoblastic cells that form the decidua basalis of the endometrium (Jenkins and Tortora., 2012). The Syncytiotrophoblast cells that form from the fusion of the underlying cytotrophoblasts consists of a continuous surface which is responsible for most of placental

functions. It is a multinucleate epithelium, while the large surface area of the placenta is surrounded by the Syncytiotrophoblast cells.

Placenta formation starts when trophoblasts (foetus cells) attaches to the uterine walls and then proceeds to the uterus tissues; as it continues attachment process until it reached the uterine walls deeply and begins to form a connection with the mother's blood vessels (Lessey *et al*., 2002. The placenta further develops to a villi around the initial connection and connects into the lacunae section. Then the foetus' circulatory system and foetal blood vessels start inside the placental villi through the umbilical cord that connects the baby to the placenta where foetal blood vessels joined to the foetus (Lessey *et al*., 2002). The placenta looks finally as pancake shape (Jenkins and Tortora, 2012).

Syncytiotrophoblast is a large surface area of the placenta. It is an epithelial surface layer that is separates the interior of the maternal blood from the villous that flows around the villi (Benirschke *et al*., 2012). Syncytiotrophoblast cells plays a protection role in the foetus, by providing a barrier against the flow of possibly maternal harmful cells and molecules which keep the foetus safe from this risk as it linked by tight junctions (Wooding and Burton, 2008). Syncytiotrophoblast cells play role in foetus' protection, by providing barrier against the diffusion of potentially harmful maternal cells and molecules to the foetus as it linked by tight junctions (Wooding and Burton, 2008).

1.10.3. Mature Placenta Structure

The placenta is composed of two parts (as shown in **Error! Reference source n ot found.**)namely, the chorionic plate which is the foetal aspect that is derived from the chorionic sac and the basal plate that is derived from the endometrium. Intervillous space separates these two parts, where the essential functional activities take place and it is surrounded by a multinucleated layer called the Syncytiotrophoblast (Benirschke and Kaufmann, 2000). Moreover, the placenta represents a basic role in the transportation of immunoglobulin G. Through this process, passive immunity occurs, and the foetus can be immunised against most diseases including malaria. (Denise., *et al* 2009).

1.10.4. Placenta Function

The placenta is vital for the foetus to survive and grow into a normal healthy during gestation, thereby reflecting on the importance of its function in a full term healthy pregnancy. Therefore, the functions of the placenta are categorized as the following (Harvey,2014): (1) **Respiratory function**, function involving the gaseous exchange of oxygen and carbon dioxide (O2 and CO2) which passes through the placenta between the conceptus and the mother and this is achieved by a simple diffusion mechanism; (2) **Nutritive function,** mechanisms which are involved in the transportation of nutrients from the mother to the foetus through simple diffusion mechanism (e.g.; for water and electrolytes), the facilitated mechanism (e.g. for the glucose, the active diffusion e.g. for amino acids), and finally the pinocytosis mechanism (e.g. for the large molecules and cells) (Harvey ,2014); (3) **Excretory function**, the mechanism where foetal waste like urea which excretes to the maternal body by simple diffusion mechanism as a result of PAPP production (pregnancy associated plasma proteins). That is used to determine healthy pregnancy from affected gestation; (4) **Barrier function**, where the placenta allows some substances, such as IgG, hormones, viruses, rubella and antibodies to pass through, while inhibiting other molecules to pass through the placenta, such as heparin and insulin (because they are large molecules). This placental barrier is the intervillous spaces (Harvey,2014); (5) **Endocrine function,** in which the placenta is responsible for a variety of hormonal production which are essential and critical for a healthy pregnancy and hence, its specific trophoblast T-cells secretes steroid hormones (progesterone, estradiol), p-peptide hormones (HCG, TRH, CRH, GnRH, GH, HPL), decidual hormones (prolactin, IGFBP, PP53), enzymes (alkaline phosphatase) and so forth (Hill, 2010); (6) **Enzyme synthesis function**, there are various enzymes secreted from the placenta such as the heat stable alkaline phosphatase group, insulinase, histaminase, monoamino oxidase, oxytocinase, and so forth, which have many functions. They are secreted by their specific trophoblast tissue (cells) and therefore, scientists and researchers are doing their best to discover more about these enzymes' structures, secretions, and function (Harvey,2014).

1.10.5. Placental membranes

The placental membrane or placental barrier is formed by a layer of cells that contain foetal blood within the core of the villi which prevents it from mixing with maternal blood that occupies the intervillous space (Benirschke et al., 2012). Four layers make up the placental membrane (Figure 6), namely, the cytotrophoblast T-cell layer, the Syncytiotrophoblast layer, the villus connective tissue layer, and an endothelium layer lining the foetal capillaries. By

the 40th week of gestation the cytotrophoblast T-cell layer that is present in many of the villi will be gradually attenuated and it finally disappears (Figure 6), leaving the noticeable thin Syncytiotrophoblast layer of cells to meet and attach itself to the foetal capillary endothelium. Hence, allowing the foetal and maternal blood to come into proximity and allow successful trans-placental exchange of oxygen, nutrients, and antibodies, without the need of direct contact between maternal and foetal circulations (Parekh, 2010). From the foetal placental circulation, oxygen-deprived blood from the foetus passes via two umbilical arteries. Then, the oxygenated foetal blood returns, via chorionic and umbilical veins, back to the foetus (**Error! Reference source not found.**a).

Figure 5a: The structure of the placenta, (*Placental structure and transport*, 2010).

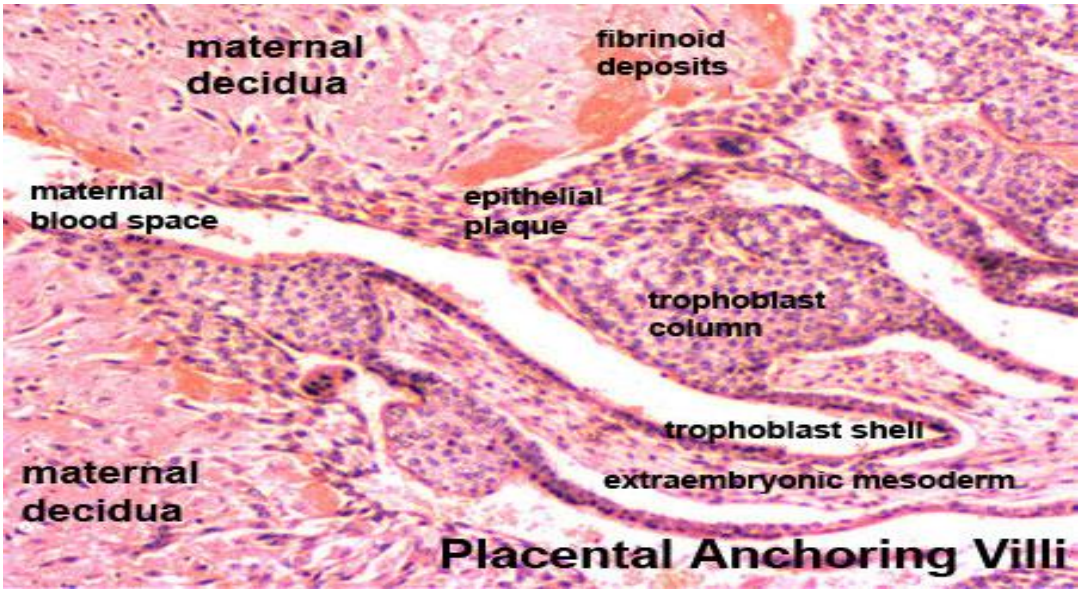


Figure 5b: Placental histology, (Hill, *Embryology Placenta anchoring villi.jpg,* August 2009)

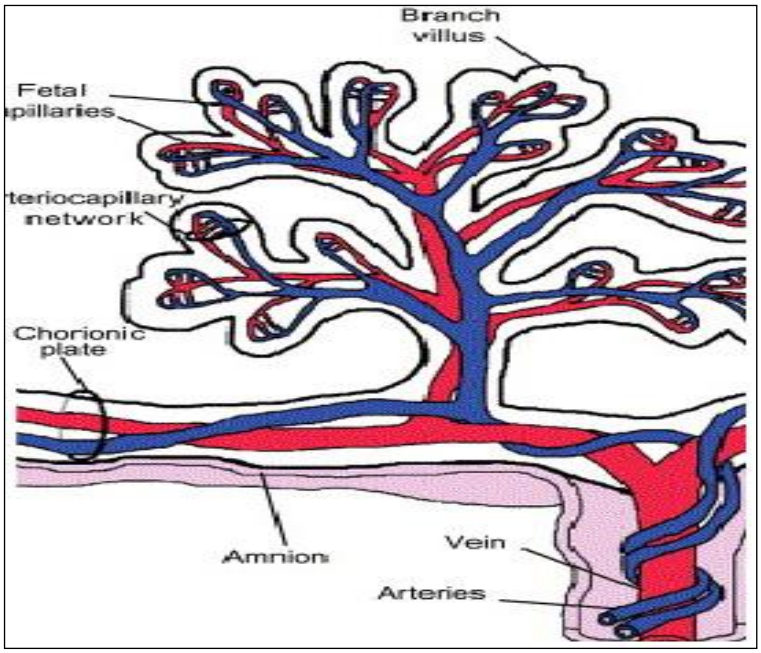


Figure 6: The foetal placental circulation Oxygen deprived blood from the foetus passes via two umbilical arteries. Then, the oxygenated foetal blood returns via chorionic and umbilical veins back to the foetus. (Blackburn, Maternal, Foetal & Neonatal Physiology: A Clinical Perspective, 2003).

The Syncytiotrophoblast, is a specialised multinucleated epithelium, which is responsible for carrying out most of the placental functions. The formation of the Syncytiotrophoblast layer takes place as the constant fusion of the underlying cytotrophoblasts, making up the bulk of the placenta and creating a constant, large surface area for continuous exchange. By the 16th week chorion frondosum forms foetal portion and decidua basalis forms the maternal portion.

The initial structure of the placenta begins from the foetal trophoblast T-cells which, attaches itself to the uterine wall, penetrating it until a connection with maternal blood vessels (Moore *et al*., 2011). The placenta then further develops by forming the villi finger-like protrusions and projects around the early connection into the mother and foetus space. During this stage, the foetal circulatory system is also developing, and foetal blood vessels form the inner placental villi, that connects the foetus to the placenta through the umbilical cord (Schoen *et al*., 2009). When fully formed, the placenta is like the shape of a pancake (Jenkins and Tortora, 2012).

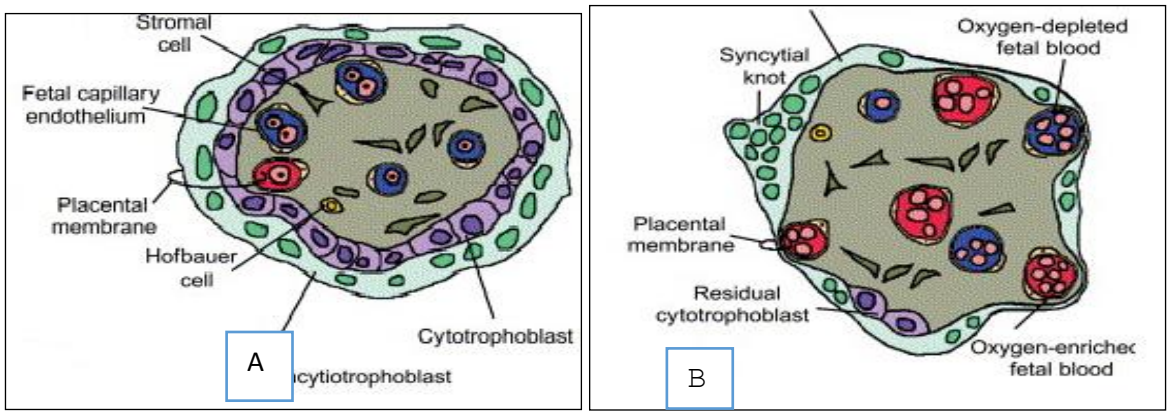


Figure 7: The layers of the placental membrane, showing sections through the chorionic villus in A: a 10-week placenta and B: the layer of Syncytiotrophoblast comes near or in direct contact with the foetal capillary endothelium (Blackburn, 2003).

1.11. Placental Malaria (PM)

Placental malaria or pregnancy - associated malaria (PAM) is one of the most significant contributors to congenital malaria. In most cases, placental malaria infections are accompanied by infiltrates of intervillous mononuclear cells (Michel Cot *et al*, 2003).

The number of physiological changes occur during pregnancy has a direct effect on the *Plasmodium* parasite invasion. Maternal immunity is noticed to be reduced or regulated as it is necessary for conceptus rejection. Moreover, cell-mediated immunity (Th1) inhabits pregnant women, but interestingly, pregnant women are protected via the increased humoral

immunity (Th2). This suppression is understood to be the main reason for pregnant women's high risk of malaria infection. On other hand, the immune response continues to prevent parasite infection (Duffy *et al*, 2003).

Furthermore, there are major differences in severity and risk in women during first pregnancy (Primigravida) and their peers are reported to have multiple pregnancies (multigravida). Therefore, risk and severity is considered to be in proportion to pregnancy number (Boel *et al*., 2012).

It is mostly associated with maternal anaemia and low birthweight. Fascinatingly, placental malaria infection is also a way of protection against perinatal death among infants with low birthweight (Alex, 2004).

Table 2: Placental pathology classification Source: Rogerson *et al*., 2007

Feature	**Description**
Non-infected	No evidence of parasite or malarial pigment (hemozoin)
Active-acute	Parasites present, with absent or minimal pigment deposition within fibrin
Active-chronic	Parasites with substantial amounts of pigments in fibrin
Past infection	Presence of pigment with no parasites

P. falciparum sequesters in the intervillous space of the placenta causes a type of malaria called placental malaria (Uneke, 2007). The infected erythrocyte is bound to the uninfected ones by a process called ‘rosettes’ (Mayor *et al*., 2005).

This sequestration causes many problems for both pregnant women and their infants, such as premature delivery, foetal growth restriction, an increased risk of low birth weight, or even a spontaneous abortion, as noted in some cases. Adverse effects amongst pregnant women are also highly noticeable, for example, maternal anaemia (Menéndez *et al*., 2000).

In previous studies, the detection of the malaria parasite in the placenta and its sequestration has been reported. Placental infection is present, without any clinical symptoms (Staalsoe *et al*., 2004). Placental histological changes, as well as monocyte infiltration, are also noticed (Suguitan *et al*., 2003).

These histological changes in the placenta are used as a classification criterion (Figure 7Table 2**: Placental pathology classification Source: Rogerson *et al*., 200** and Table 2) and are useful to diagnose malaria, especially in the cases where malarial diagnosis is missed through a peripheral blood test (Mockenhaupt *et al*., 2006).

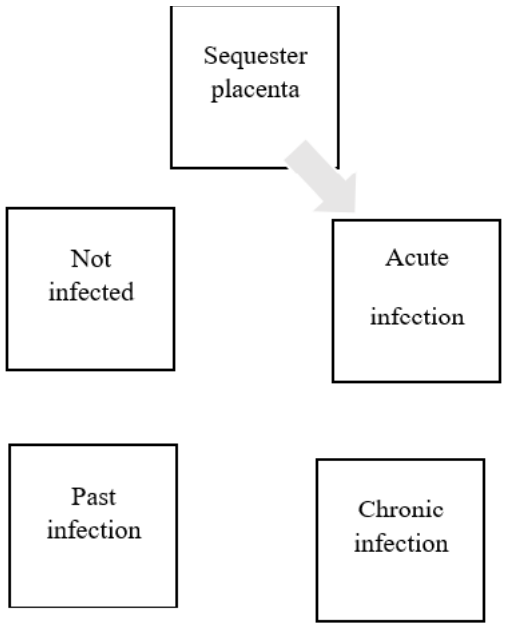


Figure 8: Placenta sequestrations, when the sequestration occurs, it becomes acute infection and passes through to become either chronic or past infection (www.nature.com/nature communications & 2013 Macmillan Publishers Limited)

Maternal malaria commonly occurs in endemic areas. It represents about 8% to 36% for low birthweight cases and around 3% to 8% of infant mortality (Steketee *et al*., 1996). All previous knowledge, regarding pathogenesis of pregnancy associated malaria, were researched amongst people in high and low transmission areas. In areas where malaria transmission is low, pregnant women from all gravidities have become susceptible to both symptomatic and severe maternal diseases, where variation depends on the severity of infection, such as stillbirth and miscarriage (Steketee *et al*.,1996). It has been noticed that congenital malaria has similar complications, including low birth weight (Steketee *et al2001*).

In addition to its direct impact, malaria also has a larger, indirect effect on mortality and disability, such as the sequestration of infected red blood cells in the placenta that can cause fatal effects in the foetus. Therefore, the results include low birth weight (<2500 g), thus noticeably decreasing the survival chances of infants (Steketee *et al*., 2001).

Pregnant women are at high risk of malaria infection, with the number of reported incidences increasing every year to reach more than 50 million, particularly in epidemic countries (WHO, 2015), reflecting the severity of the infection among pregnant women. Women in their first pregnancy (primigravidae) and babies whose age is less than 5 years old will be at risk, as well as non-immune women, for example, travellers. Still during malaria epidemics and in areas where malaria has a low incidence, it is noticed that all age groups may be at risk of severe infection (McGregor *et al*., 1984; Adam *et al*., 2005).

Understanding this vulnerability of pregnant women to malaria infection still needs more effort. It is clearly noticed that pregnant women are at higher risks of malaria infection, compared to their non-pregnant ones (Desai *et al*., 2007). For full-term successful pregnancy to occur, physiological adaptations occur in all maternal systems, including the immune system. To maintain pregnancy, especially in the first trimester, there is a state of selective immune tolerance and immunosuppression (Luppi, 2003). It has also been hypothesized that the hormonal changes in pregnancy have an effect. The placenta is considered as being a preferable place for parasite sequestration (Figure 8), to which it is linked with increased susceptibility amongst pregnant women *(Katie, 2015).*

When pregnant women become infected with *Plasmodium malaria*, their condition tends to be severe (Snow *et al*., *2003)*. The parasitised placenta with infected *P. falciparum* is different from the normal placenta (Figure 9).

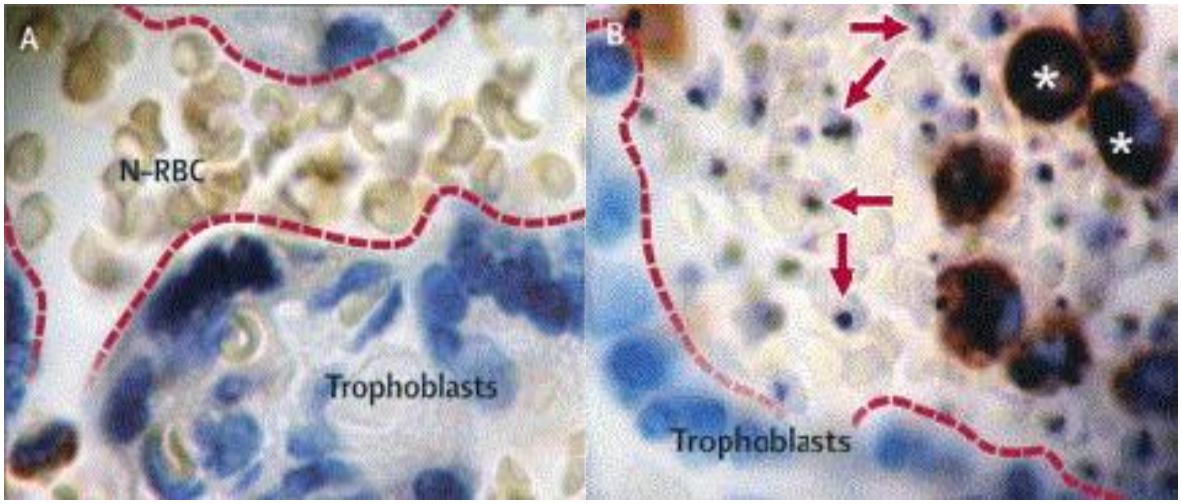


Figure 9: Sections of the placenta A: normal placenta. B: malaria-infected placenta (arrows indicate parasitized erythrocytes; lines show the layer of trophoblast). N-RBC: normal red blood cells. Source: Suguitan Jr, 2003.

There are many factors that contributes to this severity; the main factors being their immunity level, their pregnancy trimester, and their parity (Coll *et al*., *2003*). It has been noticed that adult women from stable transmission or an endemic acquire a type of immunity called the semi–immunity, usually during 10 to 15 years of life (Dorman *et al*., 2000). This is thought to be sustained through continued exposure to malaria infections. Most of the malaria diseases are symptomatic in these cases (absence of fever) (Snow *et al*., 2003).

Malaria is unstable in non-endemic transmission areas, where adult women lack immunity against malaria and they look symptomatic, especially when they are parasitemic. They are at greater risk to develop severe infection that can lead to death (WHO Report, 2008). But for the lower parity and younger age group, their susceptibility to malaria increases (Mutabingwa *et al*., 2005). It is reported that placental malaria in endemic areas can reach up to 63% among Primigravida, but a lower percentage, of 33%, was observed among multigravidae women (Goldenberg *et al*., 2003). Pregnant women have the possibility of maintaining a type of partially acquired immunity, particularly those who live in malaria-endemic areas and become asymptomatic.

1.11.1 Parasite Sequestration

P. falciparum differ from other human malarias; in a way that *P. falciparum* behaves and adapts to red blood cell surface for adherent process. Both parasite types, asexual and gametocytes, adhere to the endothelium and asexual parasites within placenta, therefore, it is a noticeable finding of the ring forms of *P. falciparum* in the circulating blood (Chen *et al*., 2000).

The *P. falciparum* trophozoite and schizont-infected RBCs are sheltered with knob-like excrescences to communicate with host-cell (Dodd *et al*., 1998). This adherence mechanism is a clever way to protect the parasite from being destructed, where all non-adherent RBCs infected with parasites, will be excreted within the spleen rapidly (Mungai *et al*., 2001). To answer whether and exactly how sequestration might lead to pathogenesis, let us look at the way parasites sequester. The adhesion of *P. falciparum* is like leukocytes' adhesion; at first most of the parasites tether and then roll, before becoming attached (Hanscheid, 1999). The diverse properties of *P. falciparum* erythrocyte membrane protein (PfEMP1) is to sequester for evasion of the immune system that contributes to the virulence and pathogenesis of *P. falciparum*, which is vital for its survival. Parasite sequestration in the placenta (Figure 10 and Figure 11) causes complications of placental malaria (PM).

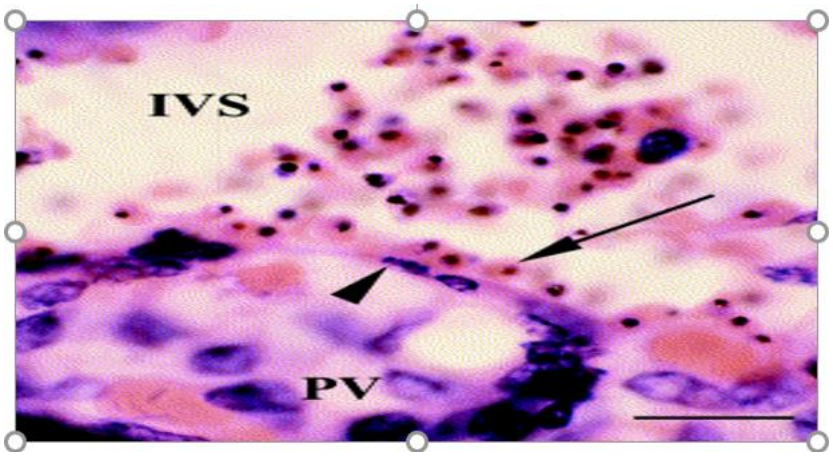


Figure 10: Section of the malaria-infected placenta, Arrow shows *P. falciparum*-infected RBCs in the intervillous space (IVS), sequestering the Syncytiotrophoblast cell layer (arrowhead) of a placental villous (PV), Source: A light and electron microscopic and immunohistologic study, American journal for tropical Medicine and. Hygiene, 41-1989.

Most host receptors are elaborated with both tethering and rolling mechanisms (Wongsrichanalai, 2001). Adhesion to host receptors is necessary to give the parasite-efficient binding power to the endothelium of different organs and preventing its elimination from the host (Warrell, 1982; Avril *et al*., 2004). Up to now, only two receptors have been discovered and proven to have essential roles in the adhesion process. These are chondroitin sulfate A (CSA) and CD36 (Wongsrichanalai, 2001).

In addition to the placenta, this parasite sequesters in a few organs such as the brain, liver, kidneys, lungs, and heart. The Syncytiotrophoblast and other organs consists of endothelial cells that have a huge variety and number of host receptors, to which the parasite can bind itself effectively, for the completion of the adherence process (Knight *et al*., 1999; Alexandra *et al*., 2009). Parasite adhesions (cytoadherence), resetting with uninfected red blood cells and platelet-mediated clumping of infected red blood cells (Figure 11).

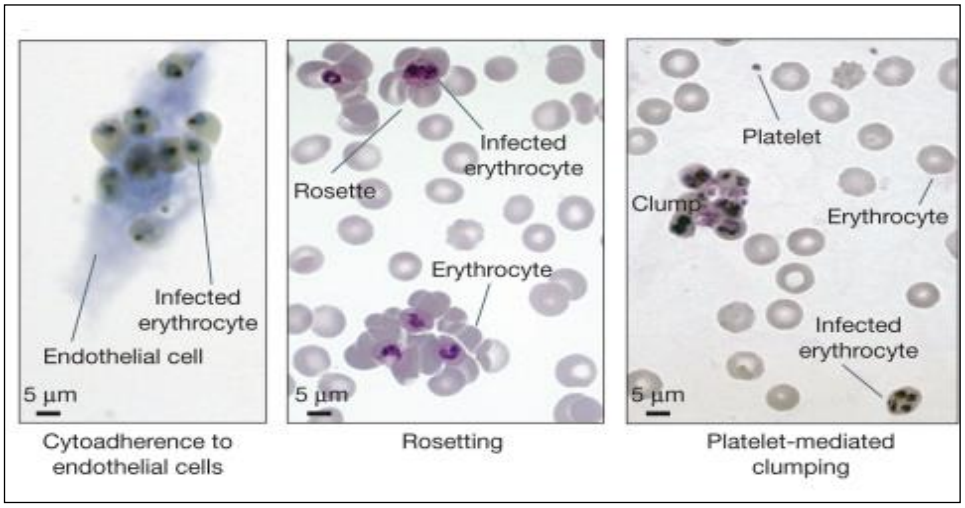


Figure 11: Adhesion process of infected erythrocytes with *P. falciparum* in different cell types in humans, (Alexandra *et al*., 2009)

The adhesion mechanism is not consistent, and the variable parasites can bind to different types and groups of host receptors at the same time (Nagel *et al*., 2001; Currat *et al*., 2002). This inconsistency is thought to have different roles in tissue distribution and parasite pathogenesis. Interestingly, a type of single parasite protein called *P. falciparum* erythrocyte membrane protein-1 (PfEMP1) that facilitates the binding to different types of receptors, in which it is expressed by infected erythrocytes (Knight *et al*., 1999; Aitman *et al*., 2000; Pain *et al*., 2001).

The RBCs that are isolated from the placenta show a unique property adhesion process that is different from ones in non-pregnant females. These parasites fail to adhere to CD36 but in meantime, they can bind to chondroitin sulphate A (CSA) (Beeson *et al*., 1999; Padhmanand, 2007).

Pregnant women are mainly vulnerable to be infected with *P. falciparum*, mainly due to the immunological response variations underwent, such as the alterations in T-cell activity (Riley *et al*., 1989), to maintain the pregnancy (Menendez, 1995).

Sequestration of the parasite in the placenta is a key marker of malaria infection among pregnant women, in which is linked with severe adverse results for both mothers and infants (Brabin *et al*., 1983; Duffy, 2003).

The erythrocyte infected with *P. falciparum* has specific features such as it looks different than those in non-pregnant women as they adhere to the receptors of glycosaminoglycan which is not otherwise occupied by other erythrocyte infections (Fried and Duffy, 1996). Placental infected erythrocytes are requisitioned in the intervillous space, whereas the sequestrations of other infected tissues are usually close to the vascular wall (Muthusamy *et al*., 2004).

The infected erythrocytes could express a few multiple adhesive ligands (

Figure 16) in which it can promote the parasite's survival and increase sequestration while, avoiding being cleared out by the immune system. Moreover, infected erythrocytes could co-express ligands for both CSA and HA (Beeson *et al*., 2000).

1.11.2 Adhesion to Glycos Amino Glycan in the Placenta

The Syncytiotrophoblast layers that line the blood space in the placenta, constitutes a broad area of foetal tissue in which communication with the maternal circulation occurs. Histopathological investigations for the placenta infected with malaria parasite demonstrates the process of infected erythrocyte adherence to the Syncytiotrophoblast, which are the mechanisms for sequestration of *P. falciparum* infected RBS in the placenta layer (

Figure 13) (Yamada *et al*., 1989). This phenomenon proposes a type of cellular adhesion to the receptors on the Syncytiotrophoblast layer which is necessary for this sequestration process. Some studies have suggested that parasites sequester in the placenta via adhesion to HA and CSA, which form essential coats on the layer of Syncytiotrophoblast (Parmley *et al*., 1984; Sunderland *et al*., 1985). A recent research proposed that chondroitin sulphate A is one of the main GAGs to exist in the uninfected blood and placenta tissue (Achur *et al*., 2000). However, the CS and other GAGs expression in adhesion, such as Syncytiotrophoblast, are not precisely examined. Some studies have suggested that 'rosette' formation which can assist parasite sequestration is infrequent, as it is not a necessary process to assist sequestration (Maubert *et al*., 1998).

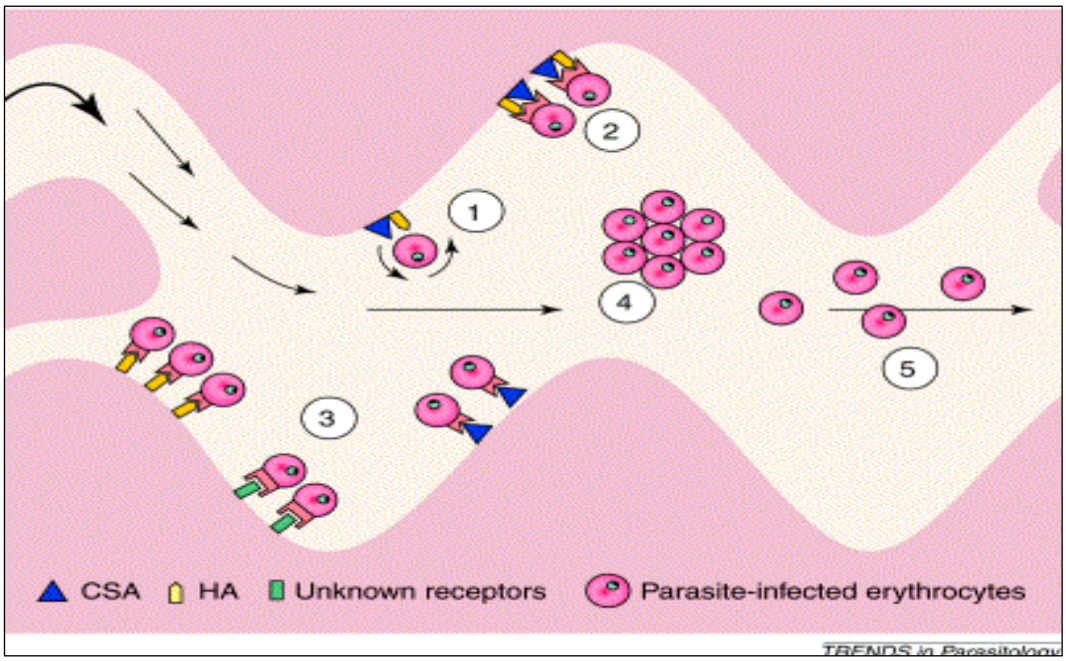


Figure 12: The mechanisms for sequestration of *P. falciparum*-infected RBS in the placenta (1) Blood enters the placenta. (2) Parasite-infected RBC becomes tethered onto the receptor by adhering to both HA and CSA. (3) Alternatively, the parasite may attach to HA or CSA only or may be to other unknown receptors. (4) Other infected RBCs may aggregate by unknown mechanisms. (5) Infected RBCs can be, with or without, adhesive ligands on cell surface that passes through the placental intervillous

space and do not adhere to the placental layer. CSA: chondroitin sulfate. A: HA: Hyaluronic acid. Source: James *et al*., 2008).

1.11.3 Expression of *Var2csa* Gene in Placental Malaria

The Syncytiotrophoblast layer represents the vital part of the placenta that has been described as a common receptor for infected erythrocytes (IEs). Sequestration mechanism is correlated with IEs that binds to CD36 and variably to other receptors expressed by the host, such as CSA (Fried *et al*., 1996).

The cyto-adhesion process is enhanced via PfEMP1 that is encoded by members of the *var* multi-gene family (Su *et al*., 1995). They are ~200–350 kDa in size (Su *et al*., 1995). This gene is composed of domains that play an essential role in binding the mechanism of CSA (Figure 17). Each parasite has several genes up to 60 *var* genes (Gardner *et al*., 2002).

The allogeneic parasite retains variety of *var* gene ranges. Few previous studies have found a correlation between several *var* genes and CSA which include *FCR3.varCSA* and var-CS2 (Smith *et al., 1995;* Emsley *et al.,* 2004*).* The binding parasite ligand for CSA is the VAR2CSA PfEMP1 discovered by Salanti *et al*. (Bai *et al., 2005).*

The extracellular portion of each PfEMP1 protein consists of two to nine domains, and other forms of individual domains that sustain binding of variant receptors of endothelial cells (Kraemer *et al*., 2006). The variant surface antigens, VSA PAM–specific IgG levels correspond with protection against PAM and parity (Staalsoe *et al*., 2004). Significantly the VAR2CSA have been recognized by plasma IgG from females, as its levels are linked to the protection against PAM and parity (Salanti *et al*., 2004). The *VAR2CSA* gene is disrupted by parasite cloning, as it has lost the ability to bind to CSA (Viebig *et al*., 2005).

Previous studies have found that some indication of maternal circulation retains *P. falciparum*-infected red blood cells (IRBCs), mainly in the placenta; this reason alone cannot clarify why parasitemia is in higher level in the placenta than in the peripheral blood (Desowitz and Buchbinder, 2016).

1.12 Placental Malaria consequences

Placental malaria causes many defects, such as increased risk of still birth and neonatal death in correlation with placental parasitemia (Newman *et al*., 2003). Malaria is one of the main causes of reduced birthweight by either local or systemic consequences (Menendez *et al*., 2000). Malaria can affect reduction in birthweight by placental infection or via malaria induced anaemia (Ibhanesebhor *et al*., 1992), in which the parasite can cause a type of mechanical cooperation of placental circulation through increased fibrinoid necrosis or, in some way, by inducing pathological lesions (Galbraith *et al*., 1980). It is very noticeable that infants mostly suffer from low birthweight, when the mother has placental malaria. The control of placental malaria does not show any hope for pregnant women (McCormick, 1985).

1.12.1 Intrauterine Growth Retardation and Pre-term Delivery

There is a relationship between preterm delivery or intrauterine growth retardation and placental malaria. Some studies have failed to prove any correlation between infected/non-infected mothers and pre-term delivery. On the other hand, some studies that have been conducted across sub-Saharan Africa have succeeded in proving that there is a significant relation between preterm delivery/intrauterine growth retardation and placental malaria (D'Alessandro, 1996).

In studies specifying the causes of low birthweight is due to high parasitemia and the mechanism, essential for intrauterine growth restriction and low weight with increased inflammatory cytokines such as TNF and IL-8.

The role of malaria-infected placenta in pre-term delivery is still unknown. But infected placenta that carries antibodies, macrophage, and cytokines can be a sign of an active immune response, because of early labour (Ismail *et al*., 2000). It appears that the intrauterine growth retardation can possibly be related to the transport of nutrient to the foetus (Guyatt *et al*., 2004).

Accumulation of parasites in the placental blood can result in the consumption of both oxygen and glucose by the parasite. Histopathological studies have found changes in the thickening of the cytotrophoblast membrane, which may obstruct the nutrient-transportation process (Ismail *et al*., 2000; Guyatt *et al*., 2004).

Parasitised red blood cells deposition and monocyte infiltration can possibly lead to physical blockage which can cause placental deficiency (Ordi *et al*., 1998). Cytokines such as IL-2, IL-

6, and IF-G are very dangerous and harmful to pregnant women as it correlates to foetal growth retardation (Ordi *et al*., 1998).

1.12.2 Foetal Anaemia

The incidence of foetal anaemia is reported to be more common in sub-Saharan Africa. It is defined as a condition in which the level of cord haemoglobin reaches levels of <12.5g/dl. The causative role of placental malaria towards foetal anaemia has been assessed in few studies which has given different results (Brabin *et al*., 2004).

Fascinatingly, in all previous studies, there is a correlation between foetal anaemia and severe maternal malaria infection (Brabin *et al*., 1997). But some studies have found that there is no major correlation between malaria parasite infection and anaemia (McElroy *et al*., 1999). All previous studies have different findings, and this may be explained by the fact that the variety of factors that affect, such as intensity of transmission, treatment method, quality of antenatal services, and resistance to drug (Chawla *et al*., 1998).

The foetal anaemia aetiology is complex, and the factors implicated in this phenomenon placental malaria may possibly play either minor or major roles, depending on epidemiological situations (Brabin, 1992). From previous studies, it has been suggested that new-borns become immunologically vulnerable to mediated haemolysis when exposed to malarial antigens due to placental damage (Brabin, 1992).

1.12.3 Low Birth Weight

Malaria during pregnancy is an independent risk factor of low birthweight. Infant low birthweight (LBW) is due to placental malaria which has been reported by most of previous studies. The rate of LBW (<2.5 kg) for new born ranges from 3.9% to 24%, as reported in sub-Saharan Africa (Okoko *et al*., 2002; Menendez *et al*., 2000). Placental malaria (PM) is thought to be one of the serious contributors to 3.5 million cases of LBW (Brabin *et al*., 1997).

Infant low birth weight due to malaria parasite infection initiated by different mechanisms. Malaria can affect birthweight via malaria-induced anaemia as well as placental infection (Ibanesebhor *et al*., 1992; Kassam *et al*., 2006), in which the parasite may cause compromise the placental circulation via interfering with placental function or widespread trophoblast T-cells, leading to the thickening and increased fibrinoid necrosis or pathological lesions (Moshi *et al*., 1995; Galbraith *et al*., 1980). Regardless of the occurrence of placental malaria infections among women's gravidities, between (5%- 52%) is associated with LBW risk

which is raised to 2/4 times in several studies (Moshi *et al*., 1995; Guyatt *et al*., 2004; Galbraith *et al*., 1980). On the other hand, infant low birth weight is measured and its vulnerability to malaria is compared to different placental malarial infection (Active or Chronic). In different studies, there are incompatible findings (Okoko *et al*. 2002) regarding the non-significant correlation that is found between LBW and increased fibrinoid necrosis and cytotrophoblast status (Menendez *et al*., 2000). Severe maternal anaemia is associated with low birthweight among primigravidae, where there is no obvious consistent association between parasite positivity and low birthweight (Brabin *et al*., 1990).

1.13 Congenital Malaria

Congenital malaria (CM) is the presence of malarial infection or parasitemia in the first week of the infant's life. It is thought to be acquired either through the placenta or due to contamination during delivery time. However, it has also been defined as the presence of asexual staged parasites in the infant's peripheral blood or cord blood in their first seven days of life (Uneke, 2007). Recent review studies have shown that congenital malaria cases are more frequent than what has been previously considered, (see Table 3) especially in sub-Saharan areas, where it is thought to be extremely rare (Uneke CJ, 2007).

1.13.1 Prevalence of Congenital Malaria

It is also reported that there is an increase in congenital cases in endemic areas. This is thought to be due to an interaction of factors, such as the parasites increased resistance to antimalarial treatment. On the other hand, this will also increase maternal parasitemia (Chabasse *et al*., 1988). In addition to some related factors, such as decreased antibody transfer rate from mothers to their infants, as mothers develop malaria chemoprophylaxis during their pregnancy (Ibeziako *et al*., 1980). Another reason for increased rate is very low number of congenital malaria cases reported in endemic areas which can be due to the difficulty in detecting its low density (Hulbert *et al*., 1992). The occurrence of congenital malaria (CM) in different studies conducted in malaria endemic areas of sub - Saharan Africa from periods (1990–2010) have supported the hypothesis that congenital malaria is more common than it is believed (see Table 3).

Recently, the current use of sensitive techniques, such as PCR, has accordingly increased the detection of congenital malaria (Adachi *et al*., 2000). In most previous research studies, the

severity and frequency of parasitemia in peripheral blood are lower than in cord blood (Mukhtar *et al*., 2006; Nyirjesy *et al.*, 1993).

Most foetal growth cases are restricted to malaria infection with specific chronic type and it is likely to occur through placental deficiency. Foetal growth is restricted by many factors, such as the release of cytokines, disturbance in placental biochemical transport, or the utero-placental blood flow destruction. Pregnancy-associated malaria possibly can cause reduced placental circulation and it can impair uterine spiral arteries remodelling (Redman and Sargent, 2005).

Moreover, placental blood flow decreases due to infected erythrocytes, monocytes, and fibrin deposition (Imamura *et al.*, 2002).

The mechanism and the timing by which the parasite is transmitted from the mother to the foetus is poorly understood. Still, more research is needed. The basic postulated hypothesis of transfer is either due to contamination during the delivery time or due to premature placental separation (Silva *et al*., 1982). A range of studies has sustained each of above hypothesis (Logie and McGregor, 1970; Malviya, 1984).

According to the hypothesis, malaria is most rare among infants, since the placenta operates as an effective barrier; it is thought that the process of malaria transmission occurs during the time of labour or at parturition, where minute quantities of maternal and foetal blood may mix (Hulbert, 1992).

Fever in pregnant women is more common. Moreover, the attacks of malaria are more familiar in infected women with low-levels of malarial immunity. In low transmission areas, the effect of congenital malaria is more frequent. Non-immune pregnant women traveling to endemic areas have significantly suffered an increased risk of malarial infection (Harvey *et al*., 1969).

Table 3: Prevalence of congenital malaria in different studies conducted in malaria endemic areas of sub - Saharan Africa from 1996–2010. SSA: Sub-Saharan Africa; CS: Cross-sectional; R: Retrospective. (Chiogzie and Nukee, 2011).

Type of the study	Study location	Congenital malaria prevalence (%)	Year of publication	Study /authors reference
CS	Lagos, Nigeria	13.6	2010	Lesi *et al*.
CS	Western Kenya	10.8	2009	Perrault *et al*.
CS	Muhez, Tanzania	19,1	2008	Mwangoka *et al.*
CS	Calabar, Nigeria	13	2008	Ekanem *et al*.
CS	Sagamu, Nigeria	10.9	2008	Sotimehni *et al*.
CS	Ibadan, Nigeria	5.1	2007	Flalade *et al*.
CS	Enugu, Nigeria	32.48	2006	Okafor *et al.*
R	Sagamu, Nigeria	17.4	2006	Abiodun et al.
CS	Lagos, Nigeria	15.3	2006	Mukhtar *et al*.
CS	IIe-Ife, Nigeria	54.2	2005	Obiajunwa *et al.*
CS	Southem, Cameroon	7.8	2005	Akum *et al*.
CS	Dar-es Salam, Tanzania	0.33	2000	Adachi *et al*
CS	Various site in SSA	23.0	1997	Fischer

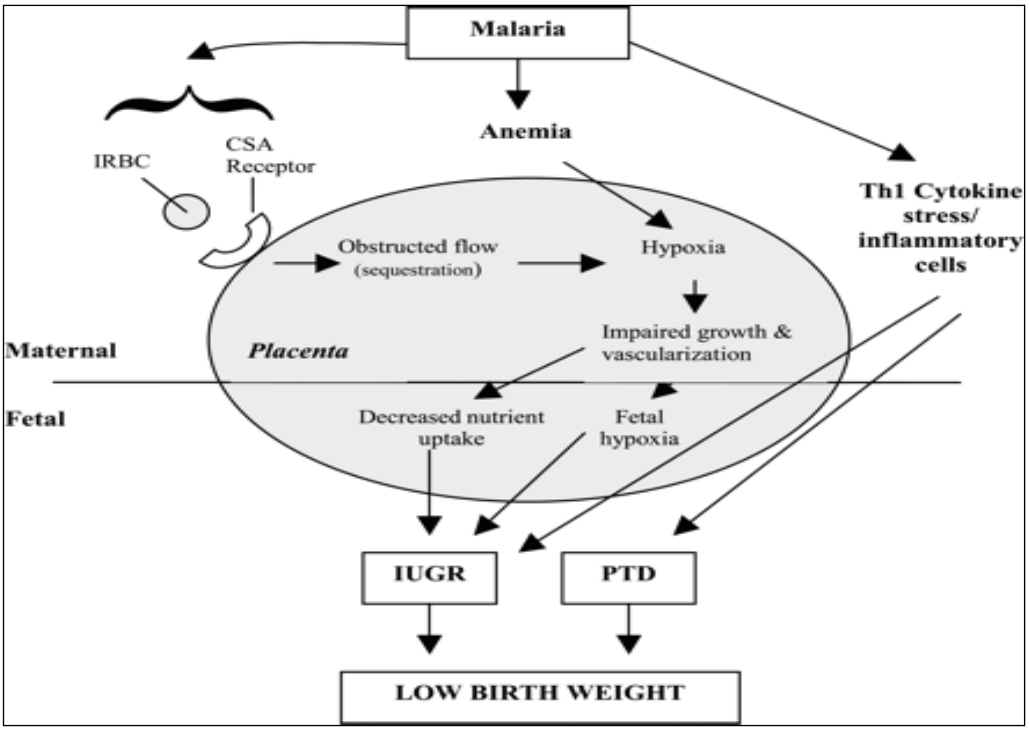


Figure 13: A potential pathogenic mechanism affecting placental functions and results in IUGR or PTD, IRBC: infected red blood cell. CSA: Chondroitin Sulfate. IUGR: intrauterine growth retardation. PTD: pre-term delivery. Source: Stephen *et al*., 2007

1.13.2 Hemozoin

Hemozoin is a malaria pigment, a haem polymer (Figure 15), formed by malaria parasites as the digestion of haemoglobin occurs (Levesque *et al*., 1999). In areas where malaria transmission is high, there are high hemozoin depositions, probably due to most recent and untreated infections (Sullivan *et al*., 2000). It is formed during the intraerythrocytic parasite growth cycle (Guzman, *et al*., 1994; Goldberg *et al*., 1990; Hanscheid *et al*., 2007). Because of its toxicity, the parasite transforms haem into an insoluble crystallized form, in which the group of haem is dimerized by iron carboxylate links and three-dimensional structures. It is stabilized by hydrogen bonds (Slater *et al*., 1991).

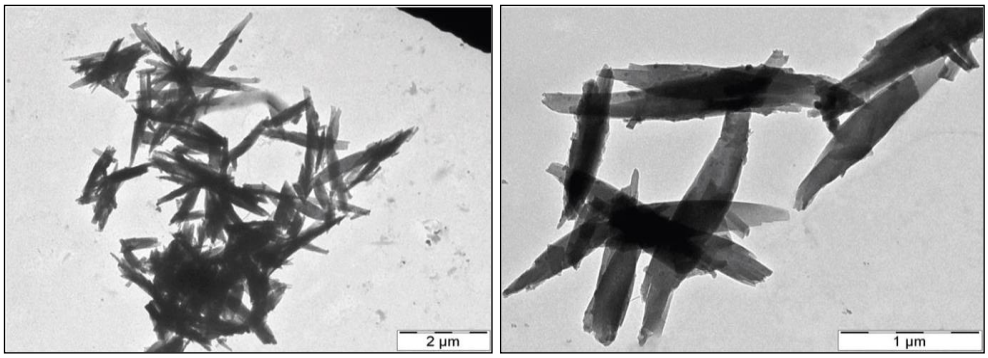


Figure 14: Electron microscopy showing the structure and morphology of typical hemozoin crystals dried from suspensions, Source: Hanscheid *et al*., 2007.

Hemozoin plays an essential role in malarial diagnosis; (see

Figure 14). Moreover, it is a basic drug target that can act as an immune modulator (Hanscheid *et al*., 2007). Hemozoin is of a crystal structure but it has a low-symmetry triclinic. The morphology of the hemozoin crystals varies, depending on the species of the parasite; however, they are typically elongated and rod-shaped, ranging in length from 300nm to 1µm (Hanscheid *et al*., 2007).

1.14 Tight Junctions

Tight junctions (TJs) are complexes of multi-proteins that basically exists at the luminal end of the intervillous space (Schneeberger *et al*., 2004). Tight junction arises in epithelial cells such as epithelial and endothelial cells, in all vertebrate species and tunicates (Lane, 1980). All the multicellular organism surfaces such as skin, gastro-intestinal tract, and respiratory tract are covered by different forms of epithelia. There are endothelial sheets in epithelial to work properly as a barrier; these intercellular spaces must be strictly sealed by these tight junctions to protect them. To connect the lateral membrane close to their external surfaces; tight junctions represents as an adhesion complex, which are responsible for sealing intracellular spaces as well as devising apical and basolateral compartments (Martin, 2013).

Tight junctions are intercellular junctions neighbouring to the apical end of the lateral membrane surface. They function to mediate adhesion of cells between endothelial or epithelial cells where they also play a role in solutes passage regulation such as ions, water, and other various macromolecules through paracellular spaces (Figure 15a) (Chiba *et al*,.2008). On other hand, epithelial cells consist of two domains of cell membranes, namely basolateral and apical membrane which is composed of protein lipids. Tight junctions have

another role in permeability of the paracellular epithelial as transport of ions and solutes. The tight junction transmits signals to prevent the intermixing of molecules in the apical membrane. This function of the tight junction is referred as the fence function (Chiba *et al*., 2008).

1.14.1 Tight Junctions in Relation to Placenta

Tight junctions (TJs) such as Claudin-4, Occludin, and the cytoplasmic scaffolding proteins ZO-1, -2, and -3 plays a basic role in paracellular pathway controlling (Challier *et al*., 2005). ZO-1 is peripheral to the tight junction and its adherens junctions. It is not clear why such junctions are needed in the placenta although a continuous syncytium that regulates the solute transfer between maternal and foetal tissues (Marzioni *et al*., 2001) However, immunoreactive TJ proteins, including Occludin and ZO-1, are present at CTB-CTB, CTB-STB boundaries, and in the syncytial line (Challier *et al*., 2005). By the first trimester Claudin-1 marks prominently the lateral edges between CTB and those between CTB and STB. Claudin-3 has an immunoreactivity character in trophoblasts, while Claudin-4 is found in the syncytial micro villous membrane and the villous CTB is found in first trimester and term tissue. Occludin also exists in the first trimester villous trophoblast (Marzioni *et al*., 2001).

Tight junction proteins (TJ) are a complex tool. It facilitates interactions of cell to cell contact in form of an epithelial sheet (Anderson, 2001; Anderson and Van Itallie,2006). Extracellular protein constituents of the TJ, including Occludin and intracellular proteins (like ZO-1, ZO-2, cingulin, and 7H6) (Anderson and Van Itallie ,2006), junctional adhesion molecule and Claudin with a molecular weight of approximately 24 kDa (Anderson, 2001, Furuse 2002).

Throughout the gestation period, the human placenta and foetal membranes provide a barrier to regulate the transfer process between the mother and the developing foetus (D'Alquen *et al*., 2005). Through the intercellular space the tight junction controls the paracellular movement of water, solutes, and immune cells by creating a boundary between the apical and basolateral sides of cellular barriers (Gruenheid and Finlay, 2003). Tight junctions (TJs) seals between adjacent sections, as cell–cell adhesion is crucial for the normal organization. The function of the organism is controlled, in response to various physiological reactions (Wong and Gumbiner, 1997). Claudin plays an essential role in the barrier function of TJs in epithelia. Epithelial-like Syncytiotrophoblast, are responsible for the barrier function of the placenta and they control the transportation of substances between the maternal fluid and the foetus.

1.14.2 Tight Junction Molecular Components

Tight junctions (TJ) have a (fibril-like) structure within the lipid bilayer, namely TJ strands (Chiba *et al*., 2008) (Figure 15a). Tight junctions are composed of transmembrane proteins and is associated with cytoplasmic proteins (Pinto and Kacher, 1982). Tight junctions are divided into 3 main groups which includes (Figure 2): integral, junctional adhesion molecules (JAMs) (Fanning *et al*., 1999). JAMs are a type of peripheral proteins containing (PDZ) domains that are responsible for facilitation of protein interactions, such as the Zona-occludens family, Par6, Par3, and afadin (Martin, 2013). A family of proteins play an essential role in regulating signalling like, rho-GTPases and cingulin (Saoudi *et al*., 2014).

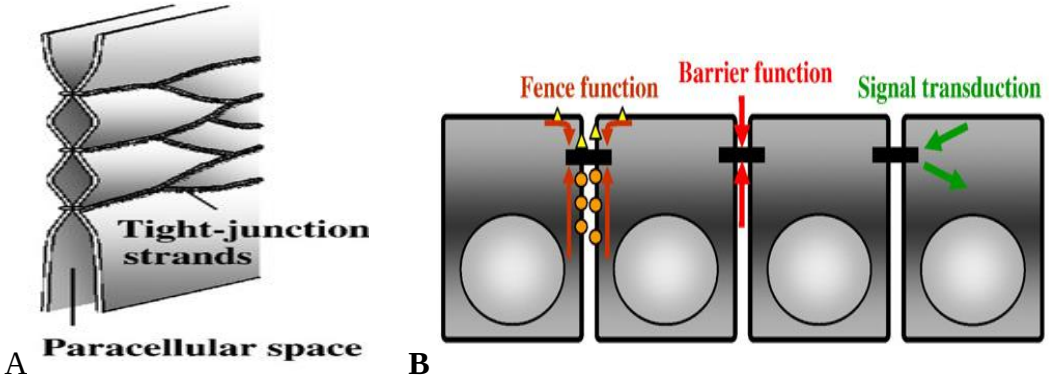


Figure 15: (A) Schematic structure of tight junction and position of paracellular space; (B) Functions of tight junctions, Source: Chiba *et al*., 2008.

1.14.3 Transmembrane Proteins of Tight Junctions

Tight junctions look like a series of fusion points between the outer leaflets of plasma membranes of adjacent T-cells (Gonzalez M *et al*.,2003). There are three groups of macromolecules where the integral membrane parts of tight junctions are considered as follows: Occludin, Claudins, and junctional adhesion molecule (JAM) (Anderson, 2001).

Occludin protein was the first integral protein known to be localized at TJ strands that is directly involved in the formation of TJ strands. (Furuse *et al*., 1998). However, further discovery of the Occludin gene showed the presence of integral proteins in TJ strands (Furuse *et al*., 1998).

Occludin is a 65-kD protein with four domains, two extracellular loops, an intracellular turn, and carboxyamino-terminal cytoplasmic domains. The first extracellular loop has

characteristics of amino acid content and contains very few charged amino acids. On the other hand, C-terminal domain is rich in serine, threonine, and tyrosine residues, where they are phosphorylated by various protein kinases. The C-terminal binds itself directly to ZO-1, which in turn is associated with the actin cytoskeleton (Furuse *et al*., 1994). JAMs consist of two extracellular domains, a single transmembrane region, and a C-terminal cytoplasmic domain. JAMs are considered as glycosylated transmembrane proteins that belong to the immunoglobulin (Ig) superfamily (Martìn-Padura, 1998).

1.14.4 Claudins

Claudins are a family of integral membrane proteins, responsible for cell adhesion, that are essential in cellular tight junction components that helps in carrying the polarity and paracellular permeability of epithelia, by forming the lining of the paracellular pores (Furuse *et al*., 1998). Claudins are basic components for the structure and function of TJs (Angelow *et al*., 2008). They maintain cell polarity as well as paracellular transport regulations (Morin *et al*., 2005)**.** Claudins support the ability of TJs through their homophilic and heterophilic interactions (Morin *et al*., 2005)**.** Through their cytosolic carboxy terminal interaction with PDZ containing proteins such as ZO-1. Claudins play a major role in a wide variety of cellular signalling processes, (Brehm *et al*., 2006).

1.14.5 Claudins Structure

In humans there are at least 24 Claudins known so far. They have a molecular mass ranging from 20–27 kDa (Singh *et al*., 2010). According to their degree of sequence similarity they have been categorised into two groups, namely, the classic Claudins (1–10, 14, 15, 17, 19) and non-classic Claudins (11–13, 16, 18, 20–24) (Krause *et al*., 2008). Claudins consist of four transmembrane domains like Occludin; however, they do not show any sequence similarity. There are two extracellular loops where the second one is significantly shorter than the first one and consist a short carboxyl intracellular tail (Singh *et al*., 2010).

Claudins span the lipid bilayer which participate in Claudin-Claudin interaction (Piontek *et al*., 2008) and a cytoplasmic COOH-terminal sequence that varies considerably in length between different isoforms (from 21–63 residues) Stabilization of the tight junction and permeability characteristics are controlled via interaction of Claudins with cytoplasmic tight junction proteins (Van. Itallie and Anderson, 2006). Most Claudins have the conserved motif

GLWxxC (Gly-Leu-Trp-x-x-Cys) (8–10 aa) in the first extracellular loop, and a PDZ domain binding motif at the carboxy-terminal (

Figure 16), in which allows direct interaction with tight junction cytoplasmic proteins, such as ZO-1, ZO-2, and ZO-3 (Itoh *et al*., 1999).

The first extracellular loop contains charged amino acids (

Figure 16), of which some are conserved in different Claudin isoforms. The C-terminal cytoplasmic tail of Claudins is required for their constancy and targeting (Ruffer and Jerke, 2004).

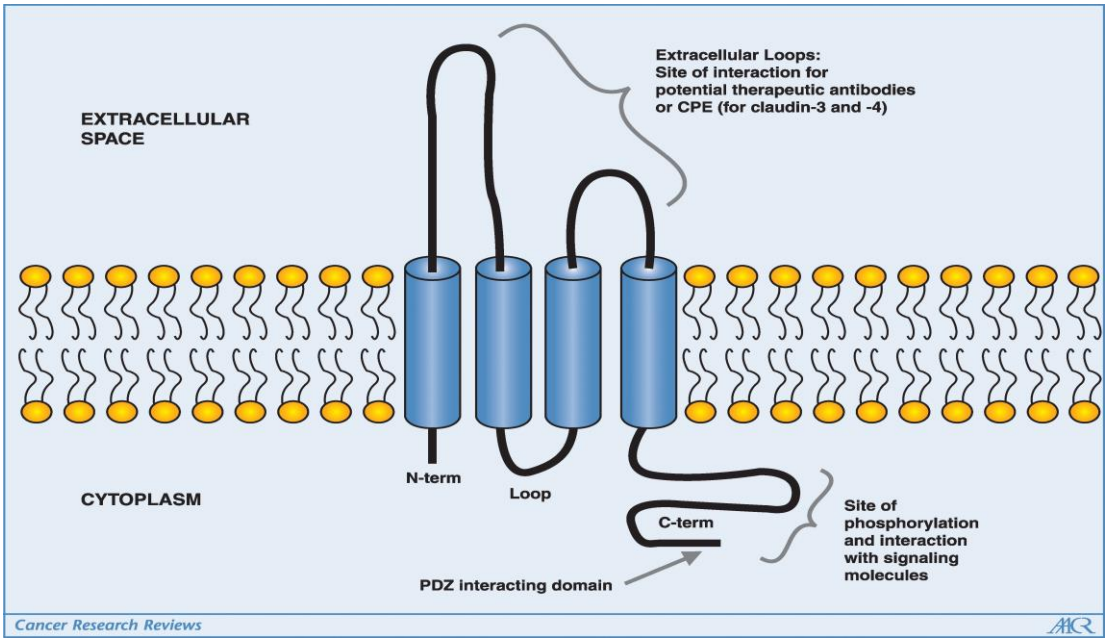


Figure 16: Claudin structure model, Source: Morin, 2005.

1.14.6 Claudins Functional Properties

Claudins functions to generate TJ strands and the structural and core of TJs within the plasma membrane by homophilic and heterophilic binding of adjacent T-cells. Claudins are essential for tight junction formation (Furuse, 1999). Majorly, physiologically barrier function via tight junctions ‘according to size selectivity and paracellular charge (Chiba *et al*., 2008).

Claudin expressions can contribute to conditions malignancies as it regulates tight junction permeability and epithelial cell polarity (Ovalle and Nahirney, 2013).

The Paracellular Sealing function is a property of Claudins in the TJ (Furuse *et al*., 2002). Claudin-1, -5, -11, and -14 have tightness role. The different tightness possessions of a given tissue and a given Claudin, however, seem to be largely dependent on the combination of the Claudins that are expressed in the way that they copolymerise (Furuse *et al*., 2006). Numerous studies have demonstrated that Claudins, particularly Claudin-4, play roles in tightness process (Itallie *et al*., 2001).

1.14.7 Interaction of Claudin to Claudin

Claudins may self-associate themselves in two orthogonal locations. Possible interactions for Claudins via their ECLs occur as a result of extracellular loops (ECLs) association between the plasma membranes of opposing cells (*trans*-interaction) as outlined in (Figure 17). Claudin-5 molecules can interact along with the plasma membrane (*cis*-interaction) (Blasig *et al*., 2006).

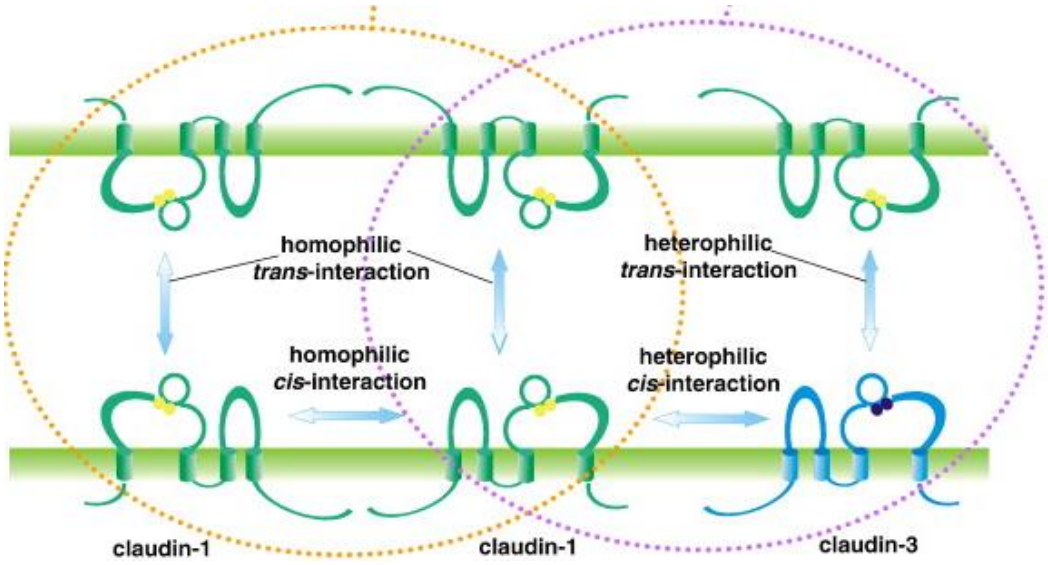


Figure 17: The homophilic and heterophilic cis- and trans-interactions based on a nomenclature, Source: Furuse *et al*., 1999

1.14.8 Zonula occludens (ZO-1*)*

Endothelial and epithelial cells attach to each other by different types of Occludin junctions (Bauer *et al*., 2010).

ZO (Zonula occludens) proteins are scaffolding proteins, providing the essential structure assembly of multiprotein complexes at the cytoplasmic surface of intercellular junctions (Bauer *et al*., 2010). Zonula occludens ZO-1 is one of the membrane-associated guanylate kinase homologs (MAGUKs). Zonula occludens (ZO) contain multiple domains (Alan *et al*., 1998).

The Zonula occludens like other proteins such as ZO-2 and ZO-3 are a family of tight junction-associated proteins anchoring the TJ strand proteins to the actin-based cytoskeleton that play a role as cross-linkers. This is encoded by the TJP1 gene. ZO-1 is implicated by the binding of both cytoplasmic and transmembrane proteins. Zonula occludens (ZO) proteins are peripheral proteins, localizing junctional sites. They are also recruiting various types of proteins to the cytoplasmic surface of the junction (Anderson *et al*., 1998; Stevenson *et al*., 1986).

The zonula occludens (ZO)family directly bind to the barrier-forming Claudin proteins of cytosolic proteins, including ZO-1, -2, and -3 which are multi-domains; that interact with other signalling and structural proteins implicated in the TJ structure (Fanning and Anderson, 2009).

1.14.9 Structural and Functional Properties of Zonula Occludens

The molecular structure and functions of ZO proteins carry three PDZ domains, one SH3, a GUK, and a proline-rich region domain (Gonzalez M *et al*., 2003). The variable domains which are termed (unique) 1 to U6 are basically located between the core domains of the ZO proteins (Fanning *et al*., 2007).

The first protein recognized is ZO-1, with a molecular mass of 220 kDa (Stevenson *et al*., 1986). ZO proteins interact directly with Occludin, Claudins, JAMs (Junctional adhesion molecule), tricellulin, and CAR (coxsackievirus and adenovirus receptor) (Cohen *et al*., 2012;

Ebnet *et al.*, 2000)

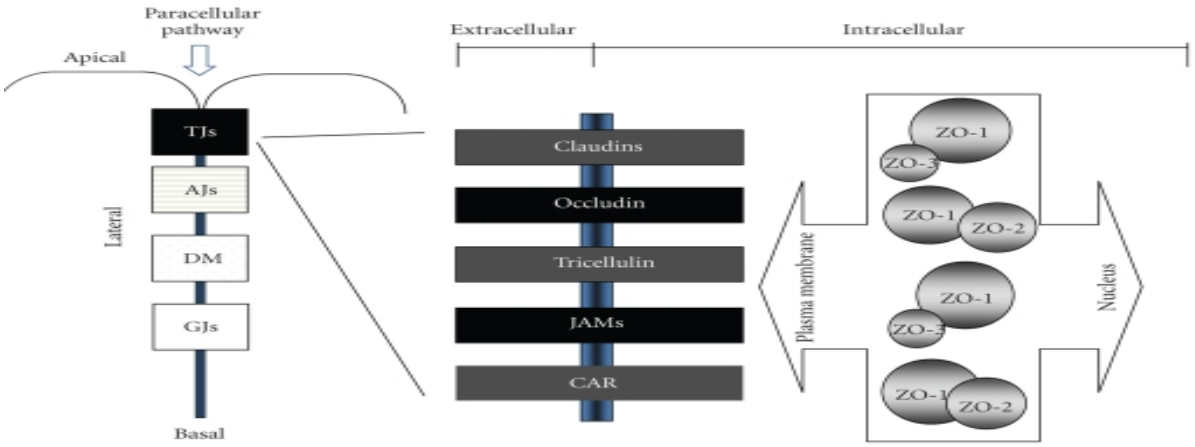


Figure 18). ZO proteins associate with a series of junctional proteins to create a complex intracellular network (Figure 19). Actin- and myosin-binding proteins, signalling molecules and transcriptional regulators of the peripheral junctional proteins. On other hand, all ZO interacts directly with actin filaments proteins through their COOH terminal regions or via a binding domain located in the N-terminal half (ZO-3) (Fanning *et al.*, 1998).

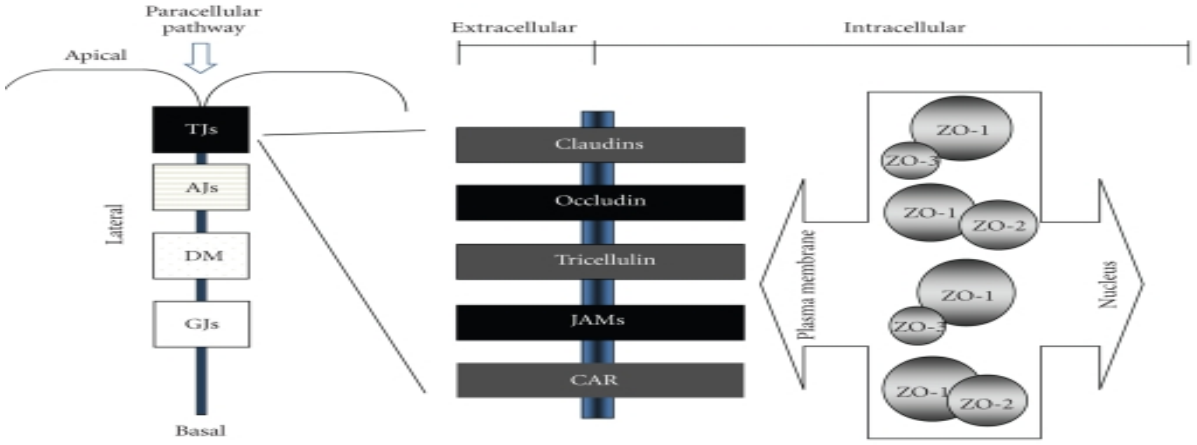


Figure 18: Zonula occludens (ZO) proteins' localization at tight junctions (TJs). Interaction of transmembrane components of TJs with at least one ZO protein. TJs: tight junctions. AJs: adherens junctions. DM: desmosomes. GJs: gap junctions. CAR: coxsackievirus and adenovirus receptor, Source: Bauer *et al.*, The Dual Role of Zonula Occludens (ZO) Proteins, 2010.

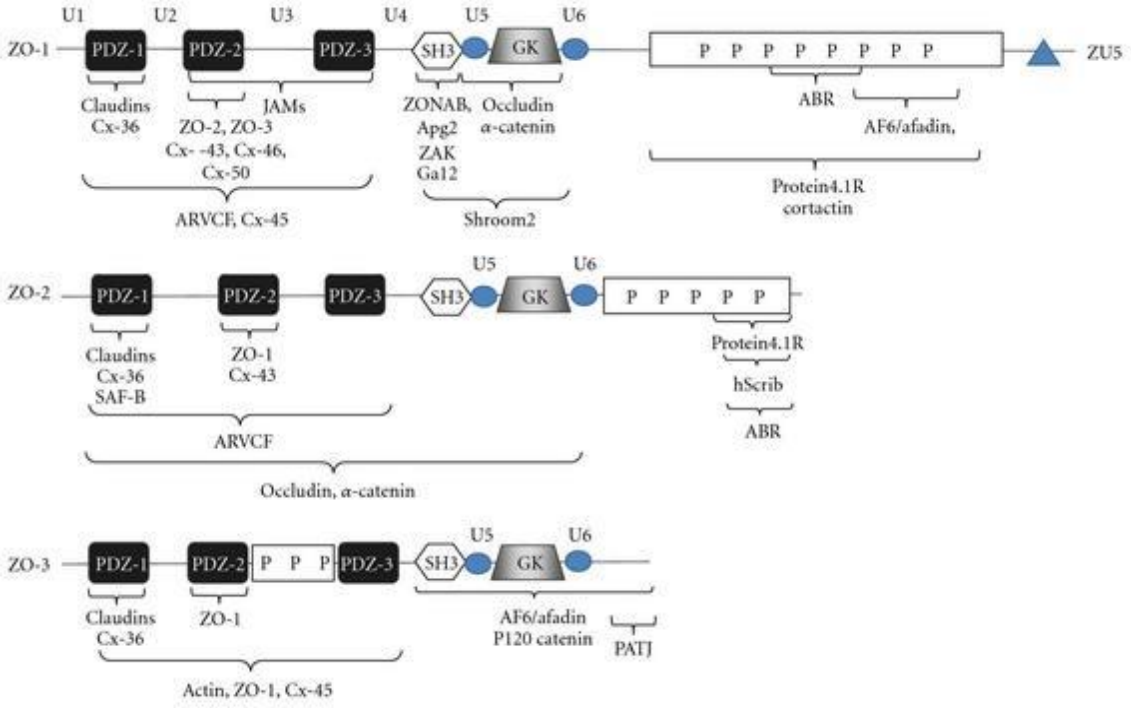


Figure 19: Interaction of ZO -1 protein with transmembrane proteins and with peripheral cytoplasmic proteins at the junctional site. Tight junctions P-P-P: Proline-rich region. PDZ: Psd95/discs large/zonula occludens-1 domain. SAF-B: Scaffold attachment factor-B. SH3: Src homology3 domain. U1-U6: Unique variable domains. ZAK: ZO-1 associated kinase. ZONAB: ZO-1 associated nucleic acid binding protein. ZU5: Domain present in ZO-1 and Unc5-like netrin receptors. Source: Bauer *et al*., *the Dual Role of Zonula Occludens (ZO) Proteins,* 2010.

Section-2

Study Rationale

Malaria in pregnancy (MIP) is a serious health problem among pregnant women particularly in sub-Saharan countries that desires effective management strategies to attain the preferred therapeutic outcomes. The pathophysiology of malaria in pregnancy is seriously due to the immunity alteration as intense breakdown of acquired immunity occurs in pregnancy.

In Sudan, malaria is one of the deadliest endemic diseases and the increased susceptibility of pregnant women to malaria is a long-standing public health problem. Clinical presentation and severity of malaria in pregnancy differ in areas of high transmission and low transmission due to differences in the level of immunity.

In Sudan, detailed data on the pattern and risk factors for placental malaria are scarce. The current study was conducted to assess the prevalence and risk factors of placental malaria and its effect on pregnancy outcomes in Sudanese women from Blue Nile State, Sudan. Furthermore, many previous research studies were carried out in different areas in Sudan such as Gadarif, Kordofan, North Sudan, Medeni Gezira, South Sudan, and Kssala. Accordingly, the current study area is different from other studies and the environmental factors were different as the endemicity is meso-endemic and the Blue Nile area is a forest. Additionally, this is the first study to assess how the parasite crosses the placental barrier and to find the relationship between tight junction proteins and the parasite transmission. Finally, the antenatal care is very poor in the current study area as the Primary health care in Blue Nile State is the worst in central Sudan. Blue Nile state lies in a tropical climate zone where malarial disease is one of the major problems. Women from rural areas in the study area usually deliver at home and tend to be less aware of the need to avoid infection because most of the education programmes are concentrated in the urban areas and there is no use of prophylaxis to protect them from severe malaria infection. Recommended prevention and control strategies in the study area are the administration of intermittent preventive treatment (IPT), distribution of insecticide-treated bed nets (ITNs) and appropriate malaria case management needed to avoid high transmission of malaria among pregnant women in the study area.

Research questions, Aims and Objectives

1. How frequent are parasitemia in mothers, placenta, cord blood, infants?
2. What are the risk factors associated with maternal malaria?
3. What are the risk factors associated with placental malaria?
4. What are the risk factors associated with positive malaria parasite in cord blood?
5. What are the risk factors associated with congenital malaria?
6. What are the risk factors associated with LBW?
7. What are the risk factors associated with maternal anaemia?

Section-3

Aims and Objectives

General Objectives

To investigate the prevalence, risk factors and pregnancy outcomes associated with malaria in Blue Nile area as well as possible involvement of placental junctional proteins in congenital malaria.

The Specific Objectives of the Study

1. To investigate the prevalence the maternal malaria infection using microscopy.
   Outcome measure: Frequency or percentage of maternal malaria infection

2. To investigate the prevalence of placental malaria infection among the women using microscopy.
   Outcome measure: Frequency or percentage of placental malaria

3. To investigate the prevalence of the umbilical cord blood malaria infection using microscopy.
   Outcome measure: Frequency or percentage of umbilical cord blood malaria infection

4. To investigate the prevalence of the neonatal malaria infection using microscopy.
   Outcome measure: Frequency or percentage of neonatal malaria infection.

5. To investigate the prevalence the maternal malaria infection (Peripheral blood) using a molecular method (PCR).
   Outcome measure: Frequency or percentage of maternal malaria infection using PCR.

6. To investigate the prevalence placental malaria infection among the women using PCR.
   Outcome measure: Frequency or percentage of placental malaria infection using PCR

7. To investigate the prevalence of the umbilical cord blood malaria infection using PCR.
   Outcome measure: Frequency or percentage of umbilical cord blood malaria infection using PCR

8. To investigate the prevalence neonatal malaria using PCR.
   Outcome measure: Frequency or percentage of neonatal malaria infection using PCR

9. To measure the sensitivity of the PCR method in detecting the malaria parasite provided that detecting the malaria parasite on blood smears by microscopy is used as a standard method.

10. To investigate the association between maternal malaria infection and neonate low birth weight (LBW), maternal anaemia, maternal age, parity, placental malaria infection, neonatal malaria infection, residence and level of education.

11. To investigate the association between placental malaria infection and neonate low birth weight (LBW), maternal anaemia, maternal age, parity, neonatal malaria infection, residence and level of education.

12. To investigate the association between neonatal malaria infection and neonate low birth weight (LBW), maternal anaemia, maternal age, parity, placental malaria infection, umbilical cord blood malaria infection, residence and level of education.

CHAPTER 2 – Section-4

MATERIALS & METHODS

2. Materials & Methods

2.1. Study Area

The study is conducted in the Blue Nile state of Sudan. The Blue Nile state is home to the Roseris Dam, the main source of electricity and hydroelectric power station in Sudan, until Merowe Dam was finished in 2010.

The study is conducted in the three main hospitals: 1) Damazin Hospital 2) El Roseris Hospital, and 3) the Surgical Complex. The Surgical Complex is the largest health facility in the state, under the umbrella of the health insurance including an obstetrics and gynaecology

department where women from different localities giving child-birth. Women are randomly selected from the three hospitals, irrespective of age, educational background, socio-economic, cultural, and religious condition.

The Blue Nile state lies in the tropical climate zone between latitude 9030° and 12030° and longitude 3305° and 3503° East. It is characterized by high temperatures and heavy rainfalls. The average annual rainfall is around 700 mm, with the southern part of the state being the wettest. The State has an area of 45,844 km² and an estimated population of 832,000, 75% of which resides in the rural areas and 25% in the four urban centres. Women represent 47% of the population in the state, with a maternal mortality rate of 258/100,000. The specific environmental, anthropological, administrative, and geographic characteristics of the Blue Nile state, which shares an international border with Ethiopia and South Sudan, impact uniquely the epidemiology and control of malaria (BNS Emergency Profile, 2014). Blue Nile state is a forest that lies in a tropical climate zone. Moreover, malarial endemicity in Sudan varies from hypo-endemic region in the north to meso-endemic central regions; with most of the population living in epidemic prone areas. Additionally, malaria is the main cause of common morbidity and mortality in the state.

The malarial transmission is seasonal and starts usually by June in the southern part of the state whereas July/August in rest of the country and lasts till November/December The most prevalent Plasmodium species in the area is *P. falciparum* which is responsible for most of the malarial infection in the areas. Primary health care in Blue Nile State is the worst in Sudan and there is no antenatal care programme to monitor pregnant women during their pregnancy. In addition, no preventive measures such as bed nets or intermittent malaria prophylaxis IMP were used in the area.

2.2. Study Design, Study Population and Sample Size

2.2.1. Study design

Study design: A prospective cross-sectional hospital-based study.

2.2.2. Study population

Study population: Pregnant women reporting for delivery services at the main maternity wards at Damazin Hospital, Roseris Hospital, and the Sheriff Centre during June 2012- July 2014 were recruited.

Inclusion criteria: Full term pregnancy and consenting parturient women with singleton pregnancies and normal vaginal were recruited. Exclusion Criteria: Pregnancy with twins and mothers with serious delivery complications including hypertension, diabetes mellitus, antepartum haemorrhage or post-partum bleeding, eclampsia and other complicated conditions were excluded (see Figure. 20 flow chart).

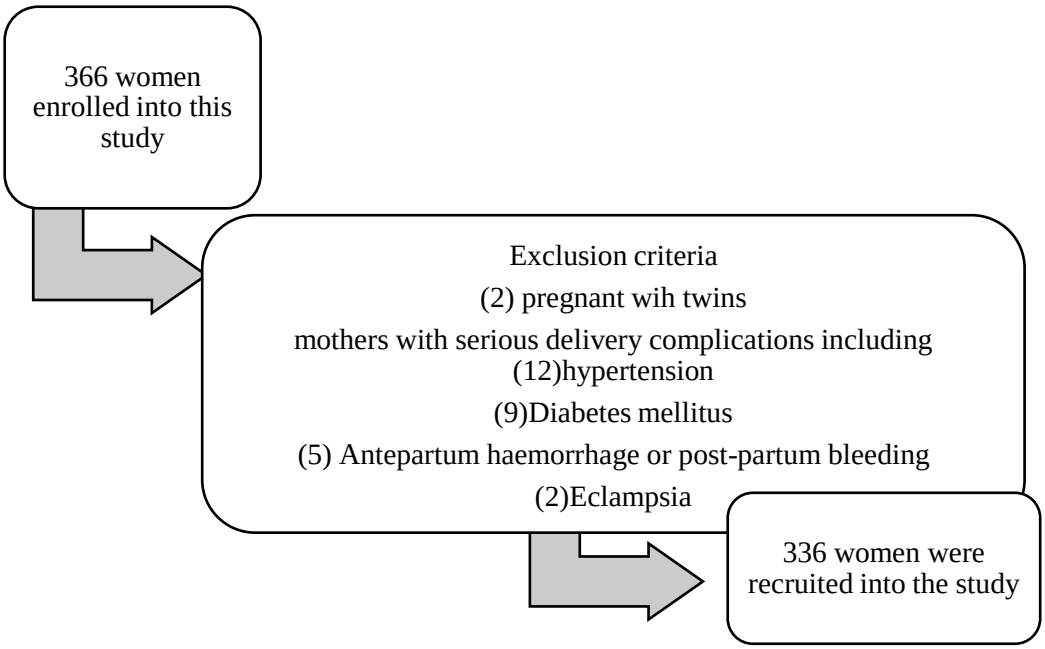


Figure 20: Flowchart of pregnant women enrolled for the current study.

Systematic random sampling technique is used to obtain the data from the pregnant women. Every hour, a pregnant woman who is registered at the labour ward for delivery is selected for the sample until the desired number is obtained.

2.2.3 Sample Size (Calculation and estimation)

The study was conducted at the same time in three main maternity units of the hospitals***:*** Damazin Hospital, Roseries Hospital and the Surgical Complex. The sample size of 336 pregnant women was obtained.

The study sites conducted around 1000 deliveries during the study period with the highest number (700) of deliveries were at the Surgical Complex and the least (300) were at El Roseries Hospital. Women were recruited after explaining to them the purpose and nature of the study and signing in consent forms. Women were included irrespective of age, educational background, socio-economic burden, cultural or religious conditions. Women were approached to participate consented to join the study and to provide the suggested 3 samples (maternal and new born peripheral samples, and placental samples).

The sample size was estimated using the formula for sample size calculation described by Swinscow as follows;

$n=Z^2 \times p\,(1-p)\,e^2$

$Z = 1.96$

p = prevalence of malaria in Blue Nile = 29%

e = error rate = 0.05

$n=1.96\ 2 \times 0.29\ (1-0.29)\ /0.05)^2$

$=316.4\approx317$

Thus, we recruited 336 participants to adjust for possible loss of samples.

2.3. Blood Sample Collection

Before delivery: blood samples were collected by finger prick technique from the mother to perform the following: 1) To prepare thin and thick blood smears on glass slides 2) To collect 50 µl on a filter paper (No. 3 Whatman, USA) 3. To measure Hb using HemoCue equipment (HemoCue Sweden) after delivers: blood samples were collected by finger prick from infant, umbilical cord blood and placenta (mother's side) to perform the following: 1) to prepare thin and thick blood smears on glass slides 2) To collect 50 µl on a filter paper (No. 3 Whatman, USA). 3) To measure Hb using a HemoCue equipment (HemoCue Sweden).

The blood smear slides are allowed to dry and were kept in slide box. Blood smears were stained with Giemsa stain within 48 hours of the collection. Each blood smear was examined by two independent examiners, of which one was an experienced laboratory technician and

the other is the author, at a magnification of X100 under oil immersion. The filter papers in which blood was collected (from mother, placenta, cord blood and infant) were sealed individually in plastic bags. They represent the samples for PCR analysis to detect and identify the presence of *P. falciparum* malaria parasite. Full thickness of placental tissues measuring between 2–3 cm was taken from each woman at birth, fixed in neutral buffer formalin, processed, and embedded in paraffin wax for analysis.

Blood samples were collected on filter papers that were obtained from pregnant women and their babies from the Damazin, the Roseris, and the Sharief centres in Sudan. An amount of 50 µl of blood is collected on No. 3 Whatman filter papers from the participants and their infants, dried, and sealed individually in plastic bags for PCR analysis to identify and detect the presence of *P. falciparum* malarial parasite.

A finger prick is made to prepare the thick and thin blood films to test for malaria using microscopy and haemoglobin (Hb) measurement. Immediately following the delivery, placental blood smears are obtained from the maternal side of the placenta, by making a punch at the placenta using a disposable lancet. Smears of the two blood specimens (peripheral and placental blood) are prepared on coded glass slides.

Identification and detection of the malarial parasite *P. falciparum* in blood samples on filtered paper are obtained from pregnant women and their babies. An amount of 50 µl of blood is collected on No.3 Whatman filter papers from the participants and their infants, dried, and sealed individually in plastic bags for PCR analysis.

The slides can dry and are kept in slide-boxes. Blood films from mothers, placenta, cords, and babies are stained with Giemsa, within 48 hours of the collection. Each film is examined by two independent examiners, of which one is an experienced laboratory technician and the other is the author, at a magnification of X100 under oil immersion.

2.4. Socio-Demographic and Data collection

The data is collected through face-to-face interview with the pregnant women at their delivery wards using a structured questionnaire to obtain information about their socio-demographic background, name, age, residence, education level, gravidity, history of malarial infection, weight of child (kg), and Hb level (g/l) of mothers.

A structured questionnaire is administered to the selected sample of pregnant women to gain information about their socio-demographic background, name, age, residence, education level, gravidity, history of malarial infection, weight of child (kg), and Hb level (g/l) of mothers. The data is collected through face-to-face interview with the pregnant women, at their delivery wards (Appendix 14, p. 174).

2.5. Staining method for blood smear and placental tissue

2.5.1. Microscopic detection of malaria parasite in Giemsa – Stained blood smear

This method considered as the gold standard procedure for malaria parasite detection. A appropriately stained blood film is critical for malaria diagnosis, especially to identify malaria species. Use of Giemsa stain is the recommended and most reliable procedure for staining thick and thin blood films. Giemsa solution is composed of eosin and methylene blue. The eosin component stains the parasite nucleus red, while the methylene blue component stains the cytoplasm blue. The thin film is fixed with methanol. De-haemoglobinization of the thick film and staining take place at the same time.

Many researchers have developed modifications to Giemsa's method to achieve better results. The Giemsa stain comprises 2 procedures, one for blood or microorganisms in smears and another for tissue sections. Giemsa stain is considered the most dependable stain for blood parasites, particularly on thick films; it is a useful tool for histopathological diagnosis of malaria. The use of Giemsa staining on thick and thin blood films is preferred diagnostic tool for malarial diagnosis; some of this work was performed by me during the summer period in Sudan, whilst the rest was done by an expert senior laboratory technician in the Khartoum Hygiene Tropical Medicine Institute.

The standard microscopy of blood and impression smears stained with Giemsa stain was conducted within 24 hours of the sample collection for the diagnosis of malaria. Thick blood smears are read for the detection of parasites. For quality assurance, all smears were read by two expert microscopists.

2.5.2 Microscopic detection of malaria parasite in the placental blood smear using Giemsa Stain

Thick blood smears from the placental biopsy sites were stained with Giemsa and were examined at x100 magnification for presence and species of parasite. A minimum of 200 fields were examined each time.

2.5.3. Microscopic examination of Haematoxylin and Eosin stained] placental tissue

The standard histology stain namely haematoxylin and eosin stain (H&E) is the most widely used stain in histology and histopathology sections. It allows the visualisation of wide range of normal and abnormal cells and tissue components. It is a comparatively simple stain and can be performed on frozen tissue sections as well as on paraffin embedded tissue and was used to assess Haematoxylin and eosin–stained sections were assessed for placental pathologic changes, such as fibrinoid as well as parasite as well as placental malaria categories.

2.5.4. Microscopic examination using Prussian Blue Stain for placental tissue

Prussian blue stains are used for placental tissues sections to assess and differentiate malarial pigment from hemosiderin, a normal by-product of haemoglobin degradation. Although hemozoin and hemosiderin stain brown with the other two stains, hemozoin stains brown and hemosiderin stain bright turquoise blue with Prussian blue stain enabling the differentiation.

2.5.5. Microscopic examination of Double Stained - (Giemsa and Prussian Blue) placental tissue

The placental biopsies were processed and embedded in paraffin wax using double stain Paraffin sections of 7 mm thickness. Under polarized light to assess the deposition of malaria parasite and pigment presence for placental malaria categorization in which classifies malaria into its respective categories. The placental malaria histology results were classified into no infection, active infection, active chronic infection, and chronic infection.

2.6. Molecular detection of malaria parasite on blood samples spotted on filter paper

2.6.1. Background

The Polymerase Chain Reaction (PCR) is an enzymatic method of synthesizing a targeted region of DNA *in vitro*. Taq polymerase, a thermostable DNA polymerase, is isolated from the thermophilic bacterium, *Thermus aquaticus*, which is originally isolated from the hot springs in Yellowstone National Park. In PCR, the products of the previous synthesis cycles

serve as template for the next cycle and the synthesis reaction is repeated numerous times, usually X30 or X40. This result is an exponential amplification of the targeted region of the DNA. In brief, (Nest one) PCR was performed, by the procedure described previously by Snounou (1993 and 2002), to obtain an initial amplification reaction. First, the DNA was purified from the sample to be analysed and was used as a template for the amplification of a large portion of the plasmodial ssrRNA genes. The oligonucleotides primer pairs that are used for this reaction are genus-specific and they will amplify the target from the rest of the specie's DNA.

To determine which parasite species is present in the sample, another two separate amplification reactions (Nest 2) was performed for the detection of the exact target species out of the four species. The template for these reactions is a small proportion of the amplification product obtained following the Nest 1 reaction. The primer sequence, specificity, and the expected size of the amplification products were obtained using all the oligonucleotides primer pairs as given in Table 6. Thus, to establish the presence or absence of the four human malarial species sequences in a sample, five separate PCR amplifications were required. However, only one aliquot from the genomic DNA template prepared from the sample was required. In the following sections, a detailed description is given for the procedures required for the collection of samples, the preparation of DNA template, setting up the nested PCR, and the analysis of the amplification results.

2.6.2. DNA extraction using Chelex

A (1 ml) 0.5% Saponin solution is used to remove haemoglobin, a heating step to release DNA from the cells, and a (150µl) 6% Chelex suspension is used to protect the Plasmodium DNA during the heating step, which is extracted according to the Chelex method protocol. In summary, three drops of blood were collected on a filter paper, from infant and the mother; other blood drops from each placental type were also collected. Blood samples are air-dried and stored at an ambient temperature. The filter papers are stored in individual sterile bags. Approximately 25 µl (equivalent to the 1/3 of the spot) is punched from the dried-up blood spots.

Sections of the blood spots are punched (at least 2 mm diameter) using a metal hole punch, treated with 70% ethanol, and flamed in between each spot. The sections are put into separate wells in a 2.0 ml deep and round well plates (each sample in a separate well). Next, 1 ml of

freshly prepared 0.5% Saponin (Qiagene) in 1X PBS was added, making sure that the filter paper was completely immersed.

The deep well plates were incubated at 37˚C overnight. On the second day, deep well plates were centrifuged at 4000 rpm for 2 minutes and Saponin and debris were removed by using a pipette with a new tip for each sample. Then, 1 ml of 1X fresh PBS was added to each well. Mixtures were centrifuged at 4000 rpm for 2 minutes again and Saponin and debris were removed. The steps were repeated until the haemoglobin was absent from the samples.

Then, 150µl of 6% Chelex 100 suspension (Qiagene) in nuclease-free water was added to each well. For better results, it is recommended to use a trimmed pipette tip and continuously stir Chelex 100 suspension using a magnetic stirrer. The 96-well plate was covered with Pierce--Lite foil and sealed and heated for 2 X 15 seconds (rotating the plate in between). The 96-well plate was then incubated in the water bath at 100°C for 30 minutes ensuring that the samples were fully covered in water. Next, the samples were centrifuged at 4000 rpm for 2 minutes to spin down the Chelex 100 and the remaining filter paper. The supernatant DNA (approximately 120µl of the top layer) was aspirated and aliquoted into a sterile pre-labelled plate. The supernatant was then spun down at 4000 rpm for 10 mins to form a pellet of Chelex 100. Finally, the extracted DNA was stored at -20^{o}C.

2.6.3. DNA Quantification by spectrophotometer

In this study, two methods were used to quantify extracted DNA samples. First, UV Visible spectrophotometry (UV-spec) is used to measure sample absorbance at wavelength between 260 nm and 280 nm, for purity check.

The amount of isolated DNA is calculated using spectrophotometer technique. A 4µl of isolated DNA was mixed with 496 µl of distilled water and absorbance was measured using wavelengths, 260 nm and 280 nm. The ratio of optical density A_{260} to A_{280} is expected to be between 1.7 and 2 for pure DNA. Much lower A_{260} to A_{280} ratios are used to indicate protein contamination, whereas too high A_{260} to A_{280} ratio (>2.0) may indicate contamination with organic solvents (e.g. phenol). Also, degraded DNA can cause an increased absorbance at 260 nm (Tony, 2013; Davies, 2001). The amount of DNA is calculated using a standard equation:

$C\ [\mu g/ml] = A_{260} \times 50 \times F$

Where:

C is the DNA concentration [µg/ml];

A_{260} is the absorbance, OD 260nm;

50[µg/ml] - OD of 1 corresponds to a concentration of 50µg/ml for double-stranded DNA;

F is dilution factor, for example DF=125, if 4µl of DNA is mixed with 496µl of distilled water for analysis.

2.6.4. PCR Procedure

- DNA template preparation from samples and their storage;
- Storage, preparation, and aliquoting of PCR reagents;
- Addition of DNA template to perform the amplification in reaction tubes;
- Addition of template from the Nest l to the Nest 2 reactions; and
- Analysis and storage of PCR product.

The reagents (see Table 4) were added to the master mix tube in the following order: water, PCR buffer, oligonucleotides primers, and the final $MgCl_2$ concentration (2 mM). The variations in the $MgCl_2$ (1-3 mM) have not been found to affect the efficiency of the amplification.

The PCR programme parameters (see

Table 5) for the PCR amplification are as follows.

Each oligonucleotides primer was first used at a final concentration of 250 nm. and, although lower amounts of oligonucleotides primers (125 nm.) have been successfully employed, decreased efficiency of amplification might be the result.

The dNTPs aliquot from the freezer is thawed and then the appropriate amount is added to the master mix tube and immediately stored back in the freezer. The final concentration of the dNTPs is 200µM).

Table 4: Amounts of reagents for Nest 1, PCR

Reagents	Volume per sample	Final concentration
Nuclease-free water	13.1 µl	
10X NH_4 Buffer	2.0 µl	1X
50mM $MgCl_2$	0.8 µl	2mM
2mM dNTPs	2.0 µl	200µM
5 mM Primer mix (Pf)	1.0 µl	250µM
5U BioTaq	0.1 µl	0.5U
Nest 1 product	1.0 µl	
Total reaction Volume:	20.0 µl	

Table 5: Cycling conditions for Nest PCR

Step 1: 95°C for 5 min (Initial denaturation)
Step 2: 58°C for 2 min (Annealing)
Step 3: 72°C for 2 mm (Extension)
Step 4: 94°C for 1 min (Denaturation)
Step 5: Repeat steps 2-4 a total of 25 times
Step 6: 58°C for 2 min (Final annealing)
Step 7: 72°C for 5 min (Final extension)
Step 8: the reaction is completed by reducing the temperature to 20°C
Total number of cycles: 25

Table 6: Amounts of reagents for Neste 2, PCR

Reagents	Volume per sample	Final concentration
Nuclease-free water	13.1 µl	
10X NH_4 Buffer	2.0 µl	1X
50mM $MgCl_2$	0.8 µl	2mM
2mM dNTPs	2.0 µl	200µM
5 mM Primer mix (Pf)	1.0 µl	250µM
5U BioTaq	0.1 µl	0.5U
Nest 1 product	1.0 µl	
Total reaction Volume:	20.0 µl	

Taq polymerase was removed them from the freezer, and appropriate amount was added to the master mix tube and stored immediately back in the freezer. The contents of the master mix tube was thoroughly mixed by vortexing.

2.6.5. Plasmodium Species Amplification

To optimise conditions for P. falciparum amplification for Nested 1 PCR the gradient temperature annealing PCR was performed in a single thermal cycler - TC-3000G (Techne).

The PCR reaction mix contain the following: 100ng DNA template, primers for Nested 1 (rplus5+rplus6) 250µM, PCR master mix (Promega) that was pre-mixed, ready-to-use solution containing approximately Bio *Taq* DNA Polymerase (0.5U), dNTPs (200µM), $MgCl_2$ (2mM), and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR Also, ddH_2O was used to make up the final volume of 20µl. For the primary standard, PCR reaction (nested PCR1), 5 µl of genomic DNA were used in a 20µl total reaction with primers rPLU5 and rPLU6 (1.0 µl). The cycling conditions that the PCR mixture was subjected to were 95°C for 5 mins, 58°C for 2 mins, 72°C for 2 mins, 94°C for 1 min forX25 cycles and 58°C for 2 mins and 72°C for 5 mins.

2.6.6. P. falciparum Gene Amplification

For *P. falciparum* gene, the amplification in Nested 2 was applied accordingly as Nested PCR 2 is performed with 5µl of the primary PCR product and species-specific primers for *P. falciparum* under the following conditions: the initial denaturation was at 95°C for 5 min, the annealing step as at 58°C for 2 min, the elongation or extension step was at 72°C for 2 m and 94°C for 1 min (Denaturation). The steps were repeated for 25 cycles and then the final annealing at 58°C for 2 min, semi-final step for final extension at 72°C for 5 mins was performed. The final extension at 72°C was for 5 mins and the reaction was completed by reducing the temperature to 20°C total for 30 cycles. This amplification was expected to produce a *P. falciparum*-DNA fragment of 205bp *P. falciparum* (

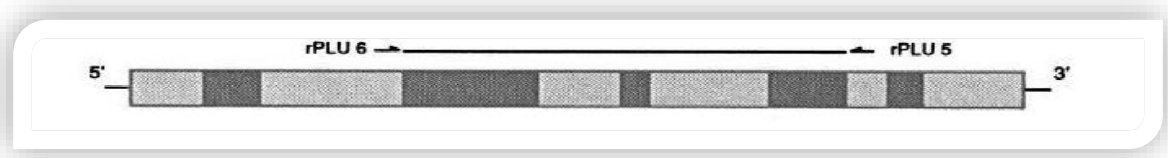


Figure 21 a and b).

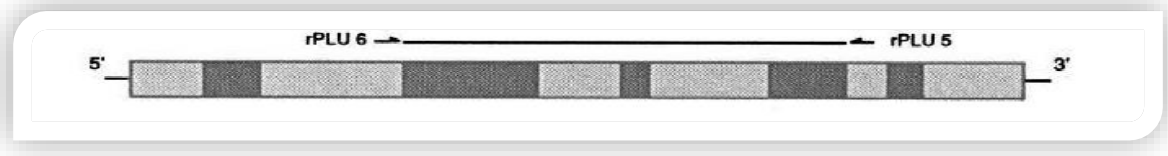


Figure 21a: Plasmodium – Specific primer (Primer for NEST 1/product size 1200bp).

Primer for NEST 2 /P. falciparum product size 205bp

Figure 21b: The specific amplification from the ssrRNA gene of the human Plasmodium species, using nested PCR.

Dark hatched areas represent regions of the genes that are specific to each of the parasite species, whereas lightly hatched areas represent sequences that are highly conserved among the species, Source: Snounou Protocol, 1993.

2.6.7. Analysis of the PCR Products by Electrophoresis

The PCR methodology is very sensitive and even the amount of amplification product from one parasite can be easily detected and visualized by safe-view staining following gel electrophoresis.

This technique was based on the rate of substance moving under the electric field. Agarose gel is formed by preparation of dry agarose in a buffer solution, either Tris-Acetate-EDTA (TAE) or Tris-Boric Acid-EDTA (TBE), and by boiling them until agarose powder is totally dissolved. It turns into a flexible gelatine-like slab when the solution cools down. During the electrophoresis procedure, the gel is immersed in a chamber that contains a TAE or TBE buffer, in addition to two electrodes, positive and negative. After the samples are loaded into individual wells and electric current is applied, DNA samples will be forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move towards the positive electrode (red) and away from the negative electrode (black), as it is DNA phosphate backbone is negatively charged.

Numbers of factors can affect the movement of DNA, such as the agarose concentration, electrical field strength, and most importantly the size of the smaller DNA molecules that move through the agarose gel faster than larger molecules. DNA, by itself, is not visible within agarose gel; to visualize it, a dye that binds to DNA needs to be used.

Gel electrophoresis is generally used for nucleic acids and proteins analysis. To determine the size and the presence of DNA, we need to run a agarose gel method. Agarose gel can be used to separate and visualize DNA of various sizes.

Amplified products were separated in 1.5% agarose gels by electrophoresis and visualised under UV light after ethidium bromide staining or Safe-view Nucleic acid stain (NBS Biologicals). Additional products are separated by the MCE-202 MultiNa Microchip Electrophoresis System for DNA analysis (Shimadzu, Japan) using the DNA-500 kit or DNA-1000 kit, depending on the size of product. There is another sensitive technique to visualize the PCR product by using *MultiNa Analysis* in which microchip Electrophoresis System for DNA analysis and MCE-202 MultiNa analysis is an alternative technique to gel electrophoresis. The advantages over traditional electrophoresis method is that MultiNa is cheaper, faster and more sensitive. Four electronic chips enable ladder calibration before each sample testing. The size of the product is estimated with a 15% error possibility. The method of detection is by SYBR Gold incorporation and results are presented as a digital gel. An internal marker consisting of a lower and upper marker serves for inter-chip normalisation and DNA ladders are used for sizing the fragments (Bekaert *et al*., 2009).

For DNA 500 kit, a 25bp ladder is used and diluted 1:100 in TE buffer. For DNA 1000 kit a ΦX174 RF DNA/*Hae* III ladder is used, diluted 1:100 in TE buffer. SYBR Gold is diluted 1:100 in TE buffer. A 5 µl of SYBR Gold was added to DNA 500 or DNA 1000 separation buffers prior to analyses. Marker solutions for DNA 500 and DNA 1000 were provided in the kits. PCR products were analysed for the presence of the genes of interests. SYBR Gold Nucleic Acid Gel Stain (Invitrogen™) was used during analyses. 10µl of SYBR Gold was diluted in 990µl of separation buffer. DNA marker reagent (100µl) was used as a calibration standard to determine size and quantity of PCR products. Migration index is directly

The method presented here remains to be the most reliable method for detecting the presence of very low number of parasites (less than 10 parasites) from the four human malarial species: *P. falciparum, Plasmodium vivax, Plasmodium malariae,* and *P. ovale*. However, in this study, only one species, *P. falciparum,* is focused on (Snounou *et al*., 2002).

Amplification of the DNA product is obtained through safe-view staining, following agarose gel electrophoresis and the exact product size is confirmed by MultiNa.

To identify this species genus and species-specific oligonucleotide, primers are designed, and nested PCR has been adapted to obtain the desired level of sensitivity. Thus, two pairs of oligonucleotide primers were required. In this project, polymerase chain reaction has been used to identify and detect *P. falciparum* parasite from a variety of samples, including the cord blood, mother peripheral blood, the infant blood, and placental blood. PCR is a preferential

method as it is at least 10-fold more sensitive than microscopy detection. It is also more reliable for determining the species in a mixed infection.

3.0. Immunohistochemistry of Claudin-4 and Zonula Occludens-1 Expressions

3.1. Avidin – Biotin Complex method (ABC) technique principle

Avidin is a 68,000 molecular weight glycoprotein found in egg white that can be labelled with peroxidase or fluorescein. Avidin has an extraordinary affinity for the small molecule vitamin biotin. Biotin is a low molecular weight vitamin that can be conjugated to a variety of biological molecules such as antibodies (Hsu *et al*., 1981).

3.2. Protocol

The expressions of Claudin-4 and Zonula occudens1 were assessed by avidin-biotin peroxidase complex technique (Vector Laboratories Inc., Burlingame, CA) according to the manufacturer's protocol. Paraffin-embedded tissue blocks were cut in 7µm sections, which were deparaffinised in 2 changes of xylene for 5 minutes each. Then, the samples were re-hydrated in 2 changes of absolute alcohol, then in 90% alcohol, and then in 70% alcohol for 3 minutes each. To block endogenous peroxidization, sections were put in 3% H_2O_2 in methanol for 10 minutes. In the next step, samples were washed briefly under running tap water and then were rinsed in phosphate buffered saline (PBS) for 2 minutes. To retrieve the epitope, samples were put for 10 minutes in boiling citrated buffer with EDTA for 2 minutes and additionally, the samples were incubated in hot buffer for another 20 minutes. In the next step, sections were washed in two changes of PBS, 5 minutes each. To block non-specific binding of immunoglobin, sections were incubated in blocking serum (normal horse serum and PBS in 1:1 proportion) for 10 minutes. Sections were then incubated with primary antibody overnight at 4ºC. For primary antibody type and dilution, see Table 7 Sections were then rinsed in PBS for 5 minutes twice and further incubated with biotinylated secondary antibody for 30 minutes at room temperature. Secondary antibodies were used according to Table 7. Samples were rinsed in PBS twice, for 5 minutes and then incubated with avidin-biotin complex (ABC) solution for 20 minutes at room temperature. Afterwards, samples were washed in PBS twice for 5 minutes. In the following step, sections were developed using 3, 3'-Diaminobenzidine (DAB) substrate solution up to 5 minutes. Then, samples were briefly rinsed in distilled water and counterstained with Gill's haematoxylin solution. Sections were differentiated in 1% acid alcohol for a few seconds, washed under running tap water for 5 minutes, dehydrated through 70% and 90% for 2 minutes and two changes of absolute alcohol

for 3 minutes each. Finally, samples were cleared in two changes of xylene, 3 minutes each and mounted with the xylene-based mounting medium before being analysed, using a light microscope (Nikon eclipse SOi). Images are captured using Image–Pro Express 6.3 programme.

3.3. Definitions

Maternal age categories: <23.3 and ≥23.3

Maternal anaemia in pregnancy is defined as a hemoglobin concentration < 110 g/L (less than 11 g/dL).

Parity categories: Primiparae, secundiparae, multiparae or grand multiparae.

Low birth weight (LBW) is defined as a birth of neonate < 2.5 Kg.

Education level categorizes: None/primary education or secondary education.

Residence categories: Urban semi-urban or rural areas.

4.0 Statistical Analysis

The collected data were analysed using SPSS V.23.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2016 – for all descriptive statistics and specificity/sensitivities. Binary logistic regressions were built where placental malaria, mother's anaemia, neonatal malarial infection, maternal malaria infection and infants low birth weight were the dependent variable, the site of samples collection and socio-demographic characteristics were the independent variables.

In order to investigate the effect of placental malaria on maternal anaemia and LBW binary logistic regression models were built where these were the independent variables and the site, socio-demographic (age, parity, residence, education,) also to investigate the effect of LBW, Placental malaria, infant peripheral malaria and education on Maternal malarial infection. $P < 0.05$ was considered significant. Parity was categorized as Primiparae, Secundiparae and multiparae (≥3). women were diagnosed as malaria positive if parasites were detected by light microscopy or PCR in the peripheral blood and placental positivity if the parasites were detected in the placenta samples collected from the placenta.

The association of tight junction markers, Claudin 4, and Zonula occludens (ZO-1) expression with both placental and congenital malaria was studied, Spearman's correlation coefficient

was used to find any relation between their expression to either placental or congenital malaria.

CHAPTER 3 – Section-5

RESULTS

This section of the chapter presents the findings from the study. The results fulfil the following objectives of the study:

3.1. Demographic Characteristics of the study

A total of 336 mothers and their new-borns fulfilled the inclusion criteria. The weight of the infants ranged between 1.9-3.9 kg, and the mean weight of neonates were (2.5 ± 0.3055). The mean maternal age was (25.13 ± 4.43). The women with age ≤ 23 years constituted 126 (37.5%) and the women aged ≥23 constituted 210 (62.5%) One hundred and thirty-two (39.3%), 90 (26.8%) and 114 (33.9%) women were recruited from Surgical Complex, El Roseires Hospital and Ed-Damazin Hospital, respectively. Primiparae represents (39.6%), Secundiparae (28.3%), and Multiparae (32.14%.). Illiteracy was seen in most of the study groups (see table 6a).

Baseline characteristics of the study population (n=336)	
Variable	Number (%)
Age groups:	
≥23 (62.5%)	210
<23	126 (37.5%)
Parity	
Primiparae	133(39.6%)
Secundiparae	95(28.3%)
Multiparae (32.14%)	108
Residence:	
Urban	204 (60.71%)
Rural	132(39.28%)
Educational level:	
None or primary	260(77.38%)
Post primary or secondary	76(22.61%)

3. 2 Prevalence of malaria parasite in maternal peripheral smear using giemsa stain

A total of 336 blood smear was examined for the detection of malaria parasites during the study period by microscopic examination using giemsa stain. The overall prevalence malaria parasite in peripheral blood smear yield was 101 (30.06%). *P. falciparum* was the only detected species. Prevalence of parasitaemia in peripheral blood smear among younger ages (≤23)was (78.2 %).

3.3 Prevalence of malaria parasite in placental blood smear using giemsa stain

A total of 336 placental blood smear was examined for the detection of placental malaria during the study period by microscopic examination using giemsa stain. The overall prevalence of placental parasitaemia was 145 (43.15%). Prevalence of parasitaemia in placental blood smear among younger ages (≤23)was (67.6 %). Two hundred and forty-six subjects (73.9%) were both positive for peripheral and placental malaria. while 47 of the mothers with negative peripheral blood had placental malaria positive. P. falciparum was the only species detected in all the positive blood smears.

3.4 Prevalence of infant peripheral malaria using giemsa stain

A total of 336 infants peripheral blood smear was examined for the detection of infant malaria by microscopic examination using giemsa stain. The overall prevalence of infant parasitaemia was 27 (8.3%). *P. falciparum* was the only detected species (***see table 6b)***

3.5 Prevalence of cord blood parasitaemia (Congenital Malaria) using giemsa stain

A total of 336 samples of cord blood were examined for the detection of cord blood (congenital malaria) presence by microscopic examination using giemsa stain. The overall prevalence of cord blood parasitaemia was171 (50.9%). *P. falciparum* was the only detected species.

Table 6b: Shows microscopic parasitaemia examination of maternal, placental, new-born and umbilical cord blood using giemsa stain

Microscopic examination (336 participants)	
Valid	Frequency/Percent
336 Maternal Peripheral blood	101+ve (30.06%) 235-ve (69.94%)
336 Placental blood	145+ve (43.15%) 191-ve (56.84%)
336 Newborns Peripheral blood	27+ve (8.3%) 309-ve (91.96%
336 Umbilical cord blood	171 +ve (50.89%) 165-ve (49.10%)

3.6. Factors associated with placental malaria infection

Only seven women were peripheral smear positive whereas placental smears negative and 2 of them (0.59%) were Primiparae. Most of the women (92.8%) who had *P. falciparum* parasitaemia by microscopy in their peripheral smears at delivery were placental malaria positive. Younger age ≤23 years old (AOR = 4.76, 95%CI (2.95-7.67); $P < 0.001$), Primiparae (AOR = 2.82, CI 1.66-4.77; $P < 0.001$), Secundiparae (AOR = 1.64, 95% CI .94-2.86; $P < 0.001$),) and peripheral blood positivity (AOR =, 95% CI.94 ; $P <$ was significantly associated with placental malaria at delivery as Logistic regression was used to investigate influencing factors on placental malaria infection. It is significantly associated with low birth weight (LBW), anaemia, parity and mother peripheral blood infection and all of them increase the risk for placental malaria parasitaemia (see Table 7) other risk factors for placental malaria such as education, residence and site of samples collected were not associated with infection.

Table 7: Association between placental malaria infection and neonate low birth weight (LBW), maternal anaemia, maternal age, parity, neonatal malaria infection, residence and level of education

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	**LBW**	**1.235**	**.360**	**11.757**	**1**	**.001**	**3.439**
	Anaemia	**1.279**	**.428**	**8.940**	**1**	**.003**	**3.595**
	Age	-.014	.049	.080	1	.777	.986
	Parity			.265	2	**.000**	**3.076**
	Parity (1)	.142	.579	.060	1	.806	1.153
	Parity (2)	-.078	.518	.023	1	.880	.925
	Residence	.164	.336	.239	1	.625	1.179
	Education	-.085	.437	.038	1	.846	.919
	Baby Blood Film	.667	1.013	.434	1	.510	1.949
	Mother peripheral infection	**3.000**	**.484**	**38.406**	**1**	**.000**	**20.083**
	Constant	-1.637	1.569	1.089	1	.297	.194

a. Variable(s) entered on step 1: LBW, Anaemia, Age, Parity, Residence, Education, Baby Blood Film, Mother Blood Film.

Table 8: Risk factors for placental malaria infection in Sudanese women using univariate and logistic regression analyses in microscopy samples.

Characteristics	N (%) of the total	Placental positive, N (%)	Univariate analyses		Logistic regression analyses	
			OR (95% CI)	P	OR (95% CI)	P
Site of collection						
Ed-Dmazen Hospital	114 (33.9%)	65 (34%)	0.835 (.501 1.39)	.489	0.580	.122
El-Roseires Hospital	90 (26.8)	45 (23.6%)	.630 (.366 1.08)	.094	0.514	.073
Surgical complex	132 (39.3%)	81 (42.4%)	Ref. category	Ref.	Ref.	
Age groups (years)[a]						
<23	150 (44.6%)	115 (60.2%)	4.76 (2.95 7.67)	0.000	2.00	< 0.001
≥23	186 (55.4%)	76 (39.8%)	Ref. Category	Ref.		
Parity						
Primiparae	133 (39.6)	91 (47.6%)	2.82 (1.66 4.77)	0.000	.485	< 0.001
Secundiparae	95 (28.3)	53 (27.7%)	1.64 (.94 2.86)	.082	.576	< 0.001
Multiparae	108 (32.1%)	47 (24.6%)	Ref.	Ref.	Ref.	
Residence						
Rural	132 (39.3%)	81 (42.4%)	1.36	0.179	NS	
Urban	204(60.7%)	110 (57.6%)	Ref.		NS	
Education						
<Secondary level	260 (77.4%)	163 (85.3%)	Ref.	Ref.	Ref.	
≥Secondary level	76 (22.6%)	28 (14.7%)	.347 (.204 .589)	0.000	.622	

Peripheral malaria						
Positive	101(30.05%)	63(18.8%)	0.02	0.000	0.019	0.000
Negative	235(69.9%)	34(10.11%)	Ref.		Ref	

3.7 Placental malaria and risk factors for adverse pregnancy outcomes

The mean (SD) of the haemoglobin among these women was (10.6 ± 3.3) g/dL. A high rate of anaemia where 98(%) women had anaemia and none of them had severe anaemia (Hb < 7 g/dL). Placental malaria was the highest risk factor for maternal anaemia (AOR = 17.94, 95% CI = 6.69; P < 0.001) (see

Table 9).

Table 9: Risk factors for placental malaria infection in Sudanese women using univariate and logistic regression analyses in microscopy samples

Characteristics	**N (%) of the total**	**Placental positive, N (%)**	**Univariate analyses**		**Logistic regression analyses**	
			OR (95% CI)	**P**	**OR (95% CI)**	**P**
Site of collection						
Ed-Dmazen Hospital	114 (33.9%)	65 (34%)	0.835 (.501 1.39)	.489	0.580	.122
El-Roseires Hospital	90 (26.8)	45 (23.6%)	.630 (.366 1.08)	.094	0.514	.073
Surgical complex	132 (39.3%)	81 (42.4%)	Ref. category	Ref.	Ref.	
Age groups (years)[a]						
<23	150 (44.6%)	115 (60.2%)	4.76 (2.95 7.67)	0.000	2.00	< 0.001
≥23	186 (55.4%)	76 (39.8%)	Ref. Category	Ref.		
Parity						

Primiparae	133 (39.6)	91 (47.6%)	2.82 (1.66 4.77)	0.000	.485	< 0.001
Secundiparae	95 (28.3)	53 (27.7%)	1.64 (.94 2.86)	.082	.576	< 0.001
Multiparae	108 (32.1%)	47 (24.6%)	Ref.	Ref.	Ref.	
Residence						
Rural	132 (39.3%)	81 (42.4%)	1.36	0.179	NS	
Urban	204(60.7%)	110 (57.6%)	Ref.		NS	
Education						
<Secondary level	260 (77.4%)	163 (85.3%)	Ref.	Ref.	Ref.	
≥Secondary level	76 (22.6%)	28 (14.7%)	.347 (.204 .589)	0.000	.622	
Peripheral malaria						
Positive	101(30.05%)	63(18.8%)	0.02	0.000	0.019	0.000
Negative	235(69.9%)	34(10.11%)	Ref.		Ref	

The overall mean (SD) of the birth weight of the neonates was (2.5 ± 0.30) kg and the overall frequency of LBW was 29.16% (n = 98). Malaria infection was significantly associated with low birthweight (LBW). Maternal anaemia (AOR = 21.25, 95% CI 6.70; $P < 0.001$), placental malaria (AOR = 13.94, 95% CI 4.326; $P < 0.001$), were significant risk factors for low birth weight (see Table 10).

Table 10: Risk factors for low birth weight in Blue Nile women's using univariate and logistic regression analyses

Characteristics	N (%) of the total	Low BW, N (%)	Univariate analyses		Logistic regression analyses	
			OR (95% CI)	P	OR (95% CI)	P
Site of collection						
Ed-Dmazen Hospital	114 (33.9%)	56 (39.7%)	1.27	.351	1.36	.405
El-Roseires Hospital	90 (26.8)	28 (19.9%)	.594	.07	.826	.631
Surgical complex	132 (39.3%)	57 (40.4%)	.76	.118	Ref.	
Age groups (years)						
<23	150 (44.6%)	100 (70.0%)	7.073	0.000	3.70	.003
≥23	186 (55.4%)	41 (29.1%)	Ref.		Ref.	
Parity						
Primiparae	133 (39.6)	76 (53.9%)	3.632	0.000	.533	.255
Secundiparae	95 (28.3)	36 (25.5%)	1.662	0.094	.889	.803
Multiparae	108 (32.1%)	29 (20.6%)	Ref.		Ref.	
Residence						
Rural	132 (39.3%)	57 (40.4%)	1.086	0.716	NS	Removed
Urban	204(60.7%)	84 (59.6%)	Ref.			
Education						

Characteristics	N (%) of the total	Low BW, N (%)	Univariate analyses		Logistic regression analyses	
			OR (95% CI)	P	OR (95% CI)	P
<Secondary level	260 (77.4%)	129 (91.5%)	Ref.		Ref.	
≥Secondary level	76 (22.6%)	12 (8.5%)	0.19	0.000	.396	0.037
Anaemia						
Yes	98 (29.2%)	85 (60.3%)	21.25	0.000	6.70	0.000
No	238 (70.8%)	56 (39.7%)	Ref.		Ref.	
Peripheral malaria						
Positive	101(30.05%)	145(43.15%)	10.79	0.000	1.976	0.083
Negative	235(69.9%)	22 (10.8%)	Ref.		Ref.	
Placental malaria						
Positive	145 (43.2%)	124(87.9%)	13.94	0.000	4.326	0.000
Negative	191 (56.8%)	17 (12.1%)	Ref.		Ref.	

Table 11: Association between maternal malaria infection and neonate low birth weight (LBW), maternal anaemia, maternal age, parity, placental malaria infection, neonatal malaria infection, residence and level of education level.

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	**LBW**	**.872**	**.441**	**3.915**	**1**	**.048**	**2.393**
	Anaemia	-.154	.467	.109	1	.741	.857
	Age	-.118	.067	3.078	1	.079	.889
	Parity			2.911	2	.233	
	Parity (1)	1.332	.790	2.844	1	.092	3.788
	Parity (2)	.868	.714	1.477	1	.224	2.381
	Placenta Blood smear	**2.922**	**.475**	**37.848**	**1**	**.000**	**18.571**
	Residence	.038	.398	.009	1	.924	1.039
	Education level	**-1.390**	**.577**	**5.805**	**1**	**.016**	**.249**
	Infant peripheral blood	**2.589**	**1.067**	**5.893**	**1**	**.015**	**13.321**
	Constant	-1.100	2.116	.270	1	.603	.333

a. Variable(s) entered on step 1: LBW, Anaemia, Age, Parity, Placenta Blood Film, Residence, Education infant peripheral Blood by microscopic using Giemsa stain

To investigate the association between maternal malaria infection and neonate low birth weight (LBW), maternal anaemia, maternal age, parity, placental malaria infection, neonatal malaria infection, residence and level of education level. Logistic regression was used to investigate influencing factors on maternal malaria

infection. Maternal malarial infection is significantly associated with the LBW, Placental blood film, Baby Blood Film and education. Higher education reduces the chance of infection. All other factors increase the chance of infection. Age and parity are significant at the 10% level, older age has slightly less risk, but the first child has more risk. (see Table 11).

3.8 Association between neonatal malaria infection and neonate low birth weight (LBW), maternal anaemia, maternal age, parity, placental malaria infection, umbilical cord blood malaria infection, residence and level of education.

Logistic regression was used to investigate influencing factors on neonatal malarial infection. Residence and mother blood film are significantly associated with neonatal malarial infection. Rural area increases the risk and positivity for mother peripheral also increases the risk by ten times. Parity is significant at the 10% level. (see Table 12).

Table 12: Association between neonatal malaria infection and neonate low birth weight (LBW), maternal anaemia, maternal age, parity, placental malaria infection, umbilical cord blood malaria infection, residence and level of education.

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	LBW	.706	.805	.770	1	.380	2.026
	Anaemia	.919	.710	1.674	1	.196	2.507
	Age	-.060	.090	.445	1	.505	.941
	Parity			4.590	2	.101	
	Parity (1)	-1.489	1.233	1.458	1	.227	.226
	Parity (2)	**-2.645**	**1.285**	**4.235**	**1**	**.040**	**.071**
	Residence	**1.353**	**.488**	**7.673**	**1**	**.006**	**3.868**
	Education level	-.456	1.205	.143	1	.705	.634
	Mother peripheral blood	**2.329**	**.960**	**5.882**	**1**	**.015**	**10.268**
	Placenta blood smear	.720	.973	.548	1	.459	2.054
	Constant	-3.347	2.841	1.388	1	.239	.035

a. Variable(s) entered on step 1: LBW, Anaemia, Age, Parity, Residence, Education level, Mother peripheral blood, Placenta blood smear by microscopic using Giemsa stain.

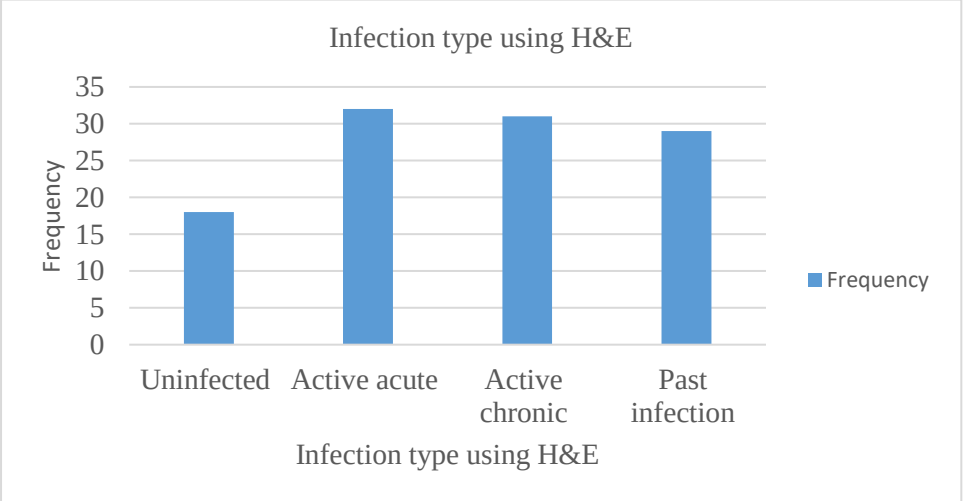
3.9 Prevalence of malaria in placental tissue by microscopic examination usingHaematoxylin and Eosin (H&E)

Stained slides were examined by the light microscope (LM) (Nikon eclipse SOi). Images were captured using the Image – Pro Express 6.3 programme. 336 women were recruited for this study. Of these, only 110 proceeded with the study because the rest were not properly prepared. Placental malaria classification was based on the criteria Rogerson *et al*., 2007. Histological evidence of malaria infection was assessed microscopically using the H&E stain, and positive results were seen

in 92 (83.7%) placentals that were studied. Of 110 placentas, 32(29.1%) showed active acute infection, 31(28.2%) showed active chronic, 29(26.4%) had post-infection, and 18 (16.4%) showed negative results (see Table 13). Presence of monocytes in foetal blood vessels was seen in three cases (2.7%). Tissue sections were examined, and digital images were captured from randomly selected cases using the Olympus BX41 light microscope (see figures 23, 24, 25and graph 1).

Table 13: Malaria infection categories frequency using H&E

		Frequency	Percent
Valid	Uninfected	18	16.4
	Active acute	32	29.1
	Active chronic	31	28.2
	Past infection	29	26.4
	Total	110	100.0



Graph 1: Shows malaria infection frequency using H&E

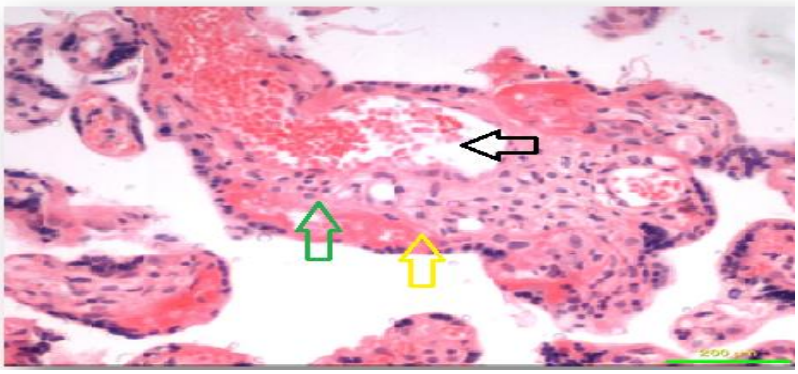


Figure 22: Uninfected placentae using H&E Magnification x20 (green arrow), Syncytiotrophoblast, (black arrow) foetal vessel and (yellow arrow)

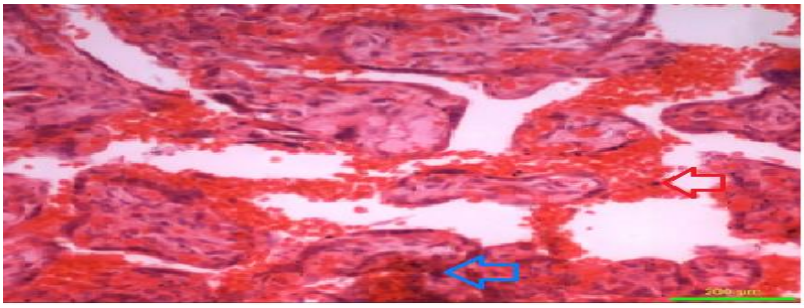


Figure 23: Active chronic using H&E present of infected erythrocyte (red arrow) and substantial malaria pigment hemozoin (blue arrow) Magnification X20

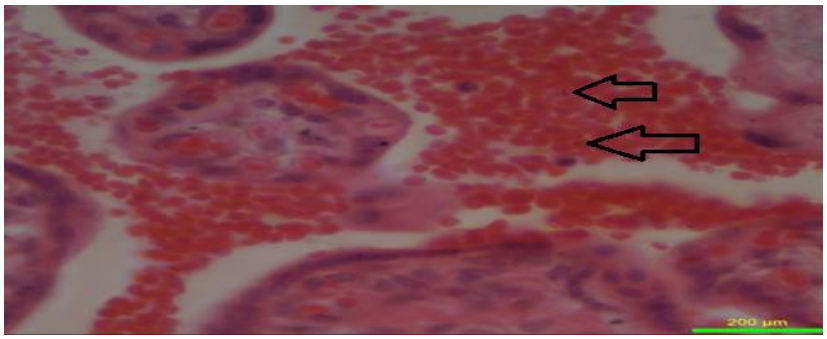


Figure 24: Active acute, the presence of infected erythrocytes (black arrows) using H&E Magnification X20

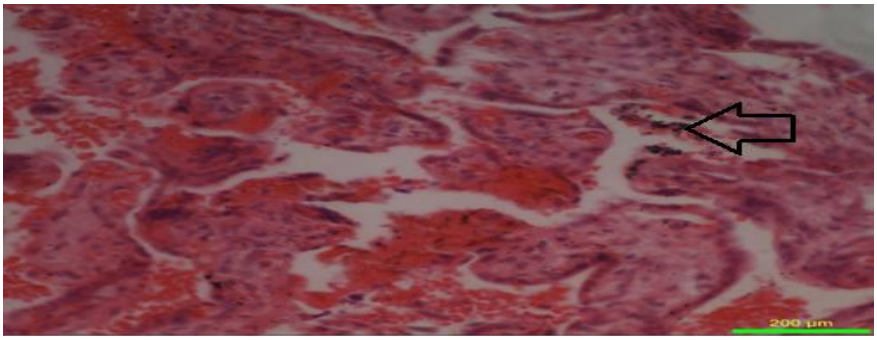


Figure 25: Past infection placentae using H&E Magnification x20 (Absence of parasite but presence of malaria pigment or hemozoin appears as brown deposits (black arrow)

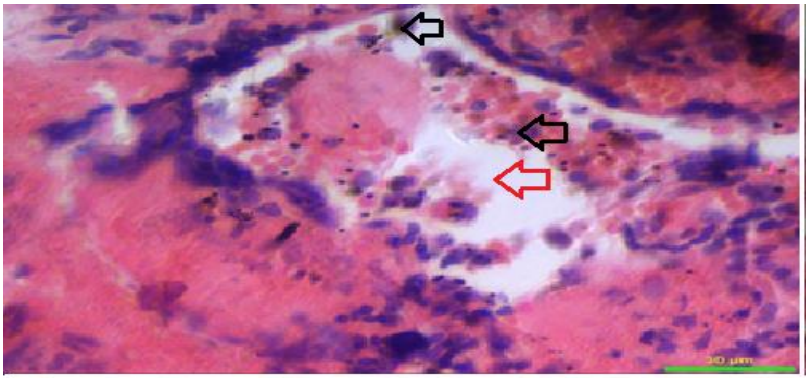


Figure 26: Large foetal vessels (red arrow) with many monocytes (black arrows) of an infant that was born with congenital malaria infection using H&E, as confirmed by a *Plasmodium. Falciparum–positive* peripheral blood smears staining within few hours of delivery magnification X40.

3.10 Results for microscopic examination using Prussian blue *for* differentiate between Haemozoin and hemosiderin

One hundred and ten Placental tissues were examined using the Prussian blue to differentiate between Haemozoin and hemosiderin (Figure 28). Histology showed that (27.27%) n 30, (30.90%) n 34(%), (13.63) n 15 had acute, chronic and past malaria infection and (28.18%) n 31 had no malaria (

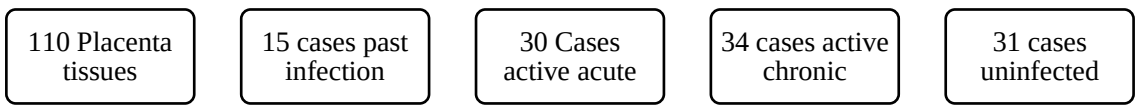


Figure 27) based on the criteria for pathologic placental malaria classification of

(Rogerson *et al*., 2007). Tissue sections were examined, and digital images were captured from ten randomly selected ×20 per slide using an Olympus BX41 microscope. Detection of monocyte was confirmed by using the Prussian blue (Figure 29).

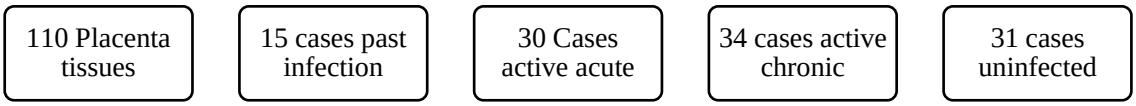


Figure 27: Flow chart showing malaria categories and number of the cases in placental tissues using Prussian Blue Stain.

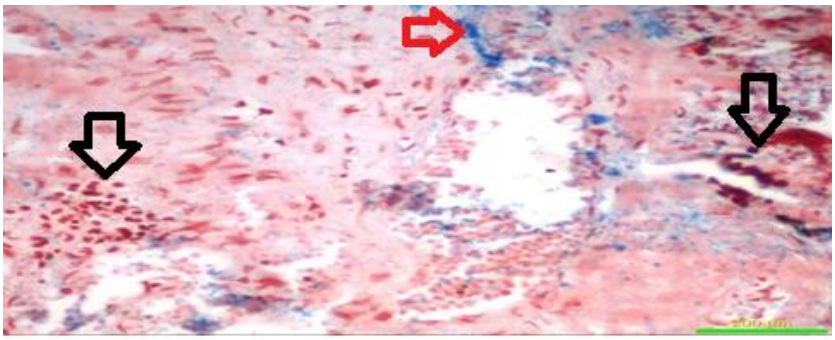


Figure 28: Prussian blue stains hemosiderin and haemozoin in placenta (hemozoin/ black arrows) and (hemosiderin blue turquoise /red arrow) Magnification X20.

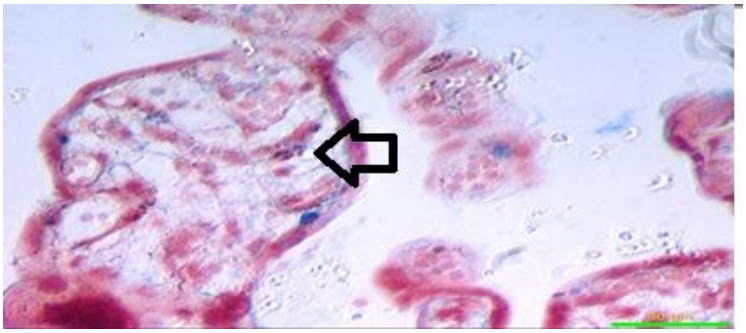


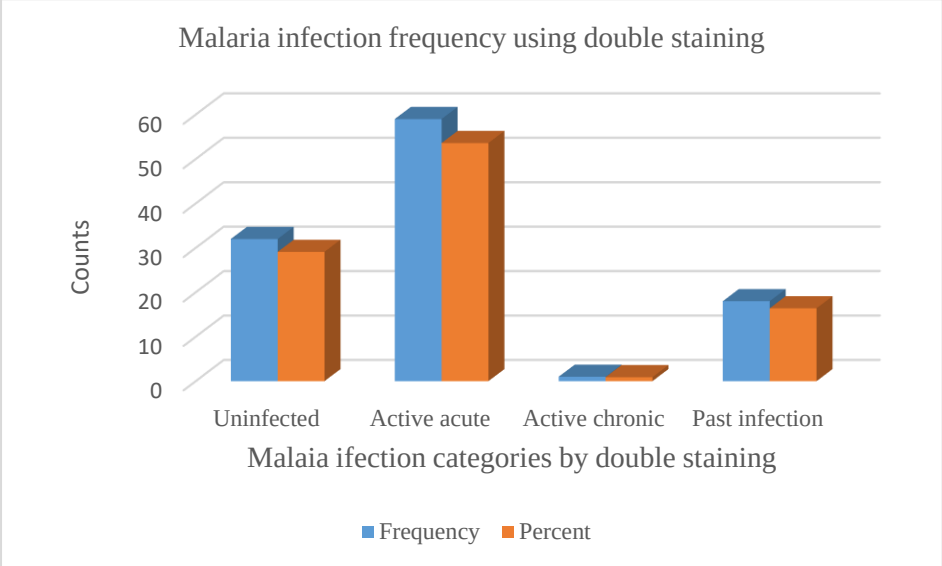
Figure 29: Pigmented monocyte using Prussian blue (black arrow) Magnification 40X.

3.11 Results for microscopic examination using ddouble staining (Giemsa & Prussian blue) for placental malaria categories

Prevalence and pattern of placental malaria (PM) in which histological evidence of malaria infection was seen in 78(70.9%) placentas were studied microscopically using the double staining. Of the 110 placentas, 59 (53.6%) showed active acute infection, and 1 (.9%) showed active chronic infection, while 18 (16.4%) showed past infection. Histological evidence of active infection was therefore seen in 60 cases, giving a prevalence of 54.5 % (see table & graph 2).

Table 14: Malaria infection frequency using double staining.

		Frequency	Percent
Valid	Uninfected	32	29.1
	Active acute	59	53.6
	Active chronic	1	.9
	Past infection	18	16.4
	Total	110	100.0



Graph 2: Malaria infection frequency using double staining

3.12 Polymerase chain reaction(PCR) results

All extracted DNA samples including the mother's peripheral blood, infant's peripheral blood, placenta blood, and cord Blood were examined using the polymerase chain reaction (see flowchart (Figure 30 & Table 15). The recorded results showed maternal peripheral and cord blood *P. falciparum* parasitemia result with the PCR. Out of the 336 maternal participants, 132 had malaria *P. falciparum* detected in their blood representing a total of 39.29% of the participants. A total number of 204 participants showed negative results. This represents a total of 60.71% of the participants. Similarly, in the placenta samples, a total of 191 samples recorded positive for *P. falciparum* representing a total of 56.87% and a total of 154 participant's placenta results samples tested negative to *P. falciparum*. This represents 43.15% of the participants. Also, out of the 336 infants' peripheral blood, 66 had malaria *P. falciparum* representing a total of 19.64% of the participants. A total of 270 infants' peripheral results samples tested negative to *P. falciparum*. This represents 80.36%. Finally, out of the 336-umbilical cord blood, 145 had malaria *P. falciparum* representing a total of 43.15% of the participants. A total number of 191 umbilical cord blood showed negative results. This represents a total of 56.85%. The detected parasite shows 205-basepair fragment for P. falciparum separated by gel electrophoresis and confirmed by MultiNa (see figures 31-34).

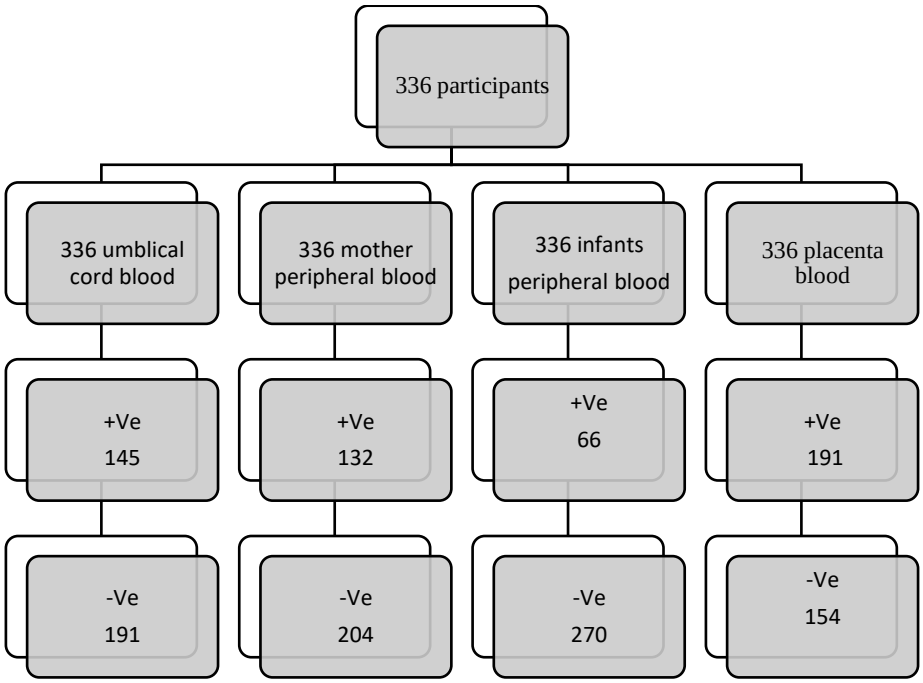


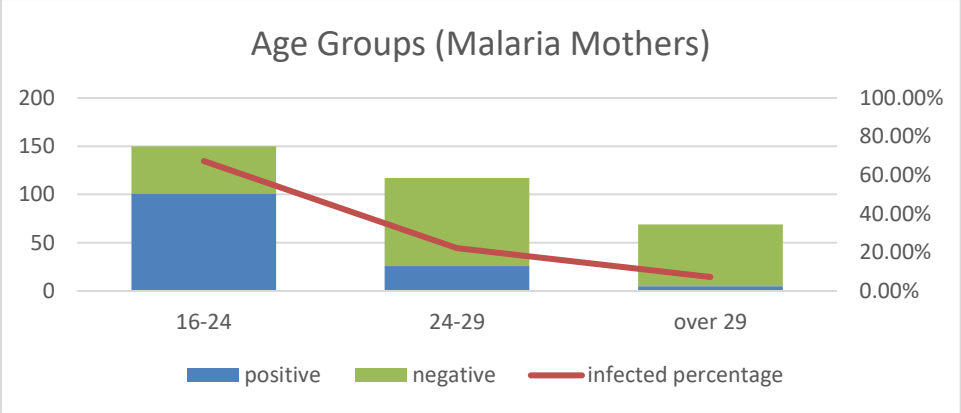
Figure 30: Flow Chart Showing PCR results for participants

Table 15: placenta, umbilical cord, maternal peripheral and baby peripheral blood using PCR.

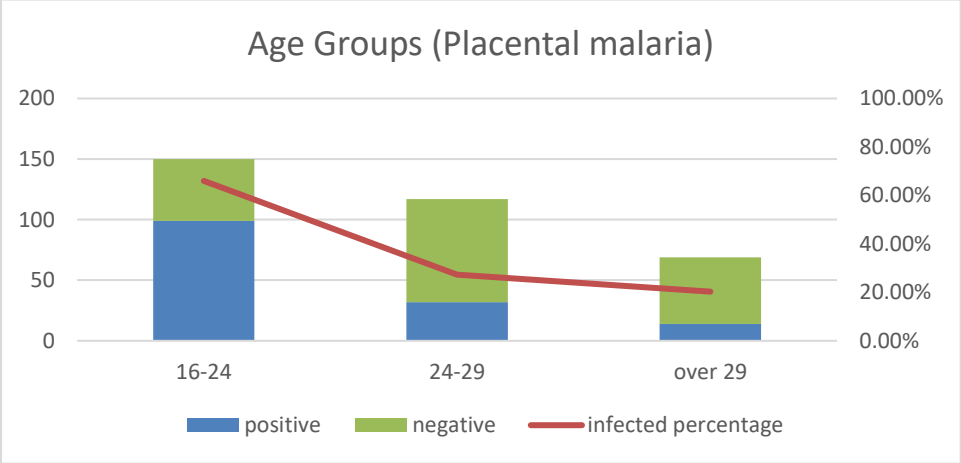
Source of blood	NO. Examined	NO. positive	% positive	NO. Negative	% Negative
Placenta	336	191	56.85%	154	43.15%
Umbilical cord	336	145	43.15%	191	56.85%
Maternal periphery	336	132	39.29%	204	60.71%
Baby periphery	336	66	19.64%	270	80.36%

	Variables	Malaria infected mothers	Malaria Infected mothers %	Non-infected mothers	Non-infected Mothers %	Placental malaria infected	Placental infected %
Age group	≥23	42	12.5%	168	50%	88	26.19%
Age group	<23	90	26.78%	36	10.71%	103	30.56%
parity	Primiparae	76	22.61%	52	15.47%	93	27.67%
parity	Secundiparae	36	10.71%	75	22.32%	54	16.07%
parity	Multiparae	20	5.95%	78	23.21%	44	13.09%
Education	None or prima	116	34.52%	144	42.85%	129	38.39%
Education	Post primary	16	4.76%	60	17.85%	16	4.76%

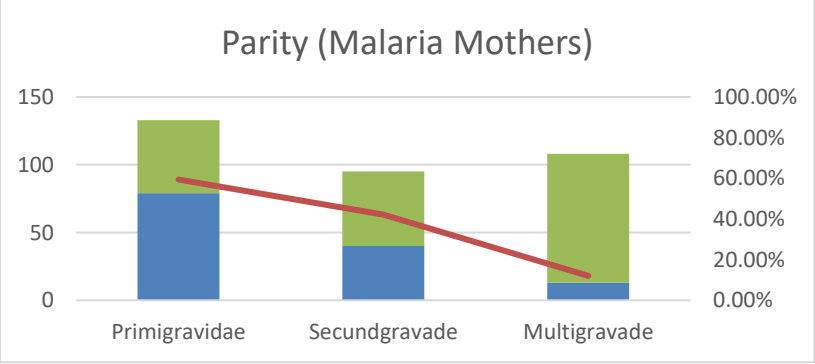
Table 16: Malaria prevalence in relation to mothers' age, parity, and education respectively for PCR



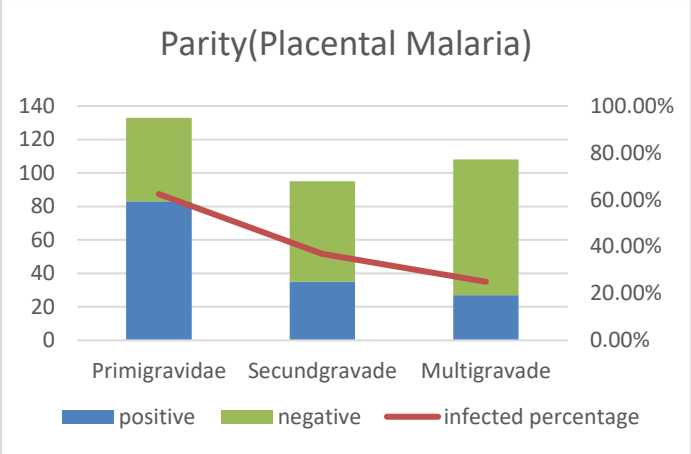
Graph 3: Peripheral malaria prevalence among different age groups



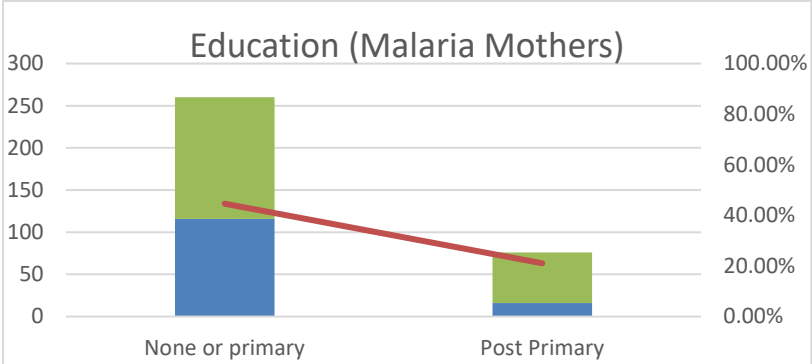
Graph 4: Placental malaria prevalence among different age groups



Graph 5: Peripheral malaria prevalence among different parity



Graph 6: Placental malaria prevalence among different parity



Graph 7: Relation between education and malaria prevalence

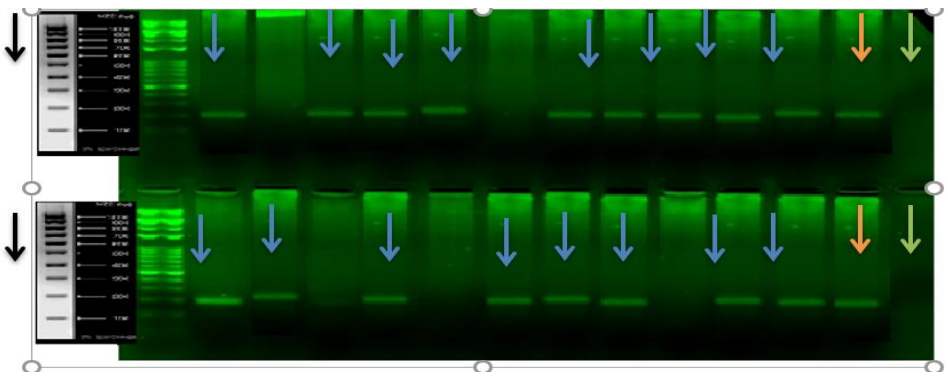


Figure 31: Amplification/analysis of *P. falciparum* malaria parasite in placental blood (blue arrows) using nested PCR. Products were analysed by agarose gel electrophoresis produced 205bp using100bp ladder, black arrows (+C orange arrows, -C green olive arrows). Samples show positive and negative controls respectively.

Figure 32: Results of malaria parasite analysis by MultiNA. PCR products are represented in lanes (A1-B2) are 205bp in size. B3 and B4 refer to positive and negative PCR controls; respectively.X1-1 represents 25 bp markers used.

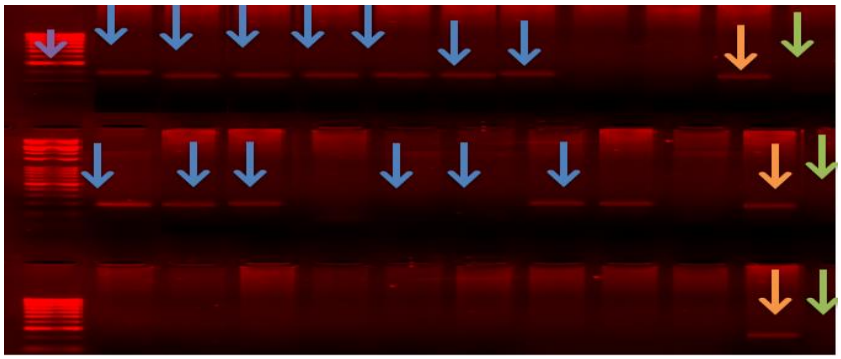


Figure 33: Amplification/analysis of *P. falciparum* Malaria parasite in umbilical cord blood using nested PCR, Products were analysed by agarose gel electrophoresis 13 samples showed a band at 205 bp (blue arrows) using100bp ladder (purple arrow) Orange arrow and green olive pointed to the positive and negative control samples, respectively.

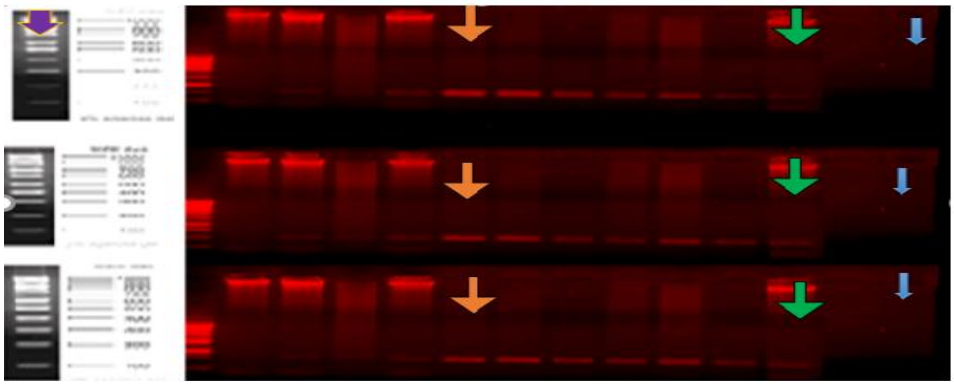


Figure 34: Amplification of *P. falciparum* Malaria parasite for baby peripheral using nested PCR. Products were analysed by agarose gel electrophoresis. Most samples showed a band at 205bp (pointed by orange arrows) using100bp ladder (L purple arrow); green and blue arrows pointed to the positive and negative control samples, respectively.

3.13 Methods comparisons

3.13.1 Sensitivity and specificity of PCR

To measure the sensitivity and specificity of the PCR method in comparison with microscopy as a standard method. In the present study, we compared PCR to microscopy (

).

Sensitivity was calculated as the number of positive results divided by the sum of positives and false negatives multiplied by 100. Also, specificity calculated as

	Maternal age (years)		Parity		p value
	≤ 23	> 23	1-2	≥ 3	
Malaria + ve Microscopy (n = 101)	66.33%	33.66%	93.06%	6.93%	0.000
Malaria + ve PCR (n = 132)	52.27%	47.72%	90.90%	9.09%	
Placenta malaria Microscopy (n = 145)	48.27%	51.72%	82.06%	17.93%	0.000128
Placenta malaria PCR (n = 191)	26.19%	30.5%	75.39%	24.60%	
Baby peripheral Microscopy (n = 26)	88.46%	11.53%	92.30%	7.69%	0.000549
Baby peripheral PCR (n = 66)	66.66%	33.33%	90.90%	9.09%	
Cord blood PCR (n = 145)	50.34%	49.65%	83.44%	16.55%	

the number of positive results divided by the sum of positives and false positive multiplied by 100. It included that the sensitivity and specificity of the samples placental Smear, cord blood, maternal blood and neonatal blood collected were 93.10% and 70.68%, 76.61% and 91.52%, 94.06% and 84.26%, 96.30% and 87.06%, respectively. Overall % rates of agreement (ORA) ranges between (98.21% -99.70%). In this study nested PCR was more sensitive compared to microscopy in which allowing the detection of Plasmodium in cases with low parasitemia accordingly. 37(28%) out of 132 negative microscopy samples were found to be positive by PCR, and all microscopy-positive samples were confirmed as positive by PCR.

Table 17: Sensitivity and Specificity for placental smear.

		Placental Blood Smear		
		+	**-**	**Total**
PCR	+	135	56	145
	-	10	135	191
	Total	145	191	

- Sensitivity = (TP/TP+FN) x 100 = 93.10%
- Specificity = (TN/TN+FP) x 100 = 70.68%
- PPV = (TP/TP+FP) x 100 = 70.68%
- NPV = (TN/TN+FN) x 100 = 93.10%
- ORA = [(TP+TN)/(TP+FP+TN+FN)] x 100 = 97.02%

Table 18: Sensitivity and Specificity for cord blood smear.

		Cord Blood Smear		
P C R		**+**	**-**	**Total**

+	131	14	145
-	40	151	191
Total	171	165	

- Sensitivity = 76.61%
- Specificity = 91.52%
- PPV = 90.34%
- NPV = 79.06%
- ORA = 88.10%

Table 19: Sensitivity and Specificity for maternal blood smear.

		Maternal Blood Smear		
		+	-	**Total**
PCR	+	95	37	132
	-	6	198	204
	Total	101	235	

- Sensitivity = 94.06%
- Specificity = 84.26%
- PPV = 71.97%
- NPV = 97.06%
- ORA = 98.21%

Table 20: Sensitivity and Specificity for neonatal blood smear.

		Neonatal Blood Smear		
P C		+	-	**Total**

+	26	40	66
-	1	269	270
Total	27	309	

- Sensitivity = 96.30%
- Specificity = 87.06%
- PPV = 39.39%
- NPV = 99.63%
- ORA = 99.70%

Table 21: Comparisons of malaria prevalence by Microscopy versus PCR

3.14 Claudin-4 (CLD4) Result

	Maternal age (years)		Parity		p value
	≤ 23	> 23	1-2	≥ 3	
Malaria + ve Microscopy (n = 101)	66.33%	33.66%	93.06%	6.93%	0.000
Malaria + ve PCR (n = 132)	52.27%	47.72%	90.90%	9.09%	
Placenta malaria Microscopy (n = 145)	48.27%	51.72%	82.06%	17.93%	0.000128
Placenta malaria PCR (n = 191)	26.19%	30.5%	75.39%	24.60%	
Baby peripheral Microscopy (n = 26)	88.46%	11.53%	92.30%	7.69%	0.000549
Baby peripheral PCR (n = 66)	66.66%	33.33%	90.90%	9.09%	
Cord blood PCR (n = 145)	50.34%	49.65%	83.44%	16.55%	

The current study investigated Claudin-4(CLDN4) expression in one hundred and ten placental tissue samples using immunohistochemistry technique to examine its possible role in congenital and placental malaria infection. The CLDN4 staining expression in syncytiotrophoblast is shown in Figure 35.

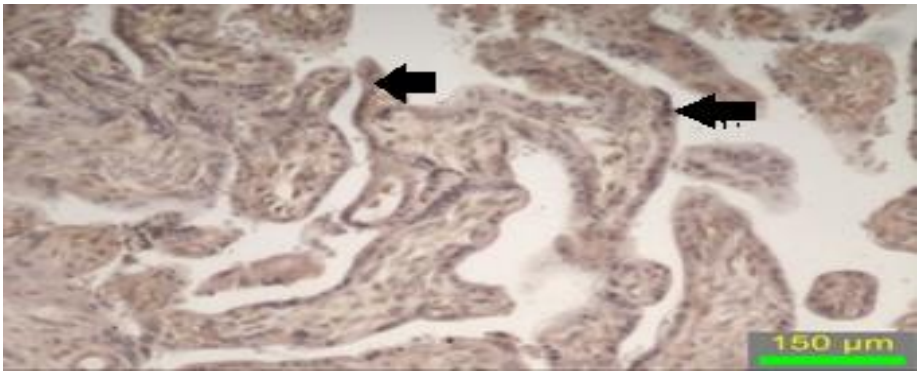


Figure 35: CLDN4 expression in syncytiotrophoblast in placental tissue (black arrows) Magnification X20.

Differences in expression of CLDN4 were analysed in placental tissues. Sections have been scored according to the intensity and presence or absence of the immunostaining into four categories: negative (0), weak 1 (+), moderate 2 (++), strong 3 (+++), very strong 4 (++++).

As illustrated in table 22, 18 out of 110 placental tissues showed a moderate expression 2 (++) for CLD4 expression, 57 out of 110 showed a strong expression 3 (+++) and 35 out of 110 showed a very strong expression 4 (++++). None of the 110 placental samples were negative for CLDN4 expression. Tissue sections were examined by two independent, blinded scorers.

Table 22: Shows CLDN4 expression according to intensity classification

		Frequency	Percentage (%)
Valid	2	18	16.4
	3	57	51.8
	4	35	31.8
	Total	110	100.0

Further Immunohistochemical analysis of CLDN4 expression in active acute malaria infection. 9 cases showed a very strong 4 (++++) expression of CLDN4. 19 cases showed a strong3 (+++) expression of Claudin-4. 3 cases showed a moderate 2 (++) expression of CLDN4 (See **Error! Reference source not found.**.)

Table 23: CLDN4expression scoring in Active – acute malaria infection

Active- Acute		
Scoring classification	Number of sample	Percentage (%)
Negative	0	0
Weak (+)	0	0
Moderate (++)	3	9.67
Strong (+++)	19	61.29
Very strong (++++)	9	29

Immunohistochemical analysis for CLDN4expression in active chronic malaria infection. 8 cases showed a very strong 4 (++++) expression of CLDN4 16 cases showed a strong3(+++) expression of CLDN4 8 cases showed a moderate8 (++) expression of CLDN4(See Table 24)

Table 24: CLDN4 expressions scoring in active chronic malaria infection

Active-chronic		
Scoring classification	Number of sample	Percentage (%)
Negative	0	0%
Weak (+)	0	0%
Moderate (++)	8	25%
Strong (+++)	16	50%
Very strong (++++)	8	25%

Immunohistochemical analysis for CLDN4 expression in past- malaria infection. 9 cases showed a very strong 4 (++++) expression of CLDN4 14 cases showed a strong3(+++) expression of CLDN4 6 cases showed a moderate (++) expression of CLDN4(See Table 25).

Table 25: CLDN4expressions scoring in past malaria infection

Past- infection		
Scoring classification	Number of sample	Percentage (%)
Negative	0	0
Weak (+)	0	0
Moderate (++)	6	20.68
Strong (+++)	14	48.27
Very strong (++++)	9	31.03

The association between CLDN4expression and presence (positive) or absence (negative) of the malaria parasite in the placental tissues was analysed using the Chi-square test. Our results showed no significant difference (χ^2 =3.012, P = 0.222) in the distribution of CLDN4expression among positive or negative placental tissues for malaria parasites (See Table 26).

Table 26: Chi Square Test for CLDN4among positive and negative placental tissue sections

(A)	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	3.012·	2	**0.222**
Likelihood Ratio	3.044	2	0.218
Linear-by-Linear Association	0.305	1	0.581
N of Valid Cases	110		

0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.22, χ^2 =3.012, *P* = 0.222

Further association between CLDN4expression and presence (positive) or absence (negative) of the malaria parasite in mothers' peripheral blood was examined using the Chi-square test. Our results showed no significant difference (χ^2 =1.720, *P* = 0.423) in the distribution of CLDN4expression among positive or negative placental tissues for malaria parasites (See Table 27).

Table 27: chi-Square tests for CLDN4 expressions in mother peripheral blood (positive &negative)

Count	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	1.720	2	0.423
Likelihood Ratio	1.725	2	0.422
Linear-by-Linear Association	1.286	1	0.257
N of Valid Cases	110		

0 cells (0.0%) have expected count less than 5. The minimum expected count is 8.51, χ^2 =1.720·, *P* = 0.423

Also, the association between CLDN4expression and presence (positive) or absence (negative) of the malaria parasite in infant peripheral blood was examined using the Chi-square test. Our results showed no significant difference

(χ^2 =0.101, P = 0.951) in the distribution of CLDN4expression among positive or negative infant blood for malaria parasites (See Table 28).

Table 28: Chi-square test for CLDN4 expression (positive &negative) in baby peripheral

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	0.101 a	2	0.951
Likelihood Ratio	0.101	2	0.951
Linear-by-Linear Association	0.001	1	0.974
N of Valid Cases	110		

a.1 cell (16.7%) have expected count less than 5. The minimum expected count is 3.27, χ^2 =0.101, P = 0. 951

Moreover, the association between CLDN4expression and presence (positive) or absence (negative) of the malaria parasite in cord blood was examined using the Chi-square test. Our results showed no significant difference (χ^2 =2.017, P = 0.365) in the distribution of CLDN4expression among positive or negative infant blood for malaria parasites (See Table 29).

Table 29: Chi-square test for CLDN4 expression in cord blood (positive & negative)

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	2.017	2	0.365
Likelihood Ratio	2.025	2	0.363
Linear-by-Linear Association	0.327	1	0.567
N of Valid Cases	110		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 8.51, χ^2 =2.017, P = 0.365)

Besides, the mean ranks for CLDN4 expression and absence (non-infected) and presence of active acute with the malaria parasite in the placental tissue was tested by using Mann Whitney U test the output of mean ranks and sum of ranks illustrated in table 30.

Then, the association between CLDN4 expression and absence (non-infected) and presence of active acute with the malaria parasite in the placental tissue was

examined using Mann Whitney U test. Our result showed that there was a significant difference (p=0.035) in the expression of CLDN4 between the two categories: non-infected and infected (active acute) placental tissue with malaria parasite (See table 31).

Table 30: Mean ranks for CLDN4 expression non-infected vs **active acute malaria infection**

	Non-infected and active acute	N	Mean Rank	Sum of Ranks
CLDN4expression	Non-infected	18	30.83	555.00
	Active acute	32	22.50	720.00
	Total	50		

a. Grouping Variable: Non-infected and active acute infect

Table 31: Mann -Whitney for CLDN4 expression for non-infected vs active acute malaria infection

	CLDN4 expression
Mann-Whitney U	192.000
Wilcoxon W	720.000
Z	- 2.111
Asymp. Sig. (2-tailed)	0.035

Besides, the mean ranks for CLDN4 expression and absence (non-infected) and presence of active chronic with the malaria parasite in the placental tissue was tested by using Mann Whitney U test the output of mean ranks and sum of ranks illustrated in table 32.

Then, the association between CLDN4 expression and absence (non-infected) and presence of active chronic with the malaria parasite in the placental tissue was examined using Mann Whitney U test. Our result showed that there was a significant difference (p= 0.153) in the expression of CLDN4 between the two categories: non-infected and infected (active chronic) placental tissue with malaria parasite (See table 33).

Table 32: Mean ranks of CLDN4for the two groups: non-infected and active chronic malaria infection

Ranks

	Non-infected and active chronic	N	Mean Rank	Sum of Ranks
CLDN4expression	Non-infected	18	28.39	511.00
	Active chronic	31	23.03	714.00
	Total	49		

a. Grouping Variable: Non-infected and active chronic malaria infection

Table 33: Mann -Whitney for CLDN4expression for non-infected vs **active chronic malaria infection**

	CLDN4 expression
Mann-Whitney U	218.000
Wilcoxon W	714.000
Z	-1.430
Asymp. Sig. (2-tailed)	0.153

Furthermore, the mean ranks for CLDN4expression and absence (non-infected) and past infection with the malaria parasite in the placental tissue was tested by using Mann Whitney U test the output of mean ranks and sum of ranks illustrated in table 34.

Table 34: Mean ranks of CLDN4for the two groups: non-infected and past infection

	Non-infected and past-infection	N	Mean Rank	Sum of Ranks
CLDN4expression	Non-infected	18	27.69	498.50
	Past infection	29	21.71	629.50
	Total	47		

Therefore, the association between CLDN4 expression and absence (non-infected) and past-infection with the malaria parasite in the placental tissue was examined using Mann Whitney U test. Our result showed that there was no significant difference (p= 0.112) in the expression of CLDN4 between the two categories: non-infected and past-infection placental tissue with malaria parasite (See table 35).

Table 35: Mann -Whitney for CLDN4expression for non-infected vs past infection

	CLDN4 expression
Mann-Whitney U	194.500
Wilcoxon W	629.500
Z	-1.589
Asymp. Sig. (2-tailed)	0.112

a. Grouping Variable: Non-infected and past-infection

3.15 Zonula occludens-1(ZO-1) Result

The current study investigated Zonula occludens-1(ZO-1) expression in one hundred and ten placental tissue samples using immunohistochemistry technique to examine its possible role in congenital and placental malaria infection. The ZO-1 staining expression in syncytiotrophoblast is shown in Figure (36).

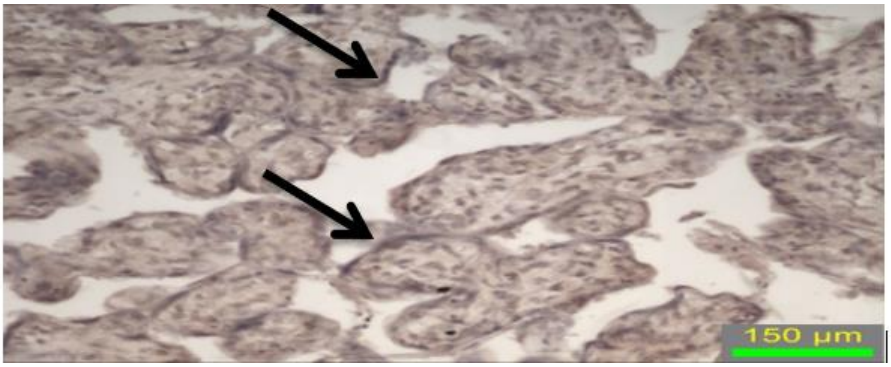


Figure 36: ZO-1 expression in Syncytiotrophoblast in placental tissue (black arrows) Magnification X20

Differences in expression of ZO-1 were analysed in placental tissues. Sections have been scored according to the intensity and presence or absence of the immunostaining into four categories: negative (0), weak 1 (+), moderate 2 (++), strong 3 (+++), very strong 4 (++++). As illustrated in table 36, 32 out of 110 placental tissues showed a moderate expression 2(++) for ZO-1 expression, 57 out of 110 showed a strong expression 3(+++) and 31 out of 110 showed a very strong expression 4 (++++). None of the 110 placental samples were negative for ZO-1 expression. Tissue sections were examined by two independent, blinded scorers.

Table 36. Shows ZO-1 expression according to intensity classification

Valid	Frequency	Percentage (%)
2	22	20.0
3	57	51.8
4	31	28.2
Total	110	100.0

Further Immunohistochemical analysis of ZO-1expression in active acute malaria infection. 3 cases showed a very strong 4 (++++) expression of ZO-1 17 cases showed a strong3 (+++) expression of ZO-1 Only one case showed a moderate 2 (++) expression of ZO-1(See Table37).

Table 37: ZO-1 expression scoring in Active – acute malaria infection

Active- Acute		
Scoring classification	Number of sample	Percentage (%)
Negative	0	0
Weak (+)	0	0
Moderate (++)	1	2.32
Strong (+++)	17	80.95
Very strong (++++)	3	14.28

Immunohistochemical analysis for ZO-1 expression in active chronic malaria infection. 17 cases showed a very strong (++++) expression of ZO-1 18 cases showed a strong3(+++) expression of ZO-1. 8 cases showed a moderate (++) expression of ZO-1(See Table 38).

Table 38: ZO-1 expressions scoring in active chronic malaria infection

Active-chronic		
Scoring classification	Number of sample	Percentage (%)
Negative	0	0
Weak (+)	0	0
Moderate (++)	8	18.60
Strong (+++)	18	41.86
Very strong (++++)	17	39

Immunohistochemical analysis for ZO-1expression in past- malaria infection. cases showed a very strong 4 (++++) expression of ZO-1 14 cases showed a strong3(+++) expression of ZO-1 4 cases showed a moderate (++) expression of ZO-1(See Table 39).

Table 391: ZO-1 expressions scoring in past malaria infection

Past- infection		
Scoring classification	Number of sample	Percentage (%)
Negative	0	0
Weak (+)	0	0
Moderate (++)	4	12.5
Strong (+++)	17	53.12
Very strong (++++)	11	34.37

The association between ZO-1 expression and presence (positive) or absence (negative) of the malaria parasite in the placental tissues was examined using the Chi-square test. Our results showed no significant difference (χ^2 = 0.111, *P* =0.946) in the distribution of ZO-1expression among positive or negative placental tissues for malaria parasites (See Table 40).

Table 40: Chi-square tests f for ZO-1 expressions in (positive & negative) placental tissue.

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	0.111 a	2	0.946
Likelihood Ratio	0.112	2	0.946
Linear-by-Linear Association	0.103	1	0.748
N of Valid Cases	110		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 7.60.

Further association between ZO-1 expression and presence (positive) or absence (negative) of the malaria parasite in mothers' peripheral blood was examined using the Chi-square test. Our results showed no significant difference (χ^2 = 1.457, *P* =0.483) in the distribution of ZO-1 expression among positive or negative placental tissues for malaria parasites (See Table 41).

Table 41: Chi-Square tests for ZO-1 expression in (Positive /negative) mother peripheral blood.

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	1.457a	2	0.483
Likelihood Ratio	1.467	2	0.480
Linear-by-Linear Association	1.377	1	0.241
N of Valid Cases	110		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 10.40.

Additionally, the association between ZO-1 expression and presence (positive) or absence (negative) of the malaria parasite in cord blood was examined using the Chi-square test. Our results showed no significant difference (χ^2 =1.4631, P =0.099) in the distribution of ZO-1 expression among positive or negative infant blood for malaria parasites (See Table 42).

Table 42: Chi-Square tests for ZO-1 expression in (Positive /negative) cord blood

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	1.4631a	2	0.099
Likelihood Ratio	4.706	2	0.095
Linear-by-Linear Association	1.377	1	0.241
N of Valid Cases	110		

Besides, the mean ranks for ZO-1 expression and absence (non-infected) and presence of active acute with the malaria parasite in the placental tissue was tested by using Mann Whitney U test the output of mean ranks and sum of ranks illustrated in table 43.

Table 43: Mean Ranks and sum of Ranks among uninfected vs **active a cute malaria infection**

	Non-infected and a active acute malaria infection-	N	Mean Rank	Sum of Ranks
ZO-1 expression	Non-infected	18	35.86	645.50
	Active a cute malaria infection	32	19.67	629.50
	Total	50		

Then, the association between ZO-1 expression and absence (non-infected) and presence of active acute with the malaria parasite in the placental tissue was examined using Mann Whitney U test. Our result showed that there was a very significant difference (p= 0.000) in the expression of ZO-1 between the two categories: non-infected and infected (active acute) placental tissue with malaria parasite (See table 44).

Table 44: Mean ranks for ZO-1 expression non-infected vs **active acute malaria infection.**

	ZO-1 expression
Mann-Whitney U	101.500
Wilcoxon W	629.500
Z	-4.040
Asymp. Sig. (2-tailed)	0.0

a. Grouping Variable: Non-infected and active acute

Besides, the mean ranks for ZO-1 expression and absence (non-infected) and presence of active chronic with the malaria parasite in the placental tissue was tested by using Mann Whitney U test the output of mean ranks and sum of ranks illustrated in table 45.

Table 45: Mean Ranks and sum of Ranks among uninfected vs **active chronic malaria infection**

	Non-infected and active chronic malaria infection	N	Mean Rank	Sum of Ranks
ZO-1 expression	Non-infected	18	33.44	602.00
	Active chronic	31	20.10	623.00
	Total	49		

Then, the association between ZO-1 expression and absence (non-infected) and presence of active chronic with the malaria parasite in the placental tissue was examined using Mann Whitney U test. Our result showed that there was a significant difference (p =0.01) in the expression of ZO-1 between the two categories: non-infected and infected (active chronic) placental tissue with malaria parasite (See table 46).

Table 46: Mann-Whitney among uninfected vs **active chronic malaria infection**

	ZO-1 expression
Mann-Whitney U	127.000
Wilcoxon W	623.000
Z	-3.421
Asymp. Sig. (2-tailed)	0.01

a. Grouping Variable: Non-infected and active chronic

Furthermore, the mean ranks for ZO-1expression and absence (non-infected) and past infection with the malaria parasite in the placental tissue was tested by using Mann Whitney U test the output of mean ranks and sum of ranks illustrated in table 47.

Table 47: Mean Ranks and sum of Ranks among uninfected vs past- infection

	Non-infected and past-infection	N	Mean Rank	Sum of Ranks
ZO -1 expression	Non-infected	18	27.83	501.00
	Past infection	29	21.62	627.00
	Total	47		

Then, the association between ZO-1 expression and absence (non-infected) and past-infection with the malaria parasite in the placental tissue was examined using Mann Whitney U. Mann-Whitney U value was found to be non- statistically significant U =192 (Z=-1.709), P =0.087(See table 48).

Table 48: Mann-Whitney for uninfected vs past infection

	ZO-1 expression
Mann-Whitney U	192.000
Wilcoxon W	627.000
Z	-1.709
Asymp. Sig. (2-tailed)	0.087

a. Grouping Variable: Non-infected and past-infection

CHAPTER 4 – Section-6

DISCUSSION

In this study the prevalence and impact of malaria infection on pregnancy and prenatal outcomes in the Blue Nile state of Sudan has been investigated. In addition, the possible involvement of the placental tight junctions, zonulaoccludens-1(ZO-1) and claudin-4 (CLDN4)**,** in the mechanism of placental and congenital malaria was also explored. The presence of the malaria parasite was examined microscopically using Giemsa stain and PCR techniques. While immunohistochemistry technique, was used to evaluate the state of placental tight junctions.

Results showed, that 30.06 % of mothers had **peripheral (maternal) parasitaemia** at parturition by microscopic examination using giemsa stain. Our findings were in accordance with those of Uneke ,2008 who stated that the range of maternal peripheral malaria range from 9% to 60% in the malaria infected areas of the Sub-Saharan Africa. Previous studies have reported lower ranges of parasitaemia, for instance, in Sudan; Omer *et al*., 2011 the prevalence of malaria among pregnant women was 26.2%. On the other hand, Adam *et al*., 2004 found a prevalence of 13.7% maternal malaria parasite in Eastern Sudan. Eastern Sudan has a low endemicity and malaria is unstable as compared to Blue Nile areas where malaria is mesoendemic (Omer *et.al* 2017). Studies from other malaria endemic parts of Africa have also reported large variations in the prevalence of malaria parasitaemia among the pregnant women. Walker-Abbey *et al*. 2005 reported that the prevalence rate in Cameroon is 82.4%.

The wide range in reported prevalence of **peripheral (maternal) parasitaemia** may be due to multiple factors. One of the main factors is the method of diagnosis. The studies that stated very high prevalence rates, that is, 70%, were those that involved the use of PCR for parasite detection. Other factors that may explain this variant include intensity of transmission, study population characteristics (age, parity, HIV status), use of preventive measures (e.g., IPT,

ITNs), and study design. Besides, many studies had small sample sizes and were restricted to a single site.

The prevalence of **placental malaria** of 43.2% in recruited women in this study was detected microscopically using giemsa stain which is lower than those reported by both Bassey *et al*., 2015 and Ezebialu *et al*., 2012.

Guyatt *et.al*. 2004, also, Sarr *et al.* 2012, have also reported a lower prevalence of 10.9% from a malaria low transmission community. These variations in prevalence may be due to variations in community-acquired immunity, sociodemographic characteristics of the study population, case selection, use and resistance to malaria chemoprophylaxis and the diagnostic tools employed in the detection of the parasite.

The most common histological outline of placental parasitization in this study was active acute, followed by active chronic, and then past malaria infection, which were marginally different from previous studies from Sudan and Tanzania (Menendez *et al.*, 2000 and Adam *et al*., 2007), where past infection cases were commonly more. Previous studies Kimbi *et al.*, 2009; Rogerson *et al*. 2003 proved that placental histological investigation is higher to microscopy placental blood thick film in identifying placental infections. This superior detection of histological placental detection could be due to the ability of histology to identify the evidence of past infections, also the quality in microscopic thick film is accredited to this finding.

In this study, the prevalence of **neonatal malaria** (**infant peripheral blood infection**) was 8.04% by using giemsa staining technique. Sotimehin *et al.*, 2008 have reported a prevalence of 10.9% in South-West Nigeria. Obiajunwa *et al*.,2005 found a Prevalence rate of 46.7 % in Nigeria. However, some researchers had reported comparatively high rates of neonatal peripheral parasitaemia, and the general acceptance was neonatal parasitaemia occurrence in endemic areas were very low, with rates ranging from 0.18% to 0.95% (Lamikanra *et al*., 1993). The present study showed that umbilical cord malarial infection prevalence was 50.9% by microscopic examination using giemsa stain. Quedrago *et al*., 2012

reported prevalence of 1.4% in Burkina Faso. Ekpuka *e al.* 2013 reported a prevalence of 9.0% in Nigeria. Malhotra A, *et al*., 2006 reported prevalence of 2.2% in Kenya. Our study was different from the other studies that showed a lower prevalence of parasitaemia in umbilical cord blood, these variations could be explained by the difference in the ways used to effectively prevent malaria infection during pregnancy, difference in seasons as Blue Nile areas where malaria is mesoendemic as well as the difference in methods used to diagnose malaria infection.

In the present study, it was found that peripheral positivity was associated with placental malaria. Most of the women who had *P. falciparum* parasitaemia by microscopy in their peripheral smears at delivery were placental malaria positive. Placental malaria was significantly associated with age; where women less than 23 years old are likely to have placental malaria, infection compared to older women. Poor **pregnancy outcome** in this study was also associated with Age, as women less than 23 years old were more likely to have placental malaria infection more than the older women. Furthermore, placental malaria also significantly associated with LBW, anaemia, parity as these factors increases the risk of plaental malaria infection. A study showed that age was not associated with placental malaria infection (Adam et al.,2009). This agrees with the findings of the study that showed no relation between age and placental malaria (Adam et al.,2017).

In consistent with a previous study (Walker et al., 2014), the present study showed that neonates of mothers with placental malaria were born with low birth weight (LBW). While Adam et al. documented no association between placental malaria and LBW in eastern Sudan (Adam et al.,2007). A previous analysis showed that a neonate is twice as likely to be born with a LBW if the mother has an infected placenta at delivery (Guyatt **et al.,2001**).

In the current study, placental malaria was significantly associated with maternal anemia. A previous study from Ubangi district of Zaire noted that placental

malaria infection had no consistent relationship to maternal anemia (Anagnos et al.,1986). Study done in Accra Ghana by Ofori showed that, placental malaria was significantly associated with maternal anemia (Ofori et al., 2009).

In our study, results showed that primipara were at higher risk of placental malaria infection compared to multipara. A previous study showed that primiparity was one of the risk factors for placental malaria infection (Falade et al., 2010). It is understood from previous studies that primipara are at a higher risk of placental malaria infection because multipara mothers develop malaria antibodies that block adhesion of parasites to CSA receptors in the placentae in subsequent pregnancies (Conroy et al.,2011).

But other risk factors for placental malaria such as education, residence and site of samples collected were not associated with placental malaria infection.

Placenta malaria infection was associated with maternal malaria infection as all women who have maternal malaria infection were placental malaria positive.

Maternal malaria remains a leading cause of low birth weight (LBW) in malaria-endemic areas of sub-Saharan Africa (Guyatt *et al*., 2004). Malaria-associated maternal illness and low birth weight is mostly the result of Plasmodium falciparum infection and occurs predominantly in Africa (Beaudrap *et al*., 2013). Maternal peripheral malaria infections contribute significantly to perinatal morbidity. Severe impairment of birthweight was observed after multiple malarial infections and in malarial infections with high parasitaemia (Muehlenbachs *et al.*, 2010).

In the current study the mean (SD) birth weight was (2.5 ± 0.30) kg; therefore, maternal malarial infection is significantly associated with the LBW, most infants from peripheral maternal malaria infected mothers were low birth weight. Maternal malaria increased neonatal mortality indirectly by affecting birth weight. In this study, maternal malaria was responsible for 29.16 percent of low birth weight. The present study found that peripheral malarial infection at delivery was associated with lower birth weight and was consistent with other studies that were carried out in Africa (Beaudrap *et al*., 2013).

Association between maternal malaria infection and neonate LBW, maternal anaemia, maternal age, parity, placental malaria infection, neonatal malaria infection, residence and level of education level were studied in this study.
Education level was also associated with maternal malaria infection. Higher education reduces the chance of infection. Infant peripheral malaria infection was also associated with maternal malaria infection. But no relation was found between maternal malaria infection and women anaemia and women age, parity or residence.

Association between neonatal malaria infection and neonate LBW, maternal anaemia, maternal age, parity, placental malaria infection, umbilical cord blood malaria infection, residence and level of education were studied in this study.
Peripheral neonatal malaria infection was associated with mother peripheral malaria infection as well as residence and parity. But there was no relation between Peripheral neonatal malaria infection and other factors such as; maternal anaemia, maternal age, placental malaria infection, umbilical cord blood malaria infection and level of education. Previous study revealed that malaria among pregnant women would have a negative impact on new-born mortality and morbidity (Bardají et al.,2011).

The impact of malaria infection on pregnant and prenatal outcomes. Malaria infection during pregnancy is a significant public health problem with extensive risks for the pregnant women, their foetus, and the new-born child. Pregnant women were highly susceptible to the infection than non-pregnant (Desai *et al.*, 2007).
Poor pregnancy outcome in this study at delivery was associated with a positive peripheral and placental blood parasitaemia by both microscopy and PCR, as mostly infected women with peripheral infection were placental infected as well. Two hundred and forty-six subjects 73.9% were both positive for peripheral and placental malaria. while 47 of the mothers with negative peripheral blood had placental malaria positive.

It was noticed that factors such as education, residence and site of samples collected were not associated with placental infection. Placental malaria considered one of the risk factors affecting pregnancy outcomes, was the highest risk for maternal anaemia. Importantly, in aparasitaemic women, with positive peripheral blood film had a risk of placental parasitaemia that was associated with anaemia, LBW and PD (Malhotra et al.,2005)

Malaria infection can be associated mostly with miscarriage or pre-term delivery. A similar association between malaria infections and anaemia has been reported in mothers in previous studies (Gready *et al*., 2012). Premature delivery is a well-known consequence of severe maternal malaria during pregnancy in areas of low malaria endemicity (Luxemburger *et al.*,2007).

Infections detected by PCR had no noticeable effect on pregnancy outcome but were still associated with reduced maternal Hb (Mockenhaupt et al.,2006). Previous studies on that subject produced disagreeing results. In Ghana, Mozambique, and Cameroon, sub-microscopic peripheral blood infections as detected by PCR were associated with low Hb levels or anaemia, *P. falciparum* as identified by peripheral blood microscopy or PCR in Burkina Faso was not associated with LBW or birth weight (Mockenhaupt *et al*.,2006). Women found to be *P. falciparum* positive by PCR show no increased risk of LBW in Malawi. One study from Kenya reported that malaria as assessed by peripheral blood film was stronger associated with intrauterine growth retardation than were peripheral PCR (Malhotra *et al.*,2005).

Histological examination of placental tissue using different types of stains was carried out in the current study to assess the prevalence of placental malaria infection. The prevalence of malaria in placental tissue was (83.7%). Presence of monocytes in foetal blood vessels was seen in three cases (2.7%). Ifeanychkwu *et al.*, 2012 in south eastern Nigeria found that a prevalence of malaria in placental tissue of 69.6% Whereas this figure was higher in a study documented by Bako et al., 2009 who reported a prevalence was 33.9% in Maiduguri, north eastern Nigeria, using placental histology. It was higher compared to Adam *et*

al.,2005 who reported the prevalence was 32% using placental histology in eastern Sudan.

Previous study by Anchang-Kimbi *et al.*,2009 have demonstrated that histological examination of the placenta is superior to placental blood thick film microscopy in identifying placental infections. The present study also supports this finding, as the prevalence of placental malaria was higher with histological examination of 83.7% than with placental blood microscopy of 43.15%. This superior detection of placental malaria by histology may be due to the ability of histology to identify the evidence of past infections, a quality that may not be seen in thick film microscopy.

The most common histological pattern of placental parasitization in this group of women was Active chronic, followed by active acute, and then past -infection. This is slightly different from observations in Sudan (Adam *et al.*,2007) and Tanzania (Menendez *et al.,* 2000), where past infection was more common. Furthermore, the subsequent aim was to addresses and explore the possible mechanism/s by which malaria parasite crosses the placental barrier and to find the role of placental tight junction proteins in relation to placental pathology and congenital malaria cases.

The prevalence of **mother's peripheral malaria** was examined using polymerase chain reaction (PCR) of 39.3%. Mockenhaupt *et al*.,2006 found the prevalence of mother's peripheral parasitaemia was 63.4% among Ghanaian women. Saute *et al*.,2002 reported the prevalence of mother's peripheral parasitaemia among rural Mozambique women was 55.8%. Adam *et al*.,2005 found that in Sudan, areas with low intensity of malaria transmission was 51.3%. Also, Omer *et al.*, 2011, found that prevalence of mother's peripheral parasitaemia in Greater Khartoum area in Sudan was 56.3%. The prevalence of malaria among pregnant women in Nchelenge – Zambia was 22.0 % (Siame *et al*., 2013). These variations could be due to numbers of factors such as methods of detections as well as endemicity.

The prevalence of **infant peripheral malaria** in this study was found to be 19.64% by using polymerase chain reaction (PCR) techniques A study in Ghana

showed that a prevalence of infant peripheral malaria detected by PCR was 12% (Christabel *et al*. (2012). Olabisi *et al.* 2011 in Nigeria reported that a prevalence of new-born was 0.0%. Whereas, Jahja *et al*. 2013, reported that a prevalence in all new-borns was 14.7% in a recently conducted study at TC Hillers Hospital in Indonesia. Our study considered the highest rate of infant peripheral parasitaemia.

The prevalence of **placental blood malaria** in this study was 56.9% using polymerase chain reaction (PCR). Elbashir *et al.*,2011 reported that the prevalence of placental malaria infections at Medani Hospital - Sudan was 31.8% by PCR.

However, Singer *et al*.,2004 reported that the parasites were detected in 47% of the samples that were tested by the placental PCR. Guin *et al*., 2012 stated that that the placental malaria positivity is 2.2 % by PCR in central India. Variations in these results could be explained by multi factors such as methods used and endemicity.

The prevalence of **Umbilical Cord Blood (Congenital malaria)** in this study was 43.15% using PCR technique while Campos *et al*. (2011), reported that cord's blood parasitaemia was 13% by PCR in Colombia.Whreas, Mwangoka *et al*. (2011), reported that cord's blood parasitaemia was 61% in Muhez District-Tanzania. Malhotra *et al*. (2006), reported that cord blood parasitaemia was 10.4% by PCR in Kenya. Different results in these studies may be due to sample sizes, endemicity, and techniques applied. Congenital malaria occurs due to transplacental transmission of parasitized erythrocytes (Desai *et al*., 2007). TI In this study, all cases of positive cord blood smears also had positive placental blood smears which was confirmed by Uneke *et al*. (2007) study.

In this study **microscopic** examination versus **PCR** method was studied. Detection of Plasmodium species by microscopy has been the golden standard for diagnosis of malaria for more than a century. Currently, the clinical diagnosis of malaria depends on the detection of parasites by light microscopy. This procedure is inexpensive and simple, but it is a labor-intensive method and requires well-trained technicians (Payne et al., 1988). Malaria diagnosis at the

early stage is challenging because of low levels of parasites. Polymerase chain reaction (PCR) is one of the molecular methods that can be used for low parasitemia detection because of greater sensitivity (Ebrahimi et al., 2015).

Sensitivity and specificity were run for all samples such as placental blood; it shows sensitivity of 93.1% and specificity of 70.7%. Cord blood; shows sensitivity of 76.6% and specificity of 91.5 %. Maternal peripheral blood; shows sensitivity of 94.1% and specificity of 84.3%. Finally, neonatal blood; shows sensitivity of 96.3% % and specificity of 87.1%.

There was (98.2% -99.7%) Overall rates of agreement (ORA) on the parasite detection in samples that were positive by both PCR and microscopy; however, a proportion of (28%, 37/132) of all microscope-positive samples were negative by PCR. Also, all microscopy-positive samples were confirmed as positive by PCR, this is similar to the findings by Adam et al. (2005) as (32%) of the 125 smear-negative pregnant women in the Eastern Sudan had submicroscopic P. falciparum (PCR) infections.

In the current study finding shows; maternal malaria parasitemia by microscopy examination among age ≤ 23 was 66.33% while maternal malaria parasitemia for the same age by PCR was 52.27%. However, Placental histology shows a proportion of (43.2%,145/336) by microscopy whereas; maternal peripheral blood shows a proportion of (30.1%,101/336).

PCR considered more reliable because it distinguished between Plasmodium species. In this study P. falciparum was the only detected species. We conclude that nested PCR is valuable as a confirmatory test. Molecular techniques are a costly procedure, including the cost of labor and access to reagents, compared to microscopical diagnosis. This characterizes a true loss for its application in reference laboratories located in poor
regions of the world, where malaria is endemic. Theoretically, since microscopy could detect only viable parasites it should be considered as the most relevant measure.

This study is the first study to investigate the role of **tight junctions Claudin-4 (CLDN4) and Zonula occludens-1 (ZO-1)** in placental pathology and congenital malaria infection. We have hypothesized that deregulation of tight junctions' proteins in relation to malaria infection can facilitate the crossing of the parasite through the placental barrier.

Expression of CLDN4 and ZO-1 were analysed in 110 placental tissues by immunohistochemical technique. Our results revealed that 16.4% of these cases showed a moderate expression of CLDN4 and 29.0% for ZO-1 expression. None of the placental samples examined showed a total absence in expression of both tight junctions' proteins.

In samples of placenta (n=32) with active acute malaria infection, CLDN4 moderate expression was found in few cases (3 cases), while for ZO-1 only one case showed moderate expression. This reflects that only few cases of placental tissues with active acute infection had minor deregulation for both tight junctions' proteins examined.

In samples of placenta (n=32) with active chronic malaria infection, CLDN4 moderate expression was found in 8 cases, and ZO-1 expression was found to be moderately expressed. There were more cases showing moderate expression of both tight junctions examined that in the case of than in the case of active acute infection. These findings can be explained that the chronicity of malaria infection may have played a role in the pathology of placental malaria and led to the down regulation of these tight junctions in this category. However, the rest of the cases had minor deregulation for both tight junctions.

In samples of placenta (29) with past-malaria infection, CLDN4 moderate expression was found in 6 cases, while for ZO-1 4 cases only showed moderate expression. It was lower than active chronic malaria infection, but it was higher than active acute malaria infection. These data suggests that malaria infection during early pregnancy may have a more impact on the status of both tight junction proteins expression and caused their deregulation in more cases than what was found in the case of active acute malaria infection cases.

The relation between CLDN4 and ZO-1 expressions and presence (positive) or absence (negative) of the malaria parasite in the 110 placental tissues was also analysed. No relationship was found between their expression in both categories. Further relationship between CLDN4 and ZO-1 expression and presence (positive) or absence (negative) of the malaria parasite in mothers' peripheral blood was studied. Also, there was no relationship was found between their expressions. in both categories. Also, the association between CLDN4 and ZO-1 expression and presence (positive) or absence (negative) of the malaria parasite in infant peripheral blood was studied. A negative correlation was found between their expressions. On the other hand, different statistical methods were used to find if there was any association between CLDN4 & ZO-1 expressions and absence (non-infected) or presence of malaria parasite in active acute and active chronic placental tissues. A significant association was found between both tight junctions' proteins expression and active acute malaria infection and active chronic malaria infection.

Additionally, the association between CLDN4 & ZO-1 expression and absence (non-infected) and past-infection with the malaria parasite in the placental tissue was studied carefully. Our finding showed a negative correlation between their expression of CLDN4 & ZO-1 and absence (non-infected) and past-infection with the malaria parasite expressions in both categories.

Data from previous studies shows that the tight junction protein CLDN4 expression has been linked to several diseases such as inflammatory bowel disease (Weber *et al*., 2008; Abdelzaher *et al*., 2011). Several pathogens invade the placenta, but few are known to actively cross the placental barrier and spread to the foetus.

It was well known that parasitized erythrocytes bind to chondroitin sulphate A expressed at the syncytiotrophoblast apical surface, where they accumulate, impeding the physiological functions of the placenta (Scherf *et al*. 2001).

The mechanism by which pathogens may crosses the placental barrier and infect the foetus remain often indefinable. Coxsackie viruses B (CVB) may be transmitted to the foetus during delivery (Moore 1982). L. monocytogenes actively crosses the placental barrier and causes severe materno-fetal infections

in humans (Mylonakis *et al*. 2002; Lecuit et al. 2007). It is not known, however, if the mechanism by which malaria parasite crosses the placental barrier and infect the foetus has similar crossing methods.

Expected down-regulation in placental tight junction's protein may occur during malaria infection among pregnant women. Hence, we aimed to find a relationship between the status of these tight junctions and presence and absence of malaria parasite infection in the placenta at delivery. We hypothesized that tight junctions' proteins could be disrupted under inflammatory conditions such as malaria infection. The impact of malaria infection on cytokines level and its disturbance to tight junction is undefined. After careful study on these tight junctions and their relation to all malaria categories. A negative correlation was found between these tight junctions' protein expression and the presence or absence of malaria infection.

Conclusion

We conclude that malaria and its impact on mother and infant health is a much-neglected areas. There is high prevalence of malaria in our study area and several risk factors and complications that are associated with malaria during pregnancy. In addition, there is a lack of information on the impact of congenital malaria and general morbidity in the infant. There are still many gaps in the literature and knowledge on how the parasite crosses the placenta as well as the timing of infection during the pregnancy. It is essential to have clear clinical guidelines for the management of congenital malaria. Besides, more work is needed to establish adequate preventive measures to avoid the consequences of malaria in pregnancy.

Limitations of the Study

The study extended from 2012 to 2014 and samples were collected to investigate the prevalence of malaria parasites at the study sites. This extended duration was due to availability of field expenses and resources. Moreover, recruitment and samples collection were not carried daily due to the limited number of midwives participated in the study. Parasite density was the existing routine practice in the laboratories of the study sites as the technicians participated the study were not researchers. Sample preparation was poor due to kept in formaldehyde for long time.

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Appendices

Appendix 1: Shows placental pathology for H & E analysis for placenta samples

Sample code	Uninfected	Active acute	Active chronic	Past infection	General comments
PL11	X				No parasite or haemozoin present/no histological changes
PL111	X				No parasite or haemozoin present/no histological changes
PL14	X				No parasite or haemozoin present/no histological changes
PL22				X	Absence of parasite but deposition of haemozoin
PL35			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL9			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL122				X	Absence of parasite but deposition of haemozoin
PL23				X	Absence of parasite but deposition of haemozoin
PL25			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL29		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small amount of haemozoin and monocyte
PL37		X			Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL38		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small amount of haemozoin

Appendix 2: Shows placental pathology for H & E analysis for placenta samples

Sample code	Uninfected	Active acute	Active chronic	Past infection	General comments
PL46	X				No parasite or haemozoin present/no histological changes
PL50				X	Absence of parasite but deposition of haemozoin
PL57				X	Absence of parasite but deposition of haemozoin
PL59			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial
PL79		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small amount of haemozoin
PL71				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PL74				X	No parasite but haemozoin present/no histological changes
PL75			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL20				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PL65			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL24			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL39			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL40			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL44				X	Absence of parasite but deposition of haemozoin
PL48	X				No parasite or haemozoin present/no histological changes
PL5		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small amount of haemozoin
PL54				X	Absence of parasite but deposition of haemozoin

Appendix 3: Shows placental pathology for H & E analysis for placenta samples

Sample code	Uninfected	Active acute	Active chronic	Past infection	General comments
PL2		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small
PL4		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small
PL33				X	Absence of parasite but deposition of haemozoin
PL1		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small
PL10			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL21				X	Absence of parasite but deposition of haemozoin
PL23				X	Absence of parasite but deposition of haemozoin
PL27				X	Absence of parasite but deposition of haemozoin
PL28 X					No parasite or haemozoin present/no histological changes
PL29 X					No parasite or haemozoin present/no histological changes
PL40			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL41			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL51 X					No parasite or haemozoin present/no histological changes
PL62			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL9				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PL125				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage

Appendix 4: Shows placental pathology for H & E analysis for placenta samples

Sample code	Uninfected	Active acute	Active chronic	Past infection	General comments
PL3		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL54		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL19				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PL28	X				No parasite or haemozoin present/no histological changes
PL33			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL45				X	Absence of parasite but deposition of haemozoin
PL55			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL17	X				No parasite or haemozoin present/no histological changes
PL18		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL21		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL24				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PL30			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL34 X					No parasite or haemozoin present/no histological changes
PL44 X					No parasite or haemozoin present/no histological changes
PL52		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin

Appendix 5: Shows placental pathology for H & E analysis for placenta samples

Sample code	Uninfected	Active acute	Active chronic	Past infection	General comments
PL60		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL67			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL7				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PL47				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PL8		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL20		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL36			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL6	X				No parasite or haemozoin present/no histological changes
PL13			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL16				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PL32		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL31			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL80		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL82			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL83		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL123				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage

Appendix 6: Shows placental pathology for H & E analysis for placenta samples

Sample code	Uninfected	Active acute	Active chronic	Past infection	General comments
PL84	X				No parasite or haemozoin present/no histological changes
PL85		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL86		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL87			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL88		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL89				X	No parasite but haemozoin present with no histological changes
PLR76	X				No parasite or haemozoin present/no histological changes
PLR6			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PLR11			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PLR9	X				No parasite or haemozoin present/no histological changes
PLR22				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PLR20	X				No parasite or haemozoin present/no histological changes
PLR17				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PLR15			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PLR16				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PL117		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL122			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin

Appendix 7: Shows placental pathology for H & E analysis for placenta samples

Sample code	Uninfected	Active acute	Active chronic	Past infection	General comments
PLR13		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PLR25			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PLR12		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PLR7		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PLR3			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PLR81		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PLR1				X	No parasite but haemozoin present with detached of syncytiotrophoblastic damage
PLR14				X	No parasite but haemozoin present with no histological changes
PL109			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL110		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL126			X		Parasitised red blood cells within syncytiotrophoblast layer with substantial amount of haemozoin
PL112		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL113	X				No parasite or haemozoin present/no histological changes
PL114		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL115		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin
PL116		X			Parasitised red blood cells within syncytiotrophoblast layer/deposition small of haemozoin

Appendix 8: Shows tight junction expression in relation to malaria categories

	Uninfected	Active Chronic	Active Acute	Past Infection	ZO-1 Expression	Claudin-4 Expression
PL11	x				++++	++++
PL111	x				++++	++++
PL14	x				++++	++++
PL22				X	+++	++++
PL35		x			+++	+++
PL9		x			+++	+++
PL122				x	++++	+++
PL23				x	+++	+++
PL25		x			++++	+++
PL29			x		++	++
PL37		x			++++	++++
PL38			x		+++	+++
PL46	x				++++	++++
PL50				x	+++	+++
PL57				x	+++	+++
PL59		x			++++	++++
PL79			x		++	+++
PL71				x	+++	+++
PL74				x	+++	+++
PL75		x			+++	+++
PL20				x	++++	++++
PL65		x			+++	++++

Appendix 9: Shows tight junction expression in relation to malaria categories

Sample	Uninfected	Active Chronic	Active Acute	Past Infection	ZO-1 Expression	Claudin-4 Expression
PL24		x			+++	++++
PL39		X			+++	+++
PL40		x			++	+++
PL44				X	+++	++
PL48	x				++++	++++
PL5			x		+++	+++
PL54				x	++++	++++
PL2			x		++	+++
PL4			x		+++	++
PL33			x		+++	+++
PL1			x		+++	++++
SPL10		x			+++	+++
PL21				X	++++	+++
PL23				x	++++	+++
PL27				x	++++	++++
PL28	X				++++	++++
PL29	x				+++	+++
PL40		x			++	++++
PL41		X			+++	++++
PL51	x				++++	+++
PL62		x			+++	+++
PL9				x	++++	++++

Appendix 10: Shows tight junction expression in relation to malaria categories

Sample	Uninfected	Active Chronic	Active Acute	Past Infection	ZO-1 Expression	Claudin-4 Expression
PL3			x		++	+++
PL54			x		++++	++
PL19				X	++	++
PL28	x				++++	+++
PL33		x			++	++
PL45				x	+++	+++
PL55		x			+++	+++
PL17	X				++++	+++
PL18			x		+++	++
PL21				x	+++	+++
PL24		x			+++	++
PL30	x				+++	+++
PL34	x				+++	++++
PL44				X	++	++++
PL52			X		++	++++
PL60			x		+++	++
PL67		x			++	+++
PL7	x				+++	++

Appendix 11: Shows tight junction expression in relation to malaria categories

Sample	Uninfected	Active Chronic	Active Acute	Past Infection	ZO-1 Expression	Claudin-4 Expression
PL47		x			+++	++
PL8				x	+++	+++
PL20			x		++	++
PL36	x				++++	++
PL6			x		+++	++
PL13		x			+++	+++
PL16				x	++++	++++
PL32	X				++++	+++
PL31		X			+++	+++
PLR14				X	+++	++
PLR				x	+++	+++
PLR81			x		++	+++
PLR3		X			++++	++++
PLR7			x		++	+++
PLR12			x		++	++++
PLR25		X			++	+++
PLR13			X		++	+++
PLR16				x	+++	+++
PLR15		x			+++	+++
PLR17				x	+++	+++
PLR20	x				++++	++++

Appendix 12: Shows tight junction expression in relation to malaria categories

Sample	Uninfected	Active Chronic	Active Acute	Past Infection	ZO-1 Expression	Claudin-4 Expression
PLS0			X		+++	++
PLS2				X	+++	+++
PLS3		X			+++	+++
PLS4		X			+++	+++
PLS5				X	+++	++++
PLS6		X			+++	++++
PLS7			X		+++	+++
PLS8	X				+++	+++
PLS9				X	++++	++++
PLR76	X				+++	+++
PLR6		x			++	+++
PLR11		x			++	+++
PLR9	x				++++	+++
PLR22				x	+++	+++

Appendix 13: Shows tight junction expression in relation to malaria categories

Sample	Uninfected	Active Chronic	Active Acute	Past Infection	ZO-1 Expression	Claudin-4 Expression
PL109		X			+++	++++
PL110			X		+++	++
PL126		X			+++	++++
PL112			X		+++	+++
PL113	X				++++	+++
PL114			X		+++	+++
PL115			X		+++	+++
PL116			x		++	+++
PL117			x		++	+++
PL122		x			++	++
PL132			X		++++	++++
PL125				X	++++	++++

Appendix 14: Shows a copy of questionnaire collected from participants

Questionnaire

Ministry of Science and Communication
National Centre for Research
Tropical Medicine Research Institute
Placental Malaria in Blue Nile State

Patient serial no: ……

DEMOGRAPHIC DATA

-Name
-Age:
Residence
Education level:

1: Illiterate …… 2: Primary school…… ..
3: Intermediate…… .. 4: Secondary……
5: University…… 6: Postgraduate……..

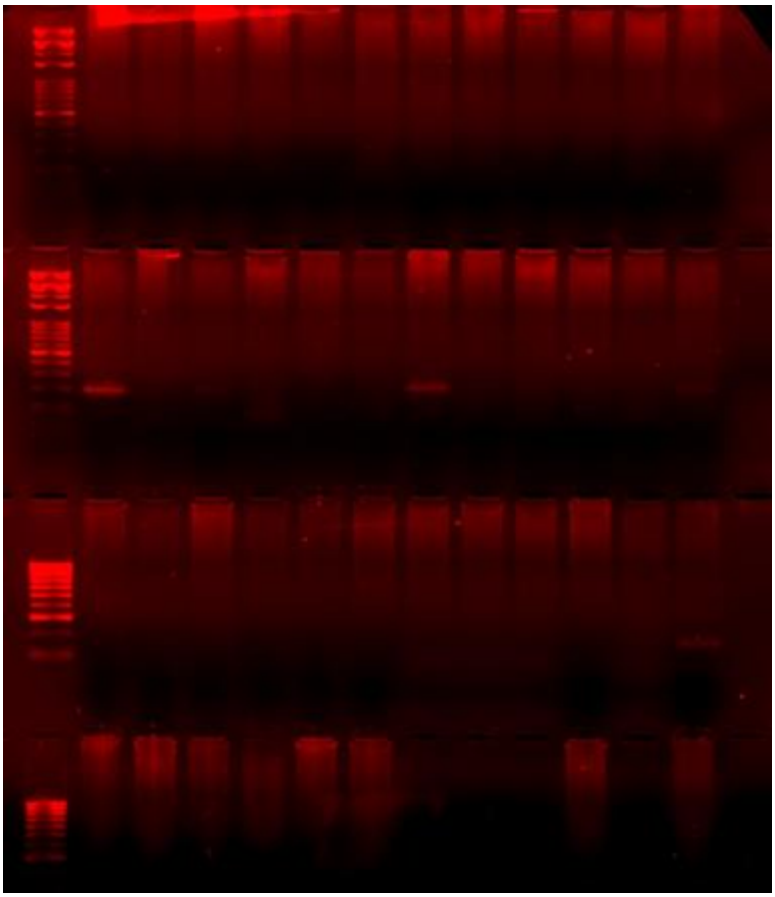
GRAVAIDITY:

(1) Primi …… (2) Secundi …… ..
(3) Multi…… (4) Grandmulti……

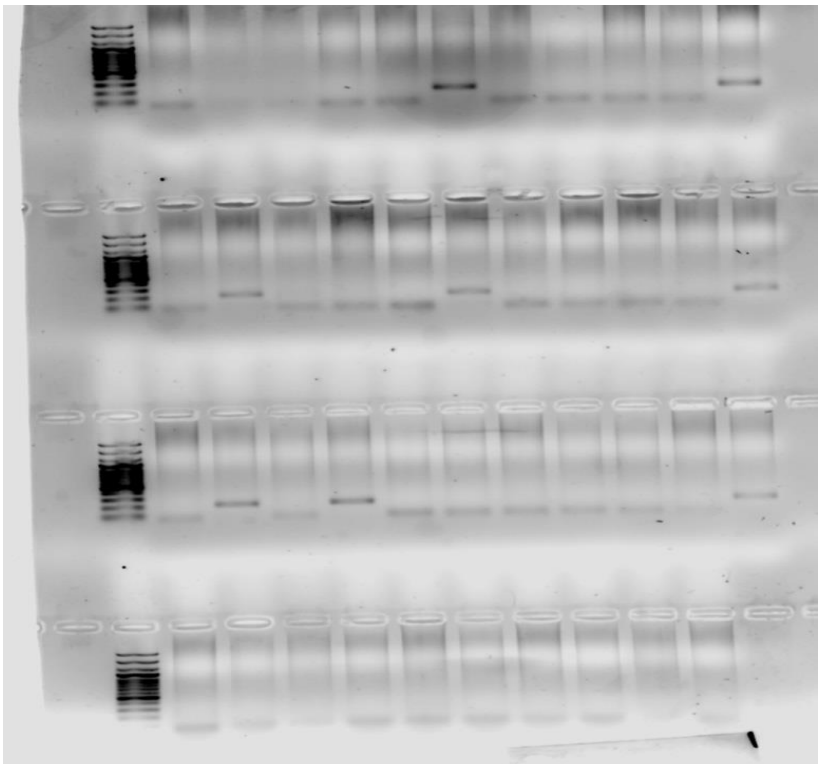
HISTORY of Malaria

Malaria attacks in pervious pregnancy: (1) Yes (2) No
No of attacks during the current pregnancy
Treatment Taken
Current malaria symptoms:
1: Fever 2: Headache 3: Nausea
4: Shivering 5: Vomiting 6: Diarrhoea
Weight of child (Kg)
Hb. (g/l) of mother
Sample collected:
1: BF 2. Dried spots 3. Placental slices
Results:
BF result
RDTs result:

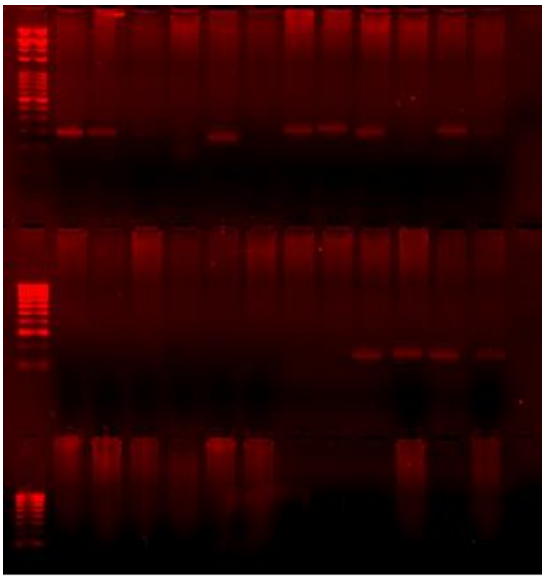
Appendix 15: Shows gel electrophoresis result for baby peripheral bloods



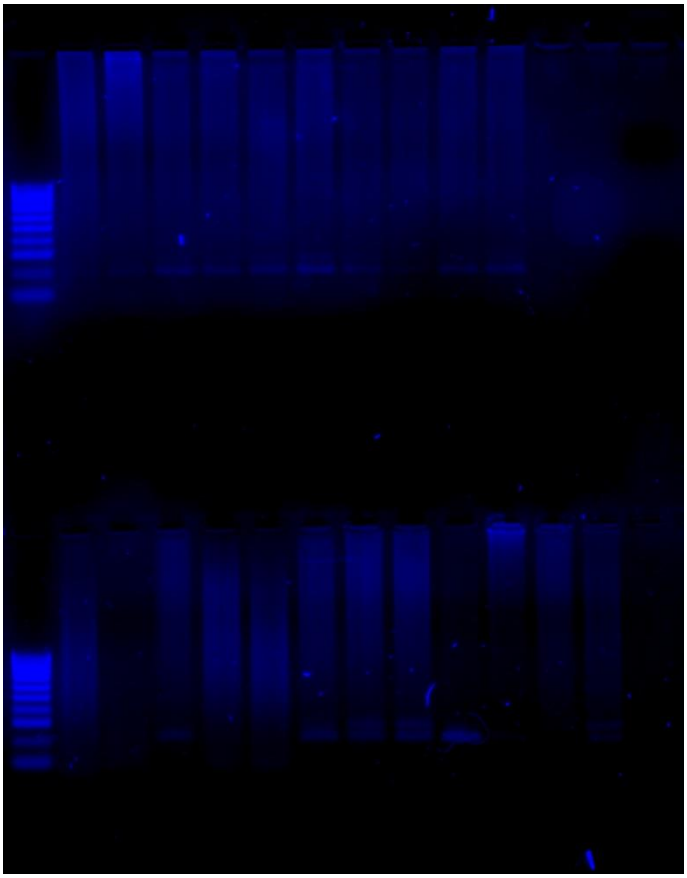
Appendix 16: Shows gel electrophoresis results for mother peripheral bloods



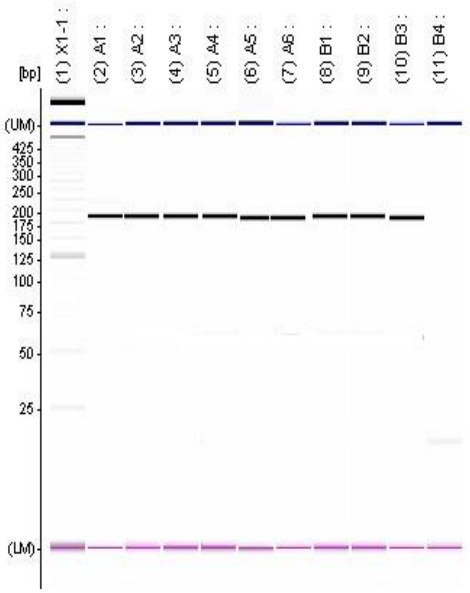
Appendix 17: Shows gel electrophoresis result for cord blood



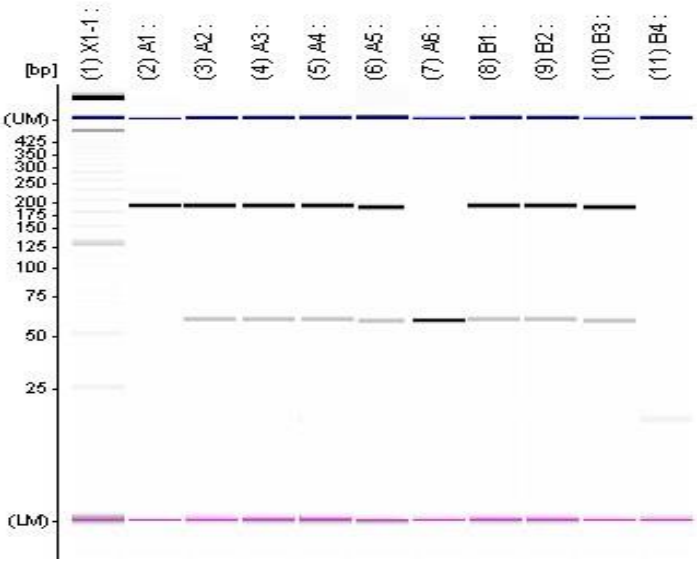
Appendix 18: Shows gel electrophoresis result for placental samples



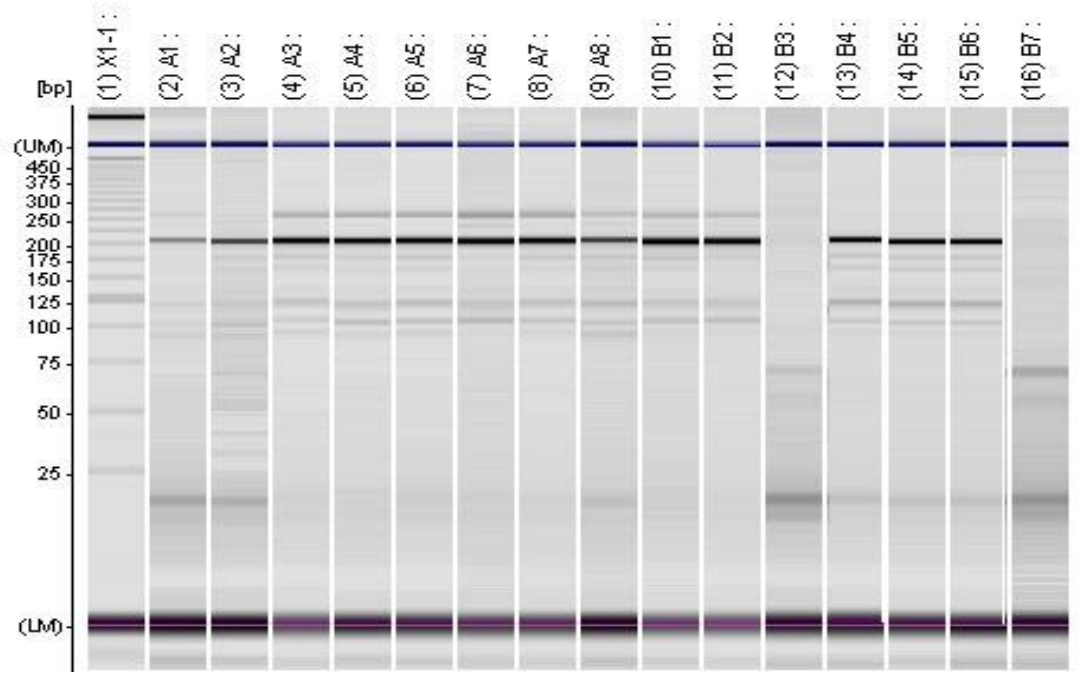
Appendix 19: Shows MultiNA results for baby peripheral bloods



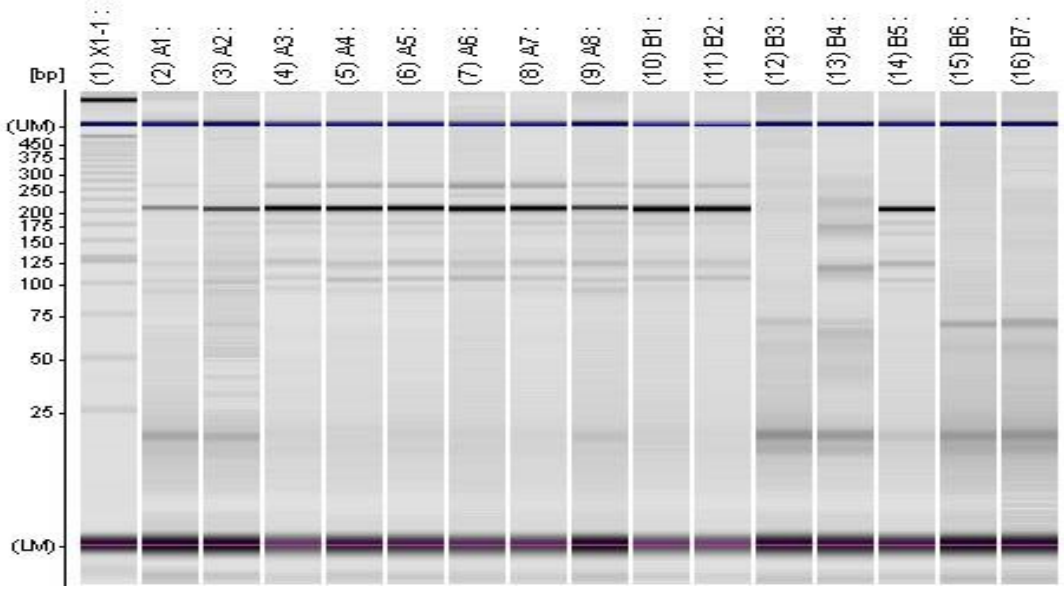
Appendix 20: Shows MultiNA results for mother peripheral bloods



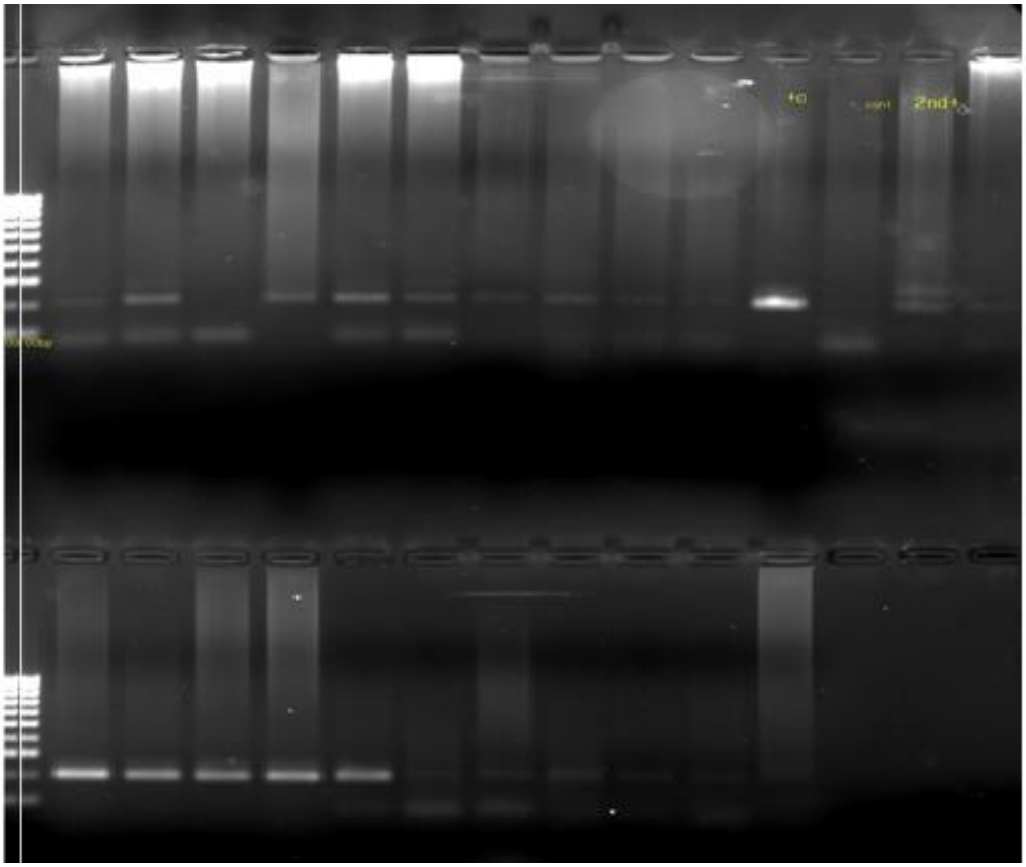
Appendix 21: Shows MultiNA confirms baby peripheral parasite



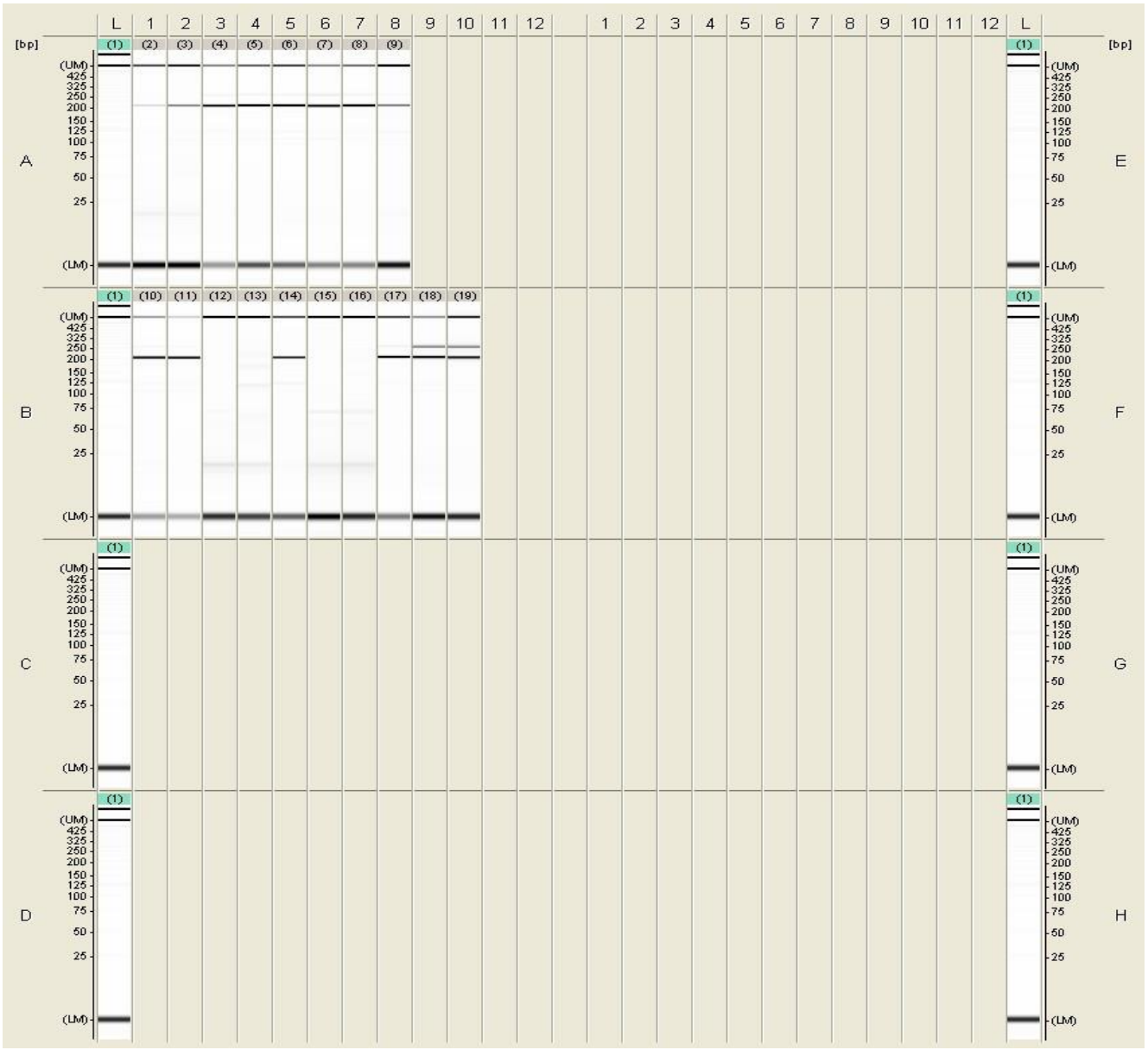
Appendix 22: Shows MultiNA confirms cord blood infection



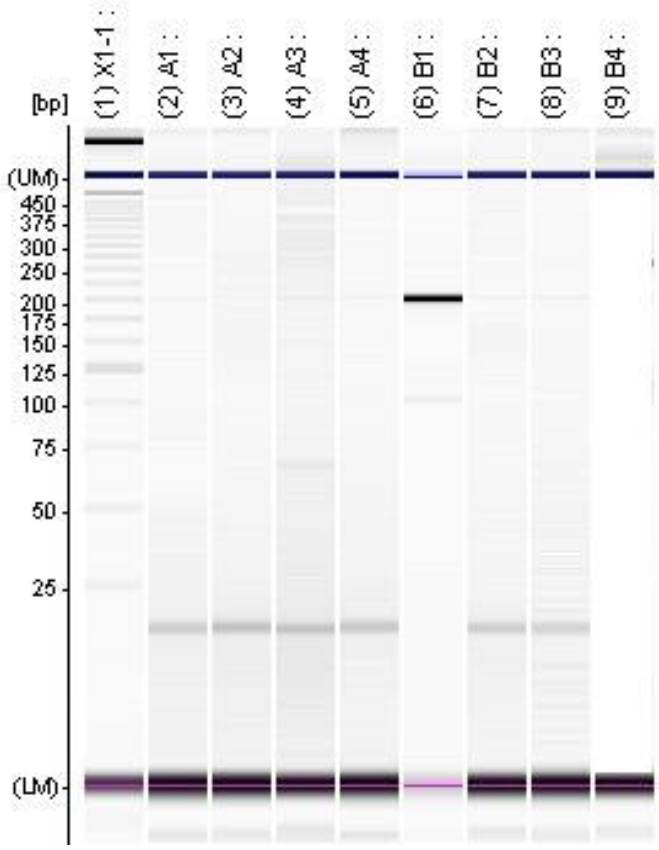
Appendix 23: Shows gel electrophoresis results for cord bloods



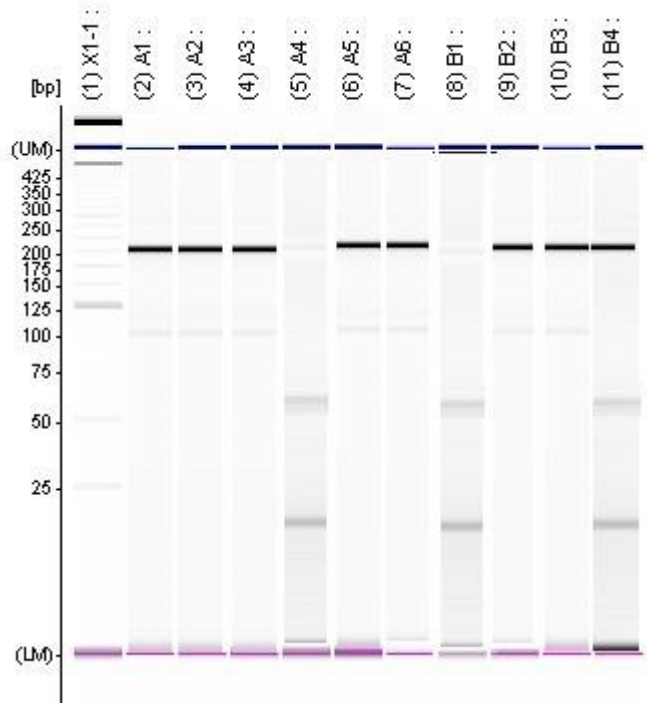
Appendix 24. Shows MultiNA results for placental blood


L 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12 L
[bp]
A
B
C
D
E
F
G
H
(UM)
425
325
250
200
150
125
100
75
50
25
(LM)

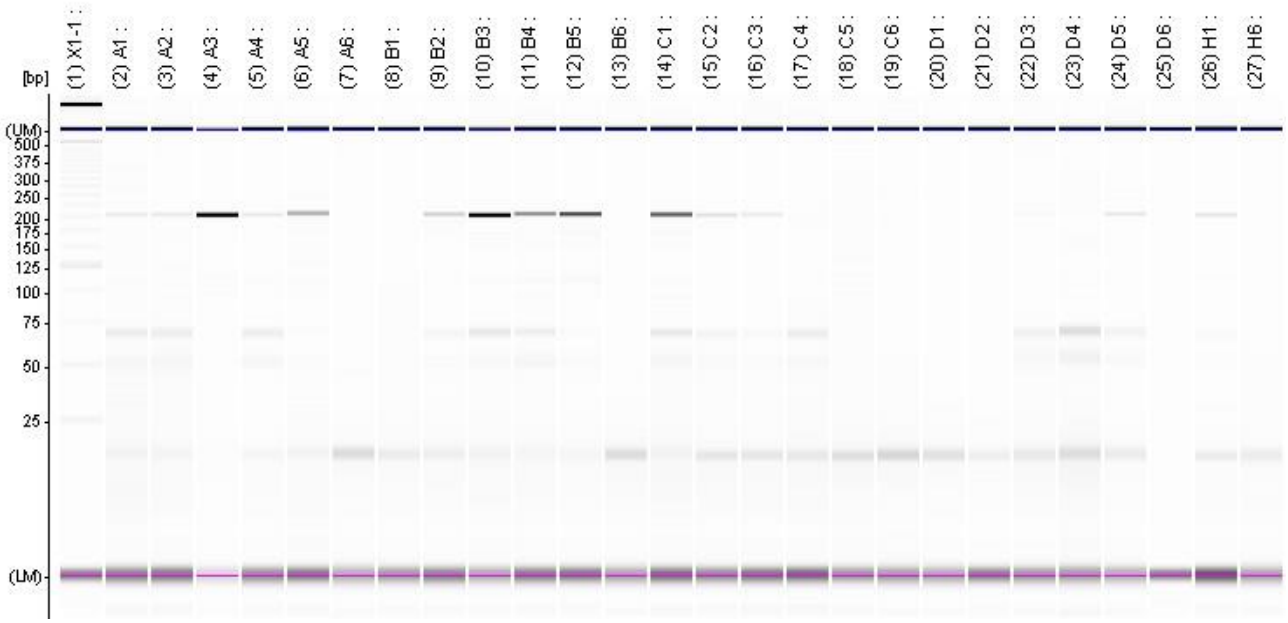
Appendix 25: Shows MultiNA results for positive control (B1)



Appendix 26. Shows MultiNA confirms cord's blood parasite



Appendix 27: Shows MultiNA confirms mother's peripheral blood



Appendix 28: Shows data sheet for study

sample number	Placenta PCR	Mother PB PCR	CordBlood PCR	Baby PB PCR	Age	Parity	Education	Hb gm/186ecilitre	ChildwKg	Mother Blood Film	Placent Blood Film	Baby Blood Film	CordBlood Film	Site
1	2	2	2	2	21	2	0	7.1	2.2	2	2	1	1	DH
2	1	1	1	1	35	4	0	11.3	3.1	1	1	1	2	DH
3	2	2	2	2	19	1	1	10.2	2.3	2	2	2	2	DH
4	2	2	2	1	17	1	1	11.2	2.1	1	2	1	2	DH
5	1	1	1	1	32	3	1	11.2	2.8	1	1	1	1	DH
6	2	2	2	1	24	2	1	11.1	2.4	2	2	1	2	DH
7	1	1	1	1	31	3	0	12.1	2.7	1	1	1	1	DH
8	2	2	2	1	20	2	1	10.2	2.2	1	2	1	2	DH
9	1	2	2	1	23	2	1	11.1	2.4	1	2	1	2	DH
10	2	2	1	1	24	3	1	12.1	2.3	1	2	1	2	DH
11	1	1	1	1	38	4	0	12.6	2.6	1	1	1	2	DH
12	1	1	1	1	21	1	3	11.8	3	1	2	1	2	DH
13	2	2	2	2	21	1	1	10.1	2.3	2	2	1	1	DH
14	2	2	2	2	20	1	1	10.3	2.4	2	2	1	1	DH
15	2	2	1	2	19	1	1	10.3	2.3	2	2	1	1	DH
16	1	1	1	1	31	3	0	12	2.9	1	1	1	1	DH
17	2	2	2	1	22	3	1	11.3	2.3	1	2	1	2	DH
18	2	2	1	1	28	3	0	11.7	2.8	2	1	1	1	DH
19	2	1	2	1	27	3	1	11.2	2.4	1	1	1	2	DH
20	1	1	1	1	18	1	1	10.3	2.4	1	1	1	1	DH
21	2	2	2	1	21	2	3	11.4	2.2	1	2	1	2	DH
22	2	2	2	1	23	2	1	10.2	2.4	1	1	1	2	DH
23	2	2	2	2	18	1	1	9.1	2.1	2	2	1	2	DH
24	1	1	1	1	31	4	1	12.1	2.9	1	1	1	1	DH
25	2	1	1	1	35	3	0	11.3	2.4	1	2	1	1	DH

26	2	2	2	1	26	3	1	11.8	2.8	2	2	1	2	DH
27	2	1	1	1	30	3	3	12.9	2.6	1	1	1	1	DH
28	1	1	1	1	36	3	3	13.5	3.5	1	1	1	1	DH
29	2	1	2	1	26	3	1	11.1	2.8	1	1	1	1	DH
30	2	2	2	1	19	1	1	10.4	2.4	2	2	1	1	DH
31	2	2	2	2	22	1	1	10.3	2.3	2	2	1	1	DH
32	1	1	1	1	37	4	0	13.8	3.2	1	1	1	2	DH
33	2	2	2	1	21	1	1	10.3	2.3	1	2	1	1	DH
34	2	2	2	2	17	1	1	11.1	2.3	2	2	2	1	DH
35	2	1	1	1	31	4	1	11.1	2.4	1	1	1	2	DH
36	2	2	1	1	23	2	0	10.2	2.4	1	2	1	2	DH
37	2	2	2	1	20	1	1	9.1	2.2	2	2	1	1	DH
38	2	1	1	1	28	3	1	11.8	3	1	1	1	2	DH
39	2	2	1	1	19	1	1	10.3	2.3	1	1	1	1	DH
40	2	1	1	1	40	4	0	11.4	2.6	1	2	1	1	DH
41	2	1	2	1	33	3	0	12.7	2.6	1	1	1	2	DH
42	1	1	1	1	35	4	1	11.4	3.2	1	1	1	1	DH
43	1	1	1	1	35	3	1	11.2	2.5	1	1	1	1	DH
44	1	1	1	1	37	4	0	12.1	2.9	1	1	1	1	DH
45	2	2	2	1	19	1	1	11.3	2.5	2	2	1	2	DH
46	1	1	1	1	26	3	1	11.9	2.8	1	1	1	1	DH
47	1	2	1	2	24	2	1	10.3	2.5	1	1	1	1	DH
48	1	1	1	1	30	2	3	12.8	3.3	1	1	1	1	DH
49	2	2	2	1	18	1	1	10.1	2.1	2	2	1	2	DH
50	1	1	1	1	23	2	0	12.1	2.6	1	1	1	2	DH
51	2	1	2	1	32	3	0	12.1	2.7	1	1	1	2	DH
52	1	1	1	1	33	3	3	15	2.9	1	1	1	2	DH
53	1	1	1	1	21	1	3	12	3.1	1	1	1	2	DH
54	1	1	1	1	31	3	0	11.7	2.8	1	1	1	2	DH
55	2	2	1	2	24	2	1	10.5	2.5	1	2	1	2	DH

56	1	1	1	1	32	3	3	12.4	3.3	1	1	1	1	DH
57	2	1	2	1	23	2	1	11.3	2.4	1	1	1	2	DH
58	2	1	2	2	19	1	1	9.3	2.4	2	2	1	2	DH
59	1	1	1	1	25	3	1	10.3	2.4	1	1	1	1	DH
60	2	1	2	2	18	1	1	10.7	2.4	2	2	2	2	DH
61	2	2	2	2	24	3	1	10.2	2.3	2	2	1	2	DH
62	2	1	1	1	26	3	1	10.2	2.4	1	2	1	1	DH
63	2	2	2	2	17	1	0	8	1.9	2	2	2	2	DH
64	2	1	1	1	28	3	1	11.2	2.3	1	1	1	2	DH
65	2	2	2	2	18	1	0	9.2	2.1	2	2	1	1	DH
66	2	2	2	1	19	2	1	9.6	2.3	2	2	1	2	DH
67	1	1	1	1	31	3	1	11.6	2.9	1	2	1	1	DH
68	2	2	2	1	20	1	1	10.2	2.3	2	2	1	2	DH
69	1	2	1	1	41	4	0	11.6	2.9	2	1	1	1	DH
70	2	1	2	1	26	3	0	12	2.6	2	2	1	2	DH
71	1	1	1	1	35	4	0	12.1	3.1	1	1	1	1	DH
72	2	2	2	2	24	2	1	11.1	2.4	2	2	1	1	DH
73	2	2	2	2	17	1	1	8.2	2.1	2	2	2	2	DH
74	1	1	1	1	20	2	3	11	3	1	1	1	1	DH
75	2	2	2	2	18	1	1	11.3	2.4	2	2	1	2	DH
76	2	1	1	1	35	4	0	12.1	2.6	1	1	1	1	DH
77	2	2	2	2	20	1	1	11.1	2.1	2	2	1	2	SC
78	2	2	2	2	22	2	1	11.2	2.4	2	2	1	2	SC
79	2	2	2	2	28	3	1	10.2	2.6	1	2	1	2	SC
80	1	1	1	1	26	2	3	12.2	3.4	1	1	1	2	SC
81	2	1	1	1	26	3	1	10.3	2.5	1	2	1	1	SC
82	2	2	2	2	23	2	1	10.7	2.4	1	1	1	2	SC
83	1	1	1	1	27	2	1	10.6	3.1	1	1	1	1	SC
84	2	1	2	1	27	3	1	10.3	2.6	1	2	1	2	SC
85	2	2	2	2	19	1	1	11	2.2	2	2	1	1	SC

86	2	1	1	1	23	2	3	11.1	2.3	1	1	1	1	SC
87	1	1	1	1	37	4	0	11.6	2.7	1	1	1	1	SC
88	2	1	2	2	28	4	1	11.3	2.2	2	2	1	2	SC
89	1	1	1	1	32	4	1	11.5	2.9	1	1	1	1	SC
90	2	1	2	1	22	2	3	11.3	2.5	1	1	1	2	SC
91	2	2	2	1	20	1	1	11.1	2.2	1	1	1	2	SC
92	1	1	1	1	41	4	0	12.7	2.8	1	1	1	1	SC
93	1	1	1	1	30	3	1	11.6	2.7	1	1	1	1	SC
94	2	1	2	1	18	1	1	9.9	2.2	2	1	1	2	SC
95	1	1	1	1	22	2	1	11.1	3.1	1	1	1	1	SC
96	2	1	1	1	26	3	0	10.8	2.5	1	1	1	1	SC
97	2	2	2	1	19	1	1	10.3	2.1	2	2	1	2	SC
98	2	2	2	1	23	2	1	10	2.2	2	2	1	2	SC
99	2	1	1	1	20	1	1	11.3	2.2	1	1	1	1	SC
100	2	1	1	2	17	1	1	8.4	2.1	2	2	1	1	SC
101	1	1	1	1	28	2	1	11.4	2.5	1	1	1	1	SC
102	1	1	1	1	37	4	1	12.2	3.4	1	1	1	1	SC
103	2	1	2	1	27	2	1	11.6	2.3	1	1	1	2	SC
104	2	2	2	1	23	3	3	11.2	2.5	1	1	1	2	SC
105	1	1	1	1	28	3	1	10.9	2.5	1	1	1	2	SC
106	1	1	1	1	31	2	1	11.4	3.3	1	1	1	1	SC
107	2	2	1	1	23	2	1	11.3	2.3	2	2	1	1	SC
108	2	1	2	1	20	2	1	10.5	2.4	1	2	1	2	SC
109	2	2	2	1	24	2	1	10.7	2.4	2	2	1	2	SC
110	2	1	2	1	26	2	1	11.1	2.7	1	1	1	2	SC
111	2	1	1	1	29	3	3	13.8	2.8	1	1	1	1	SC
112	2	2	2	2	44	4	0	11.6	2.1	2	2	2	2	SC
113	2	2	2	2	21	1	1	11.6	2.8	2	2	1	2	SC
114	2	1	2	1	27	4	1	11.6	2.7	1	1	1	2	SC
115	1	1	1	1	31	3	1	11.5	3.1	1	1	1	1	SC

116	1	1	1	1	28	4	1	11.6	2.7	1	1	1	1	SC
117	2	1	2	1	25	1	3	12.1	3.2	1	2	1	2	SC
118	2	2	2	1	20	1	1	10.9	2.7	2	2	1	2	SC
119	1	1	1	1	19	1	1	11.1	2.8	1	1	1	2	SC
120	1	1	1	1	26	2	3	12.5	2.5	1	1	1	1	SC
121	2	1	1	1	24	2	1	11.2	2.6	1	1	1	1	SC
122	2	2	2	1	23	2	1	11.1	2.9	2	2	1	2	SC
123	2	2	2	1	21	2	1	11.4	2.2	2	1	1	2	SC
124	1	1	1	1	24	3	1	11.9	2.8	1	1	1	1	SC
125	2	2	2	2	18	1	1	10.3	2.4	2	2	1	2	SC
126	2	2	2	2	20	1	1	8.1	2.2	2	2	2	2	SC
127	2	1	2	1	25	2	3	11.3	2.6	1	2	1	2	SC
128	1	1	1	1	28	1	1	11.7	2.9	1	1	1	1	SC
129	2	2	2	1	20	1	1	9.6	2.1	2	2	1	2	SC
130	2	2	2	1	18	1	1	10.3	2.4	2	2	1	2	DH
131	2	2	2	2	19	1	1	10.3	2.4	2	2	1	2	DH
132	1	1	1	1	31	3	0	11.7	2.6	1	1	1	2	DH
133	2	2	2	1	22	1	1	10.3	2.6	1	1	1	2	DH
134	2	2	2	1	18	1	1	9.5	2.4	2	2	1	2	DH
135	1	1	1	1	30	4	1	11.4	3	1	1	1	2	DH
136	2	1	2	1	24	2	3	11	2.7	1	1	1	2	DH
137	1	1	1	1	43	4	0	12.1	3.1	1	1	1	2	DH
138	2	2	2	1	19	1	1	10.3	2.2	2	2	1	2	DH
139	1	1	1	1	36	4	1	12.1	2.8	1	1	1	1	DH
140	1	1	1	1	23	1	1	12.7	2.8	1	1	1	1	DH
141	1	1	1	1	21	1	3	11.7	2.6	1	1	1	1	DH
142	1	1	1	1	28	1	3	12.2	3.1	1	1	1	1	DH
143	2	2	2	2	18	1	1	10.4	2.2	2	2	1	2	DH
144	1	1	1	1	30	1	1	11.6	3.2	1	1	1	1	DH
145	1	1	1	1	21	1	1	11.6	2.7	1	1	1	1	DH

146	1	1	1	1	22	2	1	12.1	3.3	1	1	1	2	DH
147	1	1	1	1	25	3	1	10.5	2.4	1	1	1	1	DH
148	1	1	1	1	33	4	0	11.9	2.9	1	1	1	1	DH
149	1	1	1	1	32	3	1	11.9	2.4	1	1	1	1	DH
150	2	2	2	1	20	1	1	10.3	2.2	2	2	1	2	DH
151	2	1	2	2	19	1	1	10.1	2.1	1	2	2	2	DH
152	1	1	1	1	28	1	1	13.1	2.9	1	1	1	1	DH
153	2	2	2	2	18	1	1	10.4	2.2	2	2	1	2	DH
154	2	1	2	1	25	1	0	11.8	2.7	1	1	1	2	DH
155	2	1	2	1	28	3	1	11.1	2.4	1	1	1	1	DH
156	2	1	1	1	22	1	3	10.5	2.3	1	2	1	1	DH
157	1	1	1	1	22	1	1	12	3	1	1	1	2	DH
158	2	1	1	1	29	3	0	10.5	2.4	1	2	1	1	SC
159	2	2	2	2	20	1	1	9.4	2.2	2	2	2	2	SC
160	2	2	2	2	24	1	1	10.1	2.3	2	2	1	2	SC
161	1	1	1	1	26	1	1	11.8	2.8	1	1	1	1	SC
162	2	2	1	1	29	2	3	11.9	2.6	1	1	1	1	SC
163	2	1	1	1	30	4	0	11.3	2.5	1	2	1	1	SC
164	1	1	1	1	27	3	1	11.3	2.7	1	1	1	1	SC
165	2	2	2	2	19	1	1	10	2.4	2	2	1	2	SC
166	2	2	2	1	19	1	1	10.2	2.1	2	2	1	2	SC
167	2	1	2	1	28	3	0	11.2	2.4	1	1	1	2	SC
168	1	1	1	1	35	4	0	11.9	3.1	1	1	1	1	SC
169	2	2	2	2	18	1	1	10.5	2.3	2	2	2	2	SC
170	2	2	2	1	24	1	3	11.1	2.3	2	2	1	2	SC
171	1	1	1	1	29	3	3	13.6	3.7	1	1	2	2	SC
172	1	1	1	1	23	1	1	11.3	3.1	1	1	1	1	SC
173	2	1	2	1	21	1	1	11.9	2.6	1	1	1	2	SC
174	2	2	2	2	18	1	1	10.1	2.2	2	2	2	2	SC
175	1	1	1	1	28	3	1	13.4	2.8	1	1	1	1	SC

176	1	1	1	1	30	3	1	11.6	2.5	1	2	1	1	SC
177	2	2	2	1	20	1	1	10.3	2.5	1	2	1	2	SC
178	2	2	2	2	29	3	1	11.9	2.4	1	1	2	2	SC
179	1	1	1	1	26	2	3	14.3	3.5	1	1	1	1	SC
180	1	1	1	1	26	2	3	12.1	2.6	1	1	1	1	SC
181	2	1	1	1	31	1	1	11.4	3.1	1	1	1	1	SC
182	2	1	2	1	32	3	1	11.8	2.3	1	1	1	2	SC
183	1	1	1	1	22	1	1	11.3	2.6	1	2	1	1	SC
184	1	1	1	1	25	1	3	12.8	3.5	1	1	1	2	SC
185	2	1	1	1	23	2	3	12.4	2.9	1	2	1	1	SC
186	1	1	1	1	22	1	3	11.9	2.4	1	1	1	2	SC
187	2	2	2	2	19	1	1	9.2	2.1	2	2	2	2	SC
188	2	2	2	1	26	2	3	11.2	2.6	1	1	1	2	SC
189	1	1	1	1	26	2	3	11.9	2.7	1	1	1	1	SC
190	2	1	1	1	32	3	1	10.7	2.3	1	2	1	1	SC
191	2	2	2	2	22	1	1	10.7	2.4	2	2	1	2	SC
192	2	2	1	1	21	1	1	10.2	2.2	1	2	1	1	SC
193	1	1	1	1	22	1	1	12.1	2.6	1	1	1	1	SC
194	1	1	1	1	27	2	3	13.5	3.6	1	1	1	1	SC
195	2	1	1	1	28	2	1	11.7	2.4	1	2	1	1	SC
196	2	2	2	2	19	1	1	9.2	2.1	2	2	2	2	SC
197	1	1	1	1	26	2	3	10.6	2.8	1	1	1	1	SC
198	2	2	2	2	17	1	0	8.6	2	2	2	2	2	SC
199	1	1	1	1	24	1	1	11.3	2.4	1	2	1	1	SC
200	2	2	2	2	18	1	0	9.1	2.3	2	2	2	2	SC
201	2	2	2	1	20	2	1	10.5	2.1	2	2	1	2	SC
202	2	2	2	2	17	1	0	9.4	2.2	2	2	2	2	SC
203	1	1	1	1	29	2	0	11.8	2.9	1	1	1	2	SC
204	2	2	2	2	18	1	1	10.1	2.4	2	2	2	2	SC
205	2	2	1	1	18	1	3	10.5	2.3	2	2	1	1	SC

206	2	2	2	2	22	1	3	11.2	2.6	1	2	1	2	SC
207	1	1	1	1	25	2	0	11.8	2.9	1	1	1	1	SC
208	2	2	2	2	18	1	1	10.1	2.4	2	2	2	2	SC
209	2	2	2	2	16	1	0	7.8	2.2	2	2	2	2	SC
210	2	2	1	1	24	2	3	10.2	2.3	2	2	1	1	SC
211	2	2	2	2	19	1	1	9.9	2.4	2	2	2	2	SC
212	1	1	1	1	35	4	0	12.1	3.2	1	1	1	1	SC
213	2	2	2	2	18	1	1	8.9	2.3	2	2	2	2	SC
214	1	1	1	1	22	2	3	11.4	2.3	1	2	1	1	SC
215	2	2	2	2	20	1	1	10	2.3	2	2	1	2	RH
216	2	2	1	1	24	1	1	10.3	2.4	2	2	1	1	RH
217	2	2	2	2	20	2	1	10.5	2.4	2	2	2	2	RH
218	2	1	1	1	24	1	1	11	2.5	1	2	1	1	RH
219	2	2	2	2	19	2	1	11.3	2.4	2	2	1	2	RH
220	1	2	1	1	21	1	1	11.5	2.4	2	1	1	1	RH
221	2	2	2	1	24	2	1	11.8	2.5	1	2	1	1	RH
222	1	1	1	1	20	1	1	12.7	3.1	1	1	1	1	SC
223	2	1	2	1	26	2	3	11.9	2.6	1	1	1	2	RH
224	2	2	2	1	24	2	1	11.6	3.2	2	2	1	2	RH
225	2	1	2	1	26	3	1	11.6	2.3	1	1	1	2	RH
226	1	1	1	1	31	2	0	11.4	3	1	1	1	1	RH
227	1	1	1	1	33	4	3	12.1	2.6	1	1	1	1	RH
228	1	1	2	2	20	2	1	11.7	2.4	1	1	1	2	RH
229	2	2	2	1	19	1	1	10.2	2.1	1	2	1	2	RH
230	2	2	2	2	21	2	3	10.2	2.3	2	1	1	2	RH
231	1	1	1	1	29	2	3	11.3	2.2	1	1	1	1	RH
232	1	1	1	1	37	4	0	11.6	2.7	1	1	1	1	RH
233	2	1	1	1	26	3	1	10.9	2.4	1	1	1	1	RH
234	1	1	1	1	33	3	0	11.9	3.1	1	1	1	1	RH
235	1	1	1	1	29	4	0	11.9	2.6	1	1	1	1	RH

236	1	1	1	1	22	1	1	11.8	2.8	1	1	1	1	RH
237	2	2	2	2	25	2	1	11.5	2.5	1	2	1	2	RH
238	2	2	2	1	23	1	1	10.9	2.2	2	2	1	2	RH
239	2	1	1	1	23	3	0	11.7	2.5	1	1	1	2	RH
240	2	1	1	1	36	3	0	11.2	2.6	1	1	1	2	RH
241	1	1	1	1	25	1	3	12.9	3.2	1	1	1	2	RH
242	1	1	1	1	24	1	3	12.4	3.9	1	1	1	1	RH
243	2	2	2	2	19	1	1	9.5	2.7	2	2	2	2	RH
244	1	1	1	1	26	2	3	11.9	2.8	1	1	1	1	RH
245	2	2	2	2	20	2	1	10.4	2.4	2	2	1	2	RH
246	2	2	1	1	37	4	1	11.5	2.4	1	2	1	1	RH
247	1	2	1	1	23	1	1	11.2	2.8	2	1	1	1	RH
248	1	1	1	1	20	1	1	12	2.6	1	1	1	1	RH
249	2	2	2	1	29	2	1	11.7	2.6	1	1	1	2	RH
250	1	1	1	1	24	2	3	12.3	2.8	1	1	1	1	RH
251	2	1	1	1	36	4	0	11.1	2.4	1	2	1	1	RH
252	1	1	1	1	29	1	0	11.8	2.3	1	1	1	1	RH
253	1	1	1	1	33	3	1	11.1	2.2	1	2	1	1	RH
254	2	2	2	2	20	1	1	11.1	2.5	2	2	1	2	RH
255	1	1	1	1	26	2	3	11.9	2.9	1	1	1	1	RH
256	1	1	1	1	24	2	1	12.9	3.6	1	1	1	1	RH
257	1	1	1	1	28	1	3	12.4	2.9	1	1	1	1	RH
258	2	2	2	2	37	4	0	11.1	2.7	2	2	2	2	RH
259	1	1	1	1	37	4	1	11.4	3.1	1	1	1	1	RH
260	1	1	1	1	21	1	3	11	2.5	1	1	1	2	RH
261	2	2	2	1	25	2	3	11.4	2.9	1	2	1	2	RH
262	1	1	1	1	28	3	1	12.3	2.8	1	1	1	1	RH
263	2	1	1	1	36	4	1	10.5	2.4	1	2	1	2	RH
264	2	2	2	2	22	1	1	11.6	2.6	2	2	1	2	RH
265	1	1	1	1	27	3	0	11.8	2.4	1	1	1	2	RH

266	2	2	2	1	19	1	1	10.3	2.3	2	2	1	2	RH
267	1	1	1	1	37	3	1	11.3	2.8	1	1	1	1	RH
268	2	2	2	1	21	1	1	12.2	3.3	2	2	1	2	RH
269	1	1	1	1	20	1	1	11.7	2.5	1	1	1	1	RH
270	1	1	1	1	24	2	3	12.7	2.7	1	1	1	1	RH
271	2	2	2	1	20	1	1	11.4	2.4	1	1	1	2	RH
272	1	1	1	1	27	1	3	12.9	3.1	1	1	1	1	RH
273	2	1	1	1	19	1	1	11.4	2.7	1	1	1	1	RH
274	1	1	1	1	33	3	1	11.6	2.5	1	1	1	1	RH
275	2	2	2	1	24	2	3	11.6	2.8	2	2	1	2	RH
276	1	1	1	1	31	1	3	12.1	2.9	1	1	1	1	RH
277	2	1	2	1	37	4	0	11.3	2.5	1	2	1	2	RH
278	1	1	1	1	23	1	3	11.7	2.9	1	1	1	1	RH
279	1	1	1	1	25	2	3	12.3	2.5	1	1	1	1	RH
280	2	2	2	1	20	1	1	11.5	2.7	2	2	1	2	RH
281	1	1	1	1	24	1	3	11.8	2.7	1	1	1	1	RH
282	1	1	1	1	29	3	1	12.1	2.3	1	1	1	1	RH
283	2	2	2	2	22	2	1	9.7	2.1	1	2	1	2	RH
284	1	1	1	1	27	3	1	11.7	3.1	1	1	1	1	RH
285	1	1	1	1	22	1	3	12.5	2.7	1	1	1	1	RH
286	2	1	2	1	23	2	1	10.8	2.4	1	1	1	2	RH
287	1	1	1	1	27	2	1	11.3	2.7	1	1	1	1	RH
288	2	2	1	1	24	2	3	11.4	2.9	1	1	1	1	RH
289	1	1	1	1	28	1	3	12.3	2.8	1	1	1	1	RH
290	1	1	1	1	22	2	1	11.8	2.7	1	1	1	1	RH
291	2	2	2	1	20	1	1	11.7	2.5	2	2	1	2	RH
292	2	2	2	1	21	2	1	11.6	2.4	2	2	1	2	RH
293	1	1	1	1	26	3	1	12.1	2.8	1	1	1	1	RH
294	2	1	1	1	26	2	0	12.1	2.6	1	1	1	1	RH
295	1	1	1	1	33	4	1	13.3	3	1	1	1	1	RH

296	2	1	1	1	29	2	3	11.8	2.8	1	1	1	1	RH
297	2	2	2	2	19	1	1	11.2	2.5	2	2	1	2	RH
298	2	2	2	1	23	2	1	12.1	2.3	2	2	1	2	RH
299	1	1	1	1	28	2	1	12.3	2.6	1	1	1	1	RH
300	1	1	1	1	23	2	1	11.3	2.7	1	1	1	1	RH
301	2	2	2	1	19	1	1	11.2	2.4	2	2	1	2	RH
302	1	1	1	1	25	2	1	11.3	2.7	1	1	1	2	SC
303	2	2	1	1	18	1	1	10.3	2.3	2	2	1	2	SC
304	1	1	1	1	23	2	1	11.3	2.4	1	1	1	2	SC
305	2	1	1	1	30	3	1	11.9	2.8	1	1	1	2	SC
306	1	1	1	1	21	1	3	11.8	2.7	1	1	1	2	SC
307	2	2	2	1	33	4	0	11.2	2.4	1	1	1	2	SC
308	1	1	1	1	24	2	3	12.7	3.3	1	1	1	1	SC
309	1	1	1	1	24	1	3	12.4	2.7	1	1	1	1	DH
310	2	2	2	2	18	1	1	10.2	2.1	2	2	1	2	DH
311	1	1	1	1	29	3	1	11.8	3.1	1	1	1	1	DH
312	1	1	1	1	27	2	3	12.4	3.2	1	1	1	1	DH
313	1	1	1	1	23	1	3	12.5	2.5	1	1	1	1	DH
314	1	1	1	1	29	2	3	12.7	2.7	1	1	1	1	DH
315	2	2	2	1	20	1	1	11.7	2.4	2	2	1	2	DH
316	1	1	1	1	29	3	1	11.4	2.3	1	2	1	1	DH
317	1	1	1	1	22	2	1	13.1	3.4	1	1	1	1	DH
318	1	1	1	1	30	4	0	12.1	3.1	1	1	1	1	RH
319	2	1	2	1	29	3	1	11.2	2.7	1	2	1	2	RH
320	1	1	1	1	24	2	1	12.4	2.6	1	1	1	1	RH
321	2	2	2	1	22	1	3	11.2	2.3	1	2	1	2	RH
322	1	1	1	1	20	1	3	11.8	2.8	1	1	1	1	SC
323	1	1	1	1	24	2	3	12.1	3.4	1	1	1	1	SC
324	1	1	1	1	29	1	3	11.9	2.9	1	1	1	1	SC
325	2	1	2	1	31	3	1	11.3	2.7	1	1	1	2	SC

326	1	1	1	1	24	1	3	12.5	3.5	1	1	1	2	SC
327	2	2	2	1	27	2	3	11.2	2.4	2	2	1	2	SC
328	1	1	1	1	31	4	0	11.9	2.8	1	1	1	1	SC
329	2	2	1	1	19	1	1	10.4	2.2	1	2	1	2	DH
330	1	1	1	1	28	2	3	12.7	2.8	1	1	1	1	SC
331	2	1	2	1	23	1	3	11.6	2.5	1	1	1	2	SC
332	2	2	2	2	20	2	1	10	2.1	2	2	2	2	SC
333	1	1	1	1	33	3	1	11.6	2.8	1	2	1	1	SC
334	2	2	2	1	28	2	3	11.3	2.6	1	2	1	2	SC
335	2	2	2	1	21	2	3	11.2	2.7	1	1	1	2	SC
336	1	1	1	1	29	3	1	11.9	2.8	1	1	1	2	SC

Appendix 29: Shows data sheet for study

Placental sample	Uninfected	Active acute	Active chronic	Past - infection	combinedBtoE	ZO-1 expression	Claudin - 4 expression	Placenta PCR	Mother Peipheral blood PCR	Cord blood PCR	Baby peripheral Blood PCR	Age	Gravidity	Education
PL11	1				1	4	4	2	1	2	1	27	3	1
PL111	1				1	4	4	2	1	1	1	28	2	1
PL14	1				1	4	4	2	1	1	1	35	3	0
PL22				4	4	3	4	2	1	2	1	33	3	0
PL35			3		3	3	3	2	1	1	1	28	3	1
PL9			3		3	3	3	2	2	1	2	19	1	1
PL122				4	4	4	3	2	2	1	1	24	1	1
PL23				4	4	3	3	1	1	1	1	35	3	1
PL25			3		3	4	3	1	2	1	2	24	2	1
PL29		2			2	2	2	1	1	1	1	31	3	0
PL37			3		3	4	4	2	2	2	1	20	1	1
PL38		2			2	3	3	2	1	2	1	26	3	0
PL46	1				1	4	4	1	1	1	1	27	2	1
PL50				4	4	3	3	2	1	2	1	22	2	3
PL57				4	4	3	3	1	1	1	1	37	4	1
PL59			3		3	4	4	2	2	1	1	23	2	1
PL79		2			2	2	3	1	1	1	1	30	4	1
PL71				4	4	3	3	2	2	2	2	20	1	1
PL74				4	4	3	3	2	2	2	1	20	1	1
PL75			3		3	3	3	2	2	2	1	18	1	1
PL20				4	4	4	4	2	2	2	1	20	1	1
PL65			3		3	3	4	1	1	1	1	19	1	1
PL24			3		3	3	4	2	2	2	1	19	1	1
PL39			3		3	3	3	1	1	1	1	35	4	0
PL40			3		3	2	3	2	2	2	2	24	2	1

PL44				4	4	3	2	2	2	2	2	28	3	1
PL48	1				1	4	4	2	1	1	1	23	2	3
PL5		2			2	3	3	2	2	2	1	20	2	1
PL54				4	4	4	4	2	1	1	1	26	3	0
PL2		2			2	2	4	2	2	2	2	19	1	1
PL4		2			2	3	2	2	2	2	1	24	2	1
PL33				4	4	3	3	2	1	2	2	18	1	1
PL1		2			2	3	4	2	2	2	2	21	2	0
PL10			3		3	3	3	2	2	2	1	22	3	1
PL21				4	4	4	3	2	2	1	1	19	1	1
PL23				4	4	4	3	2	2	2	1	19	2	1
PL27				4	4	4	4	2	1	2	1	32	3	0
PL28	1				1	4	4	1	1	1	1	33	3	3
PL29	1				1	3	3	1	1	1	1	31	3	0
PL40			3		3	2	4	2	2	2	2	24	2	1
PL41			3		3	3	4	1	1	1	1	20	2	3
PL51	1				1	4	3	1	1	1	1	41	3	0
PL62			3		3	3	3	2	2	2	2	21	1	1
PL9				4	4	4	4	2	2	1	2	19	1	1
PL3		2			2	2	3	2	2	2	1	17	1	1
PL54		2			2	4	2	2	1	1	1	26	3	0
PL19				4	4	2	2	2	2	1	1	23	2	0
PL28	1				1	4	3	1	1	1	1	33	3	3
PL33			3		3	2	2	2	1	2	2	18	1	1
PL45				4	4	3	3	2	1	1	1	26	3	1
PL55			3		3	3	3	2	2	2	1	23	2	1
PL17	1				1	4	3	1	1	1	1	37	4	0
PL18		2			2	3	2	2	2	2	2	17	1	1
PL21		2			2	3	3	2	2	1	1	19	1	1
PL24				4	4	3	2	2	2	2	1	19	1	1

PL30			3		3	3	3	2	2	1	2	24	2	1
PL34	1				1	3	4	2	1	1	1	26	3	1
PL44	1				1	2	4	2	2	2	2	28	3	1
PL52		2			2	2	4	2	1	2	1	18	1	1
PL60		2			2	3	2	2	2	2	1	24	2	1
PL67			3		3	2	3	2	2	2	1	21	2	1
PL7				4	4	3	2	1	1	1	1	38	4	0
PL47				4	4	3	2	2	1	2	1	27	3	1
PL8		2			2	3	3	2	2	2	2	21	1	1
PL20		2			2	2	2	2	2	2	1	20	1	1
PL36			3		3	4	2	2	2	2	1	19	2	1
PL6	1				1	3	2	1	2	2	1	23	2	1
PL13			3		3	3	3	2	2	2	2	18	1	1
PL16				4	4	4	4	2	2	2	1	19	1	1
PL32		2			2	4	3	2	1	2	2	19	1	1
PL31			3		3	3	3	2	1	2	1	23	2	1
PL80		2			2	3	2	1	1	1	1	43	4	0
PL82			3		3	3	3	1	1	1	1	23	1	1
PL83		2			2	3	3	1	1	1	1	28	1	3
PL84	1				1	3	3	1	1	1	1	30	1	1
PL85		2			2	3	4	1	1	1	1	22	2	1
PL86		2			2	3	4	1	1	1	1	33	4	0
PL87			3		3	3	3	2	2	2	1	20	1	1
PL88		2			2	3	3	1	1	1	1	28	1	1
PL89				4	4	4	4	2	2	2	1	21	2	3
PLR76	1				1	3	3	2	2	2	1	21	2	3
PLR6			3		3	2	3	2	2	1	1	24	3	1
PLR11			3		3	2	3	1	1	1	1	18	1	1
PLR9	1				1	4	3	1	1	1	1	31	3	0
PLR22				4	4	3	3	1	1	1	1	35	4	1

PLR20	1				1	4	4	2	1	1	1	28	3	1
PLR17				4	4	3	3	2	2	2	1	21	1	1
PLR15			3		3	3	3	2	1	2	1	26	3	1
PLR16				4	4	3	3	2	2	2	2	22	1	1
PLR13		2			2	2	3	1	1	1	1	31	3	1
PLR25			3		3	2	3	1	1	1	1	30	2	3
PLR12		2			2	2	4	2	2	2	1	23	2	1
PLR7		2			2	2	3	1	1	1	1	32	3	1
PLR3			3		3	4	4	1	1	1	1	36	4	1
PLR81		2			2	2	4	1	1	1	1	36	4	1
PLR1				4	4	3	3	1	1	1	1	35	4	0
PLR14				4	4	3	2	2	2	2	1	26	3	1
PL109			3		3	3	4	2	2	2	2	22	1	1
PL110		2			2	3	2	1	1	1	1	22	1	1
PL126			3		3	3	4	2	2	2	1	24	2	1
PL112		2			2	3	3	1	1	1	1	26	2	3
PL113	1				1	4	3	1	1	1	1	24	1	1
PL114		2			2	3	3	2	2	2	1	20	2	1
PL115		2			2	3	3	1	1	1	1	29	2	0
PL116		2			2	2	3	2	2	1	1	18	1	3
PL117		2			2	2	3	1	1	1	1	25	2	0
PL122			3		3	2	2	2	2	1	1	24	1	1
PL123				4	4	4	4	2	1	1	1	24	1	1
PL132		2			2	4	4	2	2	2	1	23	1	1
PL125				4	4	4	4	1	1	1	1	20	1	1

Appendix 30: Shows data sheet for study

Hbgm/d	Child Wieght	Mother blood film	Placenta blood film	Baby blood film	Mother Blood Film	Placenta Blood Film	Baby Blood Film
11.2	2.4	0	0	0	1	1	1
11.7	2.4	0	1	0	1	2	1
11.3	2.4	0	1	0	1	2	1
12.7	2.6	0	0	0	1	1	1
11.2	2.3	0	0	0	1	1	1
10.3	2.3	1	1	0	2	2	1
10.3	2.4	1	1	0	2	2	1
11.2	2.5	0	0	0	1	1	1
10.3	2.5	0	0	0	1	1	1
11.7	2.8	0	0	0	1	1	1
10.2	2.3	1	1	0	2	2	1
12	2.6	1	1	0	2	2	1
10.6	3.1	0	0	0	1	1	1
11.3	2.5	0	0	0	1	1	1
12.2	3.4	0	0	0	1	1	1
11.3	2.3	1	1	0	2	2	1
11.4	3	0	0	0	1	1	1
8.1	2.2	1	1	1	2	2	2
9.6	2.1	1	1	0	2	2	1
10.3	2.4	1	1	0	2	2	1
9.1	2.2	1	1	0	2	2	1
11.1	2.8	0	0	0	1	1	1
11.3	2.5	1	1	0	2	2	1
12.1	3.1	0	0	0	1	1	1

11.1	2.4	1	1	0	2	2	1
10.2	2.6	0	1	0	1	2	1
11.1	2.3	0	0	0	1	1	1
10.2	2.2	0	1	0	1	2	1
10.8	2.5	0	0	0	1	1	1
10.2	2.3	1	1	1	2	2	2
11.1	2.4	1	1	0	2	2	1
10.7	2.4	1	1	1	2	2	2
7.1	2.2	1	1	0	2	2	1
11.3	2.3	0	1	0	1	2	1
10.3	2.3	0	0	0	1	1	1
9.6	2.3	1	1	0	2	2	1
12.1	2.7	0	0	0	1	1	1
15	2.9	0	0	0	1	1	1
11.7	2.8	0	0	0	1	1	1
11.1	2.4	1	1	0	2	2	1
11	3	0	0	0	1	1	1
12.7	2.8	0	0	0	1	1	1
11.6	2.8	1	1	0	2	2	1
10.3	2.3	1	1	0	2	2	1
11.2	2.1	0	1	0	1	2	1
10.8	2.5	0	0	0	1	1	1
10.2	2.4	0	1	0	1	2	1
15	2.9	0	0	0	1	1	1
10.7	2.4	1	1	1	2	2	2
10.3	2.5	0	1	0	1	2	1
10	2.2	1	1	0	2	2	1
13.8	3.2	0	0	0	1	1	1
11.1	2.3	1	1	1	2	2	2
10.3	2.3	0	0	0	1	1	1

11.3	2.5	1	1	0	2	2	1
10.5	2.5	0	1	0	1	2	1
10.2	2.4	0	1	0	1	2	1
10.2	2.6	0	1	0	1	2	1
9.9	2.2	1	0	0	2	1	1
10.7	2.4	1	1	0	2	2	1
11.4	2.2	1	0	0	2	1	1
12.6	2.6	0	0	0	1	1	1
10.3	2.6	0	1	0	1	2	1
10.1	2.3	1	1	0	2	2	1
9.1	2.2	1	1	0	2	2	1
9.6	2.3	1	1	0	2	2	1
11.1	2.4	0	1	0	1	2	1
9.1	2.1	1	1	0	2	2	1
10.4	2.4	1	1	0	2	2	1
9.4	2.4	1	1	0	2	2	1
11.3	2.4	0	0	0	1	1	1
12.2	3.1	0	0	0	1	1	1
12.7	2.8	0	0	0	1	1	1
12.2	3.1	0	0	0	1	1	1
11.6	3.2	0	0	0	1	1	1
12.1	3.3	0	0	0	1	1	1
11.9	2.9	0	0	0	1	1	1
10.3	2.2	1	1	0	2	2	1
13.1	2.9	0	0	0	1	1	1
11.2	2.7	0	0	0	1	1	1
11.2	2.7	0	0	0	1	1	1
12.1	2.3	0	1	0	1	2	1
10.3	2.4	0	0	0	1	1	1
12	2.9	0	0	0	1	1	1

11.4	3.2	0	0	0	1	1	1
11.8	3	0	0	0	1	1	1
10.3	2.3	0	1	0	1	2	1
11.1	2.8	0	0	0	1	1	1
10.3	2.3	1	1	0	2	2	1
12.1	2.9	0	0	0	1	1	1
12.8	3.3	0	0	0	1	1	1
10.2	2.4	0	0	0	1	1	1
11.2	2.8	0	0	0	1	1	1
12.1	2.8	0	0	0	1	1	1
12.1	2.8	0	0	0	1	1	1
11.3	3.1	0	0	0	1	1	1
11.8	2.8	1	1	0	2	2	1
10.7	2.4	1	1	0	2	2	1
12.1	2.6	0	0	0	1	1	1
11.6	3.2	1	1	0	2	2	1
10.6	2.8	0	1	0	1	2	1
11.3	2.4	0	1	0	1	2	1
10.5	2.1	1	1	0	2	2	1
11.8	2.9	0	0	0	1	1	1
10.5	2.3	1	1	0	2	2	1
10.8	2.9	0	0	0	1	1	1
10.3	2.4	1	1	0	2	2	1
11	2.5	0	1	0	1	2	1
10.9	2.2	1	1	0	2	2	1
12.7	3.1	0	0	0	1	1	1