STUDY ON CONGENITALLY ACQUIRED *PLASMODIUM FALCIPARUM* INFECTION IN NEONATES IN MUHEZA DISTRICT, TANZANIA

BY

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ABSTRACT

The study aimed to determine if infants diagnosed with malaria parasites in the age below three months acquire the infection congenitally from their mothers through the placenta. Placenta blood, cord blood samples and blood from infants born of mothers diagnosed with placental malaria by blood smear and presented clinical malaria below three months of age were examined by PCR amplification, PCR-RFLP and sequenced. Prevalence of *Plasmodium falciparum* by PCR in the placenta and cord were 9.2% and 65%, respectively and 18 (19.1%) of infants born from mothers diagnosed with placental malaria developed clinical malaria below three months of age. Placental blood and cord blood sample, and placental blood and blood samples of infants below three months that shared the same band size by PCR and fragments size by PCR-RFLP were considered to be genetically related. Though sequencing results confirm differently that, sharing band size and fragments size between samples does not confirm that the parasites are genetically related. Six pairs (40%) out of 14 pairs of placental blood and cord blood samples that shared band size and fragments size, after sequencing were genetically unrelated while eight pairs (60%) were genetically related which is an indication of transplacental transmission of malaria parasites to the cord. One pair (14.3%) of sequenced placental blood samples and blood samples of infants below three months were genetically related. This showed that the malaria parasite that crossed from the placenta to the infants through the cord caused congenital malaria. Over three-fourths (79.8%) of newborn infants delivered from mothers with placental malaria were below normal gestation age and 14.9% of newborn infants had low birth weights. Some 42.5% of primigravidae were found to be parasitized with *P.falciparum* in the placental. A higher proportional of infants from primigravid were frequently infected with malaria while infants from

multigravid observed to acquire malaria infection early in life. Placental malaria, which leads to cord malaria, observed to significantly decrease (P <0.001) as gravid increases.

DECLARATION

I, Grace Mwangoka, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and has not been submitted for a degree award in any other University.

Signature:.....G. W. Mwangoka.....

Date:.....7th November 2006.....

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DEDICATION

To my beloved parents, Wynn Mwangoka and Aipya Kileo for their love, prayers and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS AND SYMBOLS

^{o}C	Degree Celcius
%	Percentage
µl	microliter
µM	micromole
bp	Base pair
CD 4	Cluster of differentiation antigen 4
CD 36	Cluster of differentiation antigen 36
Cm	Centimeter
CSA	Chondroitin sulfate A
DDH	District Designated Hospital
DNA	Deoxyribonucleic acid
RR	Relative Risk
g	gram
ICAM-1	Intercellular adhesion molecule 1
IgG	Immunoglobulin of class G
IgM	Immunoglobulin of class M
IPT	Intermittent presumptive therapy
iRBC	Infected Red blood cells
KCl	Potassium Chloride
Kda	Kilo Dalton
m	meter
mg	milligram
$Mgcl_2$	Magnesium Chloride
ml	milliliter

min	Minutes
mm	millimeter
mM	milli mole
MSP-1	Merozoites Surface Protein 1
MSP-2	Merozoites Surface Protein 2
MOMS	Mother Offspring Malaria Study
PAM	Pregnancy associated malaria
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
RFLP	Restriction Fragments length Polymorphism
SBRI	Seattle Biomedical Research Institute
SUA	Sokoine University of Agriculture
Taq	Thermus acquaticus
Tris HCl	Tris Hydrogen Chloride
USA	United State of America
U	Unit
Vs	Versus
VSA	variant surface antigens
WHO	World Health Organization

CHAPTER ONE

1 INTRODUCTION

1.1 Background information

Malaria is a systemic, febrile disease mainly caused by *Plasmodium falciparum* in Sub-Saharan African countries. Malaria is endemic in 103 countries where more than 2000 million people live exposed to the infection (Menendez, 1995). It is estimated that there are between 300-500 million cases of malaria globally each year and these result in about 1-2 million deaths, mainly in children less than 5 years of age living in Sub-Saharan Africa (Snow *et al.*, 2001). Malaria in pregnancy is a major cause of poor outcomes for both mother and fetus and malaria induced low birth weight, is a major risk factor for death during early life. It is estimated that between 62,000-363,000(38%) deaths in Sub-Saharan African infants alone each year are caused by malaria (Murphy *et al.*, 2001). Besides children, some 24 million women who become pregnant each year in malaria endemic regions are at increased risk of being infected with *P. falciparum* malaria and its associated complications (McGregor *et al.*, 1983). In some Sub-Saharan African countries endemic for malaria, almost half of all primigravidae will be parasitaemic at their first antenatal visit (Menendez, 1995). The reasons for increased susceptibility to malaria for this particular group are not clearly known. However, several mechanisms are likely to be involved as suggested by Fried and Duffy (1996).

Generally pregnant women are more vulnerable to malaria infection that may induce maternal anemia, low birth weight, fetal death or premature labor and infrequently clinical malaria of the neonate (Ahmed *et al.*, 1998; White *et al.*, 1998). The

Plasmodium parasite tends to sequester and multiply within the placenta even though undetectable in a peripheral blood smear of the pregnant woman (Leke *et al.*, 1999). Transplacental transmission of the *Plasmodium* parasite occurs when the placenta is infected, and even though the neonate seems healthy and asymptomatic, parasitaemia in the umbilical cord blood is commonly found (Lehner and Andrews, 1988; Redd *et al.*, 1996). Low levels of maternal peripheral blood parasitaemia is strongly correlated with placental parasitaemia, which may indicate a greater risk for umbilical cord blood parasitaemia and higher probability of mother to child parasite transmission (Redd *et al.*, 1996). There are also studies documenting fetal malarial infections as a result of maternal malaria during pregnancy (Lehner and Andrews 1988; Larkin and Thuma 1991; Fischer 1997).

In a holoendemic area of Kenya Aaron *et al.*, (2000) detected *Plasmodium* single species infection in maternal peripheral blood (3.4%) using microscopy and there were no detection of *Plasmodium* species in cord blood. In contrast, by PCR maternal blood samples showed a prevalence of 48% for *P. falciparum*, 25% for *P. malariae*, and 24% for *P. ovale* and cord blood samples showed a prevalence of 32%, 23% and 21%, respectively.

Malaria is known to impact the transfer of antibodies from mother to fetus. In developed countries, IgG levels are higher in cord blood than maternal blood (Kohler and Farr., 1966), but in many studies from Africa, due to the presence of malaria maternal blood IgG levels were found to be higher than cord blood IgG levels (Ibeziako *et al.*, 1980). Women who have been pregnant before, develop anti-adhesion antibodies that limit the accumulation of the parasite in the placenta, though

these antibodies are not found in primigravidas, who are therefore more likely to have placental infection and significantly higher levels of parasitaemia (Fried *et al.*, 1998).

1.2 Justification

Congenital malaria infection is diagnosed when parasites are found in the newborn within seven days of birth, or later if there is no possibility of postpartum infection by mosquito bite, transmission in the parturition or transmission by a blood transfusion, which are designated as neonatal malaria (Laosombat and Dharmasakti, 1981). The mechanism of transplacental passage of this infection is disputed. Many reports on congenital malaria have been published, but the reported incidence has recently increased, although it has varied according to the sample materials involved, such as peripheral blood of neonates or cord blood. However, from congenital reported case it is difficult to determine whether this represents infection of the fetus before delivery or contamination by maternal blood at birth to produce a replicating infection in the newborn. Also infants below three months have been reported to have lesser incidence, severity, parasitemia and death from malaria (Morley *et al.*, 1964).

This study aimed to address the hypothesis that first *Plasmodium falciparum* infection of infants below three month of age arises from congenitally acquired placental infection and applying Polymerase Chain Reaction (PCR) and subsequent sequence analysis of *P. falciparum* infection from Muheza North-Eastern Tanzania, an area of intense transmission. The entomologic inoculation rate in the area has

been described as approximately 400 infected mosquito bites each year (Ellman *et al.*, 1998). This study will provide useful information on congenital malaria and as such it could be of value in further studies that will try to evaluate as to whether the presence of congenital parasites to infants is associated with prognosis in terms of protective immunity, hemoglobin status and weight gain and in return will have an implication in therapy and vaccine development.

1.3 Hypothesis

The first *Plasmodium falciparum* infection in neonate born from infected mothers arises from congenitally acquired infection through the placenta.

1.3.1 Main objectives

- Determine the genetic relatedness of cord blood and placental *Plasmodium falciparum* parasites
- Determine the genetic relatedness of *Plasmodium falciparum* parasites of first infection in young infants and those detected in placental blood.
- Determine frequency of *Plasmodium falciparum* parasites in cord blood by PCR.
- Determine significance of cord malaria outcome in the neonate

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Malaria epidemiology

Malaria is one of the most prevalent human infections worldwide. Over 40% of the world's populations live in malaria endemic areas (WHO, 1994). An estimated 300 to 600 million cases and 1.5 to 2.7 million deaths occurs each year (WHO, 1994). Ninety percent of deaths occur in sub- Saharan Africa, the majority involving children less than five years of age. Malaria disproportionately affects the poor, in whom higher morbidity and mortality can be largely attributed to lack of access to effective treatment; 60% of malaria deaths worldwide occur in the poorest 20% of the population (The World Bank, 2001). In additional to children, pregnant women (particularly primigravidae) and nonimmune people (example, travelers, foreign workers) are at highest risk of the severe disease (The World Bank, 2001). However, all the age groups may be at risk of severe disease during malaria epidemics, which occur either when changes in the physical environment (caused by climatic variation, agriculture projects or mining,) increase the capacity of mosquitoes to transmit the disease.

Plasmodia species are the parasites responsible for malaria. Only four of the over 100 species of plasmodia are infectious to humans. The majority of cases and almost all deaths are caused by *Plasmodium falciparum. Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* cause less severe disease (Suh *et al.*, 2004). Malaria is present to varying degrees in 105 countries, the majority of which contain drug

resistant strains. Over 90% of all malaria cases occurs in Africa, and, and most are caused by *Plasmodium falciparum* (Greenwood & Mutabingwa, 2002).

2.1.1 Malaria in pregnancy

Each year, about 24 million African women become pregnant in areas where malaria has devastating impact (Steketee *et al.*, 1996). These women are at an increased risk of contacting malaria infections, which increases the risk of poor pregnancy outcome (Steketee *et al.*, 1996a). This susceptibility to malaria and the severity of its clinical manifestations in pregnancy can be partly explained by the observed immunosuppression mediated by pregnancy associated hormones and proteins and the cyto-adherent properties of the sub- population of malaria parasites infecting the human placenta (Moormann *et al.*,1999;Beeson *et al.*, 2000). It has been estimated that as many as 300,000 fetal and infants deaths and 2,500 maternal mortality deaths may be attributed to increased malaria susceptibility during pregnancy (Steketee *et al.*, 2001).

Pregnancy malaria, a distinct clinical entity, is a major cause of pregnancy related complications in malaria endemic areas. It is associated with premature delivery, intrauterine growth retardation, perinatal mortality in the infant, anemia, abortion, premature birth and death of the mother and low birth weight. (McGregor, 1987; Brabin, 1991; Menendez, 1995). After years of exposure to malaria, women in malaria endemic areas acquire immunity to *Plasmodium falciparum* equivalent to their male counterparts (Brabin, 1983). However pregnant women have a unique susceptibility to malaria infection, which diminishes, as their gravid status increases (McGregor, 1987).

Parasites infecting the placenta have a distinctive adhesive and antigenic phenotype (Duffy, 2003). Naturally occurring antibodies directed against placental parasites are associated with resistance to pregnancy malaria and improved pregnancy outcomes (Duffy, 2002). In the absence of evidence for other mechanisms, maternal malaria has been attributed to the immunosuppression of pregnancy (Weinberg, 1984), which is thought to lead to decrease in transmission of maternal immunity to the child.

2.1.2 Pathogenesis of placental malaria during pregnancy

Clinical immunity of malaria is acquired during childhood in areas of intense *Plasmodium falciparum* transmission, and adults healthy are generally protected against malaria (Riley *et al.*, 1994). Pregnant women, in whom malaria is both more prevalent and more severe than in non-pregnant women, constitute an important exception to this rule (Brabin, 1983). Pregnancy associated malaria (PAM) is characterized by marked accumulation of parasites in the intervillous space of the placenta, and is frequently the cause of maternal anemia as well as low birthweight, prematurity and increased infant mortality (McGregor *et al.*, 1983). In endemic areas, PAM is concentrated among primigravid women, which indicates that protective immunity to PAM is acquired as a function of parity (Brabin, 1983)

Red blood cells infected by the late developmental stages of *Plasmodium falciparum* parasites are not found in the peripheral circulation, as they adhere to receptors on the endothelial lining of blood vessels. This adhesion, called sequestration, is mediated through parasite-encoded, clonally variant surface antigens (VSA) inserted into the membrane of the infected red blood cells (iRBC) and is thought to be an immune evasion strategy, possibly evolved to avoid splenic clearance (David *et al.*,

1983; Howard and Barnwell, 1984; Berendt *et al.*, 1990). The proteoglycan chrodroitin sulphate A (CSA) can mediate parasite adhesion in vitro (Robert *et al.*, 1995: Rogerson *et al.*, 1995) and although CSA- adhering parasites are rarely found in non- pregnant hosts, placental parasites preferentially or perhaps even exclusively bind to CSA (Fried and Duffy, 1996; Achur *et al.*, 2000). Therefore, primigravidas have had little or no immunologic experience with the CSA- binding parasite and are most susceptible to infection and protection develops over successive pregnancy (McGregor, 1984).

The predisposition, which occurs in primigravidae, is evident even at quite low parasite prevalence, which suggests that a low incidence of malaria in first pregnancies would be sufficient to reduce susceptibility in multigravidae. This effect is considered to be largely parity specific but there may be a small maternal age dependent component (Brabin and Brabin, 1992). Placental as well as peripheral parasitemia occurs more frequently in first pregnancies indicating that immunity acquired with parity reduces placental parasites (Brabin, 1983). Chronic infection (detection of placental parasites and of pigment in leucocytes and or fibrin) is more frequent in primigravidae (Ismail *et al.*, 2000), prevalence and malaria parasite density decreases with increasing parity (Diagne *et al.*, 1997; Steketee *et al.*, 1996a). At low incidence with less than one malaria infection per pregnancy, proportions of primigarvidae remain uninfected and as a result should have greater susceptibility as multigravidae.

2.1.3 Fetal and infants outcomes related to pregnancy malaria

2.1.3.1 Effects related to time of infection

Garner and Brabin (1994) reported some of the outcome of fetal and infants related to placental malaria in pregnancy, which includes altered implantation when placental malaria occurs at time of conception. Maternal toxicity and or intrauterine malaria following primary pregnancy malaria infection or recurrent *P. falciparum* may results into abortion, stillbirth and late fetal infection. However, perinatal malaria infection may cause cord parasitemia, early neonatal malaria as well as later neonatal malaria (Congenital malaria). Mortality in babies would be expected to increase related to the increased low birth weight risk attributable to malaria in pregnancy. Both *P. falciparum* and *P. vivax* malaria are associated with increased low birthweight, (Brabin *et al.*, 1990a; Nosten *et al.*, 1999; Singh *et al.*, 1999). The increased low birthweight prevalence in primiparae, attributable to *Plasmodium falciparum* malaria, is substantial, ranging from below 10% in low endemic areas to over 50% in holoendemic areas (Brabin *et al.*, 1990b).

Low birthweight related to malaria is a massive public health problem, which is almost wholly preventable by adequate malaria control in pregnancy (Greenwood *et al.*, 1989).

Infants' malaria may be increased in babies born to infected mothers (Brabin, 1990c; Le Hasran *et al.*, 1997), and placental malaria is associated with a higher risk of infant anemia (Mangochi Malaria Research Project, Africa Child Survival Initiative, 1995), although it is uncertain if these observations are related to increased exposure of mother to child malaria transmission.

2.2 Congenital malaria

Congenital malaria was reported shortly after Laveran (1881) discovered the malaria parasite and early studies describing malaria in pregnancy commented on its effects on the placenta (Edmonds, 1899). Later numerous other case reports of congenital malaria have filled the literature (Covell, 1950), the frequency of its occurrence remained sticking, particularly in high immune populations.

Congenital malaria is diagnosed when parasites are found in the newborn within seven days of birth, or later if there is no possibility of postpartum infection by mosquito bite, transmission during parturition or transmission by a blood transfusion, which are designated as congenital malaria (Laosombat and Dharmasakti, 1981).

Cases of children born in non endemic countries from mothers with travel history or origin in malaria endemic countries and who, several days or weeks after delivery, present malaria symptoms without a clear differentiation between congenital malaria (occurring during gestation) or perinatal malaria (before delivery from the 28^{th} week of gestation through the first seven days after delivery) have been reported (Ahmed, 1998; Williams *et al.*, 1999).

The congenital malaria caused by transplacental transmission in semi-immune mothers could be more frequent than other reports suggest because it is difficult to detect (Malviya and Shurin, 1984). To differentiate between transplacental transmission and perinatal infection as the origin of infection in the newborn, molecular techniques must be employed. This allows a sure characterization of vertical malaria transmission as congenital malaria due to transplacental transmission

of the parasites (Rubio *et al.*, 2000). Congenital malaria has been considered to be rare, even in malaria endemic areas but the disease can result in significant clinical malaria in neonates because of its rarity, the disease may go undiagnosed for a prolonged period in a seriously ill infant (Covell, 1950; Viani and Bromberg, 1999). The mechanism by which malaria parasites are transferred from mother to fetus is not clear. Transplacental passage of maternal white blood cells has been demonstrated in a number of studies, and 10.8-38% of cord blood samples may contain maternal cells (Hall *et al.*, 1995; Poli *et al.*, 1997; Scarasavou *et al.*, 1996). Maternal erythrocytes might be transferred to fetal blood either by an active transport mechanism or during labor. These studies indicate that mixing of fetal and maternal blood can also occurs during normal pregnancy.

The presence of Plasmodium parasites in cord blood may be attributed to ability of erythrocytes to cross from maternal to fetal circulation (Lo *et al.*, 1996) through small breaks in the syncytiotrophoblastic membrane to gain access to fetal circulation during pregnancy. Infected maternal erythrocytes may then persist until the time of delivery or lead to the infection of fetal erythrocytes (Lehner *et al.*, 1988; Ibhanesebhor, 1995).

Rates of placental infection ranges from 16% to 47%, while congenital infection rate is just around 7% and occurs mainly in newborns from non-immune mothers (McGregor, 1984; Fischer, 1997).

2.2.1 Case report of congenital malaria

Congenital malaria is a rare disease. So far, 300 cases have been reported in the literature. There are some arguments about the definition of congenital malaria. Normally, for congenital malaria symptoms occur 10 to 30 days postpartum. However, the disease can be seen in a day- old baby or be delayed for weeks or months. The most common clinical features in 80% of cases are fever and anemia (Remington and Klein, 1995) other signs and symptoms includes jaundice, regurgitation, loose stool, and poor feeding; occasionally, drowsiness, restlessness and cyanosis can also be seen (Remington *et al.*, 1995; Viraraghavan and Jantausch, 2000).

Torpin *et al*, (1941) reviewed 27 cases of malaria in pregnant women where maternal mortality rate was 4% and fetal mortality was 60% in India. From Delhi, Dhatt *et al.*, (1979) reported 15 cases of malaria in the first four months of infants born from mothers diagnosed with placental malaria at delivery. In malaria endemic areas the incidence of congenital malaria is expected to be 1-4% following overt attacks of malaria in the mother. Santhanakrishman *et al.* (1985) from Madras, India reported two cases of congenital malaria diagnosed in the first four months of life in a study that involved 221 children diagnosed with malaria. Congential malaria is rare. However, several studies have indicated an increase in its incidence, although it varied according to the sample materials involved (peripheral blood of neonates or cord blood) (Covell, 1950; Kortmann, 1972). This variation could be due to variation in malaria endemicity in the areas where the studies were conducted.

Polymerase Chain Reaction (PCR) has been successfully employed increased as a highly sensitive diagnostic method for the detection of low density of malaria parasites in blood (Roper *et al.*, 1996; Vu *et al.*, 1995, Barker *et al.*, 1992; Wataya *et al.*, 1993; Wooden *et al.*, 1992) and for investigating the genetic diversity of specific loci such as the merozoites surface protein (MSP-1) (Kimura *et al.*, 1990; Snewin *et al.*, 1991) and merozoites surface protein (MSP-2) (Marshall *et al.*, 1994; Snewin *et al.*,1991). Motoi *et al.*, (2000) when working on neonates admitted at Muhimbili Medical Center in Dar es Salaam, Tanzania detected congenital malaria on one baby out of 298 neonates (prevalence of 0.33%) using a Giemsa staining method. However, using PCR techniques two cases of congenital malaria were detected, one of which was negative as determined by the Giemsa staining method. It is therefore clear that malaria parasites are occasionally detected in umbilical cord blood using Giemsa stain method which is not as sensitive as PCR technique (Larkin and Thuma, 1991; Lehner *et al.*, 1988). Whether this represents infection of the fetus before delivery or contamination by maternal blood at birth to produce a replicating infection in the newborn is not well understood. Balaka *et al.*, (2000) did a research at a University Hospital in Lome, Togo and detected 40 newborns were diagnosed as congenital malaria disease and 91 were considered as congenital malaria infection. The main clinical manifestations were related to cerebral (100%), respiratory (95%) and hemodynamic (90%) systems. The level of parasitemia varied from 700 to 3,000 parasites/μL in congenital malaria disease and from 360 to 870 parasites/μL in congenital malaria infection. Death occurred in 25% of congenital malaria disease. In Spain, Rubio *et al.* (2000) reported three cases of congenital malaria involving two malaria immune mothers. Diagnostic PCR and genotyping PCR for merozoites surface protein 1 and 2 were essential to show that mothers and the newborns had

different *Plasmodium* population parasites at the moment of the delivery, and that the infection was acquired earlier in gestation by transplacental transmission. In the first case the *Plasmodium* species found in both, mother and child were different while there are two cases of malaria which involved twins showed a mixed infection (*P.falciparum* and *P. malariae*) while the mother presented a *P.falciparum*. Blood samples of the newborns were analyzed a half an hour after delivery and this indicating a vertical transmission during pregnancy.

Malaria manifestation during the first few months of life may result from acquisition during pregnancy, at the time of delivery or by mosquito bite after birth. Both congenital and perinatal malaria are acquired by the transmission of parasitized maternal erythrocytes across the placental (Hindi and Amizi, 1980). An infants developed intermittent fever at fifth week of age and presented with anemia and hepatosplenomegaly at three month of age at which time *Plasmodium falciparum* were found on thick smears of the infants' blood. IgG and IgM antimalaria antibodies were detected in maternal blood, but only IgG antibodies were found in the infants blood at delivery and at the time of diagnosis. These transplacentally transmitted antibodies may confer transient protection for the infant and thus delay the onset of clinical manifestations. Due to the absence of an exoerythrocytic life cycle in congenitally acquired malaria, antimalaria drug should be used to clear the infection (Hindi *et al.*, 1980).

2.2.2 Malarial immunity

Reports of neonatal malaria resistant to chloroquine have increased during the last decade. About 25% cases of congenital malarial from Nigeria were found resistant to

chloroquine (Ibhansebhor, 1995). In Yaounde, Cameroon, parasite- specific immunoglobulin M (IgM) was detected in 14% of cord blood samples. The IgM antibodies reacted with a wide range of sexual stage antigens, with each newborn having its own unique pattern of IgM reactivity. PCR based detection and genotyping of cord blood parasites found that the prevalence, total number of parasite genotypes, and complexity of infection were higher in newborns who had produced antimalarial IgM than those who had not. The presence of *Plasmodium falciparum* IgM in cord blood suggests that the fetus was infected in utero and B-cell activation occurred. Alternatively, malaria antigens, perhaps as immune complexes, could have crossed the placental barrier and stimulated the response (Desowitz *et al.*, 1992; Jakobsen *et al.*, 1998). Malaria antigens like MSP 2, which is also considered as a vaccine candidate seems to stimulate antibody against malaria.

2.2.3 Merozoite surface protein-2 as a marker gene

The merozoite surface protein 2 is one of well-characterized surface protein with highly conserved carboxyl and amino terminal regions flanking central variable regions that are composed of non-repetitive semi-conservative sequences surrounding repetitive highly variable repetitive sequences (Anders and Smythe, 1989; Smythe *et al.*, 1990).

Merozoite surface protein-2 gene encodes Merozoite surface protein -2 (MSP-2) one of the smaller of the two well-characterized surface proteins of *P. falciparum*. MSP-2 is a 45kDa integral membrane protein with highly conserved carboxyl and amino terminal regions flanking a central variable region that is composed of non-repetitive sequences (Anders and Smythe, 1989). MSP-2 like many other malarial proteins is

antigenically diverse among different *P. falciparum* isolates. The sequence relationships among diverse MSP-2 genes allow them to be classified in two allelic families FC27 and 3D7 (Smythe *et al.*, 1988; Fenton *et al.*, 1991).

The diversity is due to an allele specific central region, which comprises tandem repeats of varying sizes. Difference in the number of copies of these repeats results in length polymorphism, which has been exploited in several genotyping studies, which include a Polymerase Chain reaction (PCR) amplification of central part of MSP-2 gene. The MSP-2 gene has been used as a polymorphic marker gene in field studies in different geographical locations with either a multilocus analysis or single locus analysis (Felger *et al.*, 1999a). All studies showed polymorphism of MSP 2, even in areas of low endemicity.

The use of MSP-2 as a marker has proven to be of great use when individual *Plasmodium falciparum* infections need to be identified. PCR-RFLP genotyping of parasites for MSP-2 makes it possible to distinguish the individual parasite infections concurrently present in a blood sample. The possibility of tracing individual parasite clones overtime allows detailed studies of infection dynamics. Most importantly genotyping makes it possible to determine the multiplicity of infection, which can be used as an outcome measurement of interventions such as drug trials, vaccine trials or exposure-reducing interventions (Felger *et al.*, 1999b).

There are about 82 different MSP-2 alleles have been detected so far in Tanzania and polymorphism is of such an extent that super infection with the same genotype as the previous one is highly unlikely (Smith *et al.*, 1996). This fact allows the use of MSP-

2 as a marker to discriminate between new infections and recrudescence in drug trials (Irion *et al.*, 1998).

2.2.4 PCR- RFLP of MSP -2

Use of MSP-2 gene to determine the number of concurrent infections per blood sample (multiplicity of infection), or to discriminate whether an allele belongs to 3D7 or FC27 allelic family, only a single restriction digest (*Hinf*1) is usually performed on the nested PCR product of the MSP-2 gene (Felger *et al.*, 1999a). The dimorphic region of the MSP-2 shows family specific *Hinf1* restriction sites which are located in the variable non-repetitive region and which are shared by most alleles of the same family (Felger *et al.*, 1999a). The family specific restriction fragments resulting from *Hinf*1 digests of the FC27 type alleles are two fragments with lengths of 137bp and 115bp, and of 3D7 type alleles, are two fragments of 70bp and 108bp (Felger *et al.*, 1999a). Alleles, which represent recombination between FC27 and the 3D7 type allelic families, have also been found in Tanzanian blood samples (Felger *et al.*, 1999b). These recombination are revealed in *Hinf*1 restriction fragment analysis by their unusual fragment lengths (Felger *et al.*, 1999b).

Sometimes not only, multiplicity and allelic family are required to be determined, but also it is necessary to trace individual alleles in consecutive blood samples, comparing infections from different blood samples, determine new recombinants or 3D7 type-alleles. Here further restriction digests using *DdeI, RsaI, or ScrI* are necessary which yield smaller and allele specific fragment sizes compared to *Hinf*1 fragment 3D7 type alleles which are too large to be identified by gel

electrophoresis (Felger *et al.*, 1994). Hence PCR-RFLP typing is useful in discriminating recrudescent and new malarial infections (Irion *et al.*, 1998).

However PCR-RFLP fails to detect alleles resulting from recombination between the allelic families if no obvious alteration in restriction pattern had occurred. Furthermore intrinsic limitation of the PCR technique include the fact that the number of concurrent infections detected by PCR technique is always a minimum estimate of the number of different parasite clones which are present in a carrier. Some clones might be missed owing to detection limit of the PCR technique, which might miss infections of very low density or sequestered parasites at the time of sampling (Färnert *et al.*, 1997).

placental blood. Blood samples from healthy infants were collected by heel prick every two weeks and at the time of any illness through mobile clinics until when they present with first infection. Thick and thin blood smears were prepared for all samples; thin smears were fixed with ethanol and stained with 10% Giemsa, washed in tap water and air- dried, and then examined using light microscopy at 100x magnification. In order to avoid missing the malaria parasite in the placental and infants, ten thousand red cells were examined in the thin smear before concluding that a placental blood smear was negative, and 100 high power fields were examined in the thick smear before concluding that a peripheral blood slide of an infant was negative.

Only placental samples tested *Plasmodium falciparum* positive in their thick smears and all infants born from mothers diagnosed with placenta malaria and presented with parasitemia below three months of age were selected for further analysis by PCR. In order to analyze the phenomenon of malaria infections of neonates via transmission across the placental blood, matched umbilical cord blood samples were examined in thick blood smear and further analyzed regardless of being negative by blood smear. The few cases where only one of the compartments was infected were omitted from the study. Blood samples were bloated in spots on 0.5x2cm strips of 3MM Whatman filter papers, which after drying in the lamina flow hood were packed in small individual plastic bags to avoid contamination and stored until needed for further analysis

Forty cycles of amplification composed of 94°C for 1 min, 50.3°C for 1 min, and 72°C for 45 seconds, followed by a final prolonged extension step at 72°C for 5 min, was carried out in a PTC -100 Peltier thermal cycler.

- Second-round amplification carried out in 50-µl reactions using nested conserved region 5' and 3' primers, to amplify the central region of the MSP 2 gene. One microliter of the amplification products from the first-round reaction used as the template for the nested reaction using the same reaction mix as specified above. Thirty-five cycles of amplification composed of 94°C for 1 min, 55.8°C for 1 min, and 72°C for 45 s and then one cycle of 72°C for 5 min was carried out for this and all other nested PCR.

The nested PCR products was loaded onto 2% agarose gel (Seakem, Sigma) at 100V for 25 minutes, stained with ethidium bromide and visualized by ultraviolet light. The expected lengths of the bands were 509bp and 750bp and the previously established DNA positive control sample used was 3D7. In all PCR reactions a negative control sample with no template DNA was used.

Placental- cord pair or placental – infants first parasitemia and or infants first parasitemia, placental and cord samples were run in parallel in the agarose gel so as to determine the similarities and the differences of the bands size. Nested PCR amplification for the presence of MSP-2 gene as previously described was carried and the product with the single bands were purified by QIAquick PCR product purification kit protocol using microcentrifuge before sequencing. Samples presented more than one band (more than one genotype) and the samples which were run in

parallel shared the bands size, the gel were cut along those bands and purified by QIAquick Gel extraction kit protocol using centrifuge and then sequenced.

3.2.2 Restriction fragment length polymorphism

In general a 25µl reaction mix was used containing 10x buffer provided by the manufacturer, 2.5U enzyme and the appropriate amount of nested PCR products. For MSP-2 genotyping nested PCR products was digested with *Hinf I* (NEB) for 24 hours at 37°C and the DNA fragments was separated on 10% agarose gels at 100 V for 30 minutes. Fragment sizes were estimated using a DNA size marker (10µl of 100kb ladder, GeneRuler^{TM}DNA ladders).

3.2.3 Sequencing method.

DNA sequencing was done on presenting nest 2 amplicons (Mehlotra *et al.*, 2000). In preparation for sequencing, excess dNTPs and unincorporated primers were eliminated from DNA by purification using QIAquick PCR purification kit (Qiagen). DNA sequencing was performed by fluorescence-based methodologies with an Applied Biosystems (Foster City,CA) 377 automated DNA sequencer. The purified PCR products one prepared templates was sequenced for each MSP2 and the second templates were sequenced if differences were found between the two derived sequences.

3.3 Data analysis

Data were analyzed using STARTVIEW statistical package. Descriptive analysis used to evaluate the means of the different variables. Paired t- tests were used to

analyze and determine if means of the different variables are equal to some hypothesized value. Regression determined if there were any linear relationship among dependent variable and independent variable. Regression plots that showed the variability around means of different variables and P value < 0.05 in the analysis, the observation considered to be significant. Chi-square test determined the placental status (parasitemia or no parasitemia among gravity (primigravid, secundigravid and multigravid). a DNASTAR Program was used to analyzed the sequencing results and all sequencing results were compared with MSP 2 sequences in PLASMODB by Basic Logical Alignment Search Tool (BLAST) so as to determine if each individual sequence matched with concordant MSP 2 gene of *P. falciparum* sequences previously deposited.

CHAPTER FOUR

4 RESULTS

During the study period (September 2002 to September 2005), a total of 1022 babies were born. Placental and umbilical cord blood samples were collected from the mother and the newborn infants, respectively. Only placental samples that tested *Plasmodium falciparum* positive in thick smears were selected and analyzed by PCR. All matched umbilical cord blood samples were examined in thick blood smear and were included and analysed by PCR, regardless of being negative by blood smear. Also all infants born from mothers diagnosed with placenta malaria and presented with parasitemia below three months of age were selected and further analysed by PCR. A total of 100 (9.8%) placenta blood samples collected from study women and four (0.4%) of cord blood samples were malaria positive by blood smear. However five placental samples and their respective cord samples were excluded from the study due to incomplete inclusion criteria. Following PCR amplification, the prevalence of *Plasmodium falciparum* infection in placental blood samples were 95 (9.3%) and 61(6%) of cord blood samples as compared to 9.8% and 0.4% detected by blood smear, respectively. It was observed that 0.5% (5/1017) of placental blood samples which were positive by blood smear were negative by PCR regardless of repeat procedure. During the study, 18 (19.1%) of infants born from mothers diagnosed with placental malaria developed clinical malaria below three month of age.

4.1 Characteristics of the study population

Table 2 shows the characteristics of the study population. Age of the study mothers ranged from 18- 42 years with the mean age of 23 years (Std Dev.4.5) and the parity groups ranged from 0- 5 pregnancy (primigravid to fifth pregnancy).

Table 2:Characteristics of the study populations

Characteristics	Mean	Standard Deviation
First infants parasitemia	11 weeks	15.070
Birthweight	2.93 gm	0.436
Gestation age	36.2 weeks	0.868
Age of the mother	23 years	4.528
Previous pregnancy	1 (number of previous pregnancy)	1.153
Placental parasitemia	9.8%(Blood smear)	13.142

Placenta malaria was identified in 95 of the mothers and out of these 40 (42.1%) from primigravidae, 31 (34.7%) from secundigravidae and 23 (24.2%) from multigravidae. The mean weight of newborn infants were 2.9kg with the range of 2-4.3kg (SD=±. 0.436) and 14.9% babies were below average birth weight while the mean gestation age were 36.3 weeks (range= 32-40 weeks. These results show that 79.8% of the newborn infants born from mothers with placental *Plasmodium falciparum* were born before the normal gestation age was attained, with the relative risk (RR) of 1.12. Infants born from mothers diagnosed with placental malaria during delivery, presented with the first clinical malaria at the mean age of 11 weeks (SD ± 15) and maximum of 64 (8- 64) weeks.

The prevalence of placental malaria which leads to cord malaria significantly decrease as the gravid increases (Regression coefficients, $t = 4.8$, $P < 0.001$) (Figure 1).

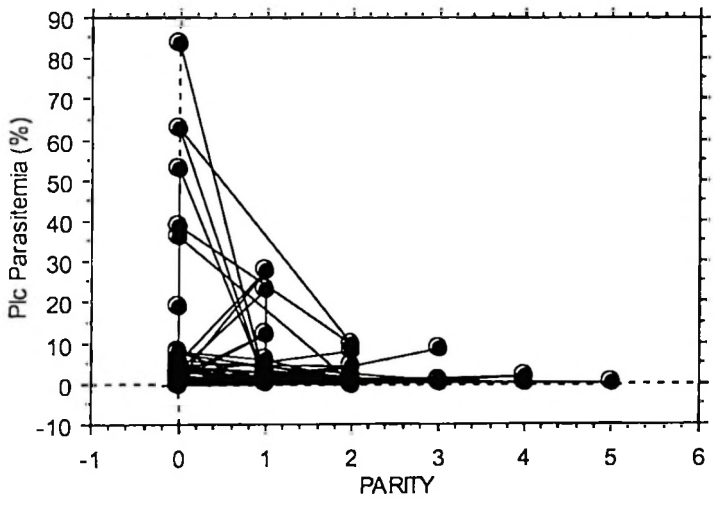


Figure 1: Relationship between placental malaria and parity

During the study it was observed that infants born from primigravidae were significantly more likely to be infected with *Plasmodium falciparum* (regression coefficients, $t = 5.5$, $P < 0.001$) as compared to infants born from secundigravidae or multigravidae. It was observed that infants from multigravidae mothers got *P. falciparum* infection early than those from secundigravidae and primigravidae, with the relative risk of 1.43 (Figure 2).

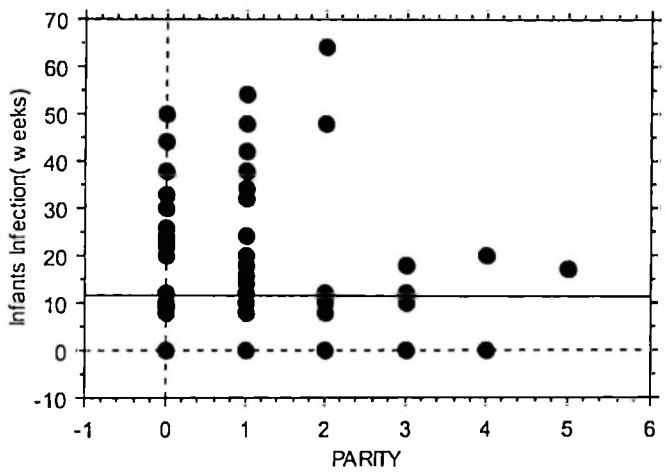
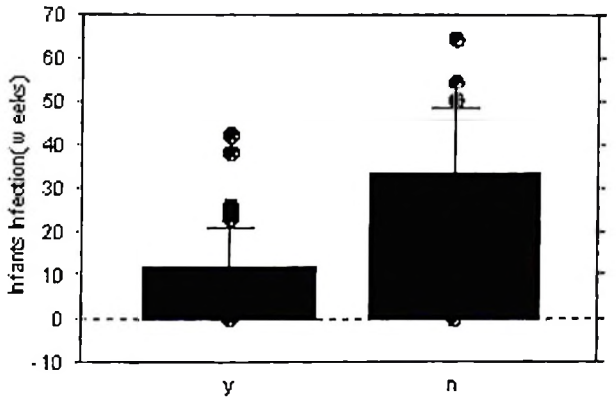


Figure 2: Frequency of malaria infection in infants in relation to parity status

Newborn infants diagnosed with cord malaria by PCR were observed to be more susceptible to *P. falciparum* infection in their early life as compared to the infants that were cord negative, with the relative risk (RR) of 2.6) (Figure 3).



y = cord PCR positive

n = Cord PCR negative

Figure 3: Infants first parasitaemia against cord malaria

However, it was observed that infants diagnosed with cord malaria at delivery got multiple infections as early as 11 weeks and decreases as the child got older (144 weeks), as indicated in Figure 4.

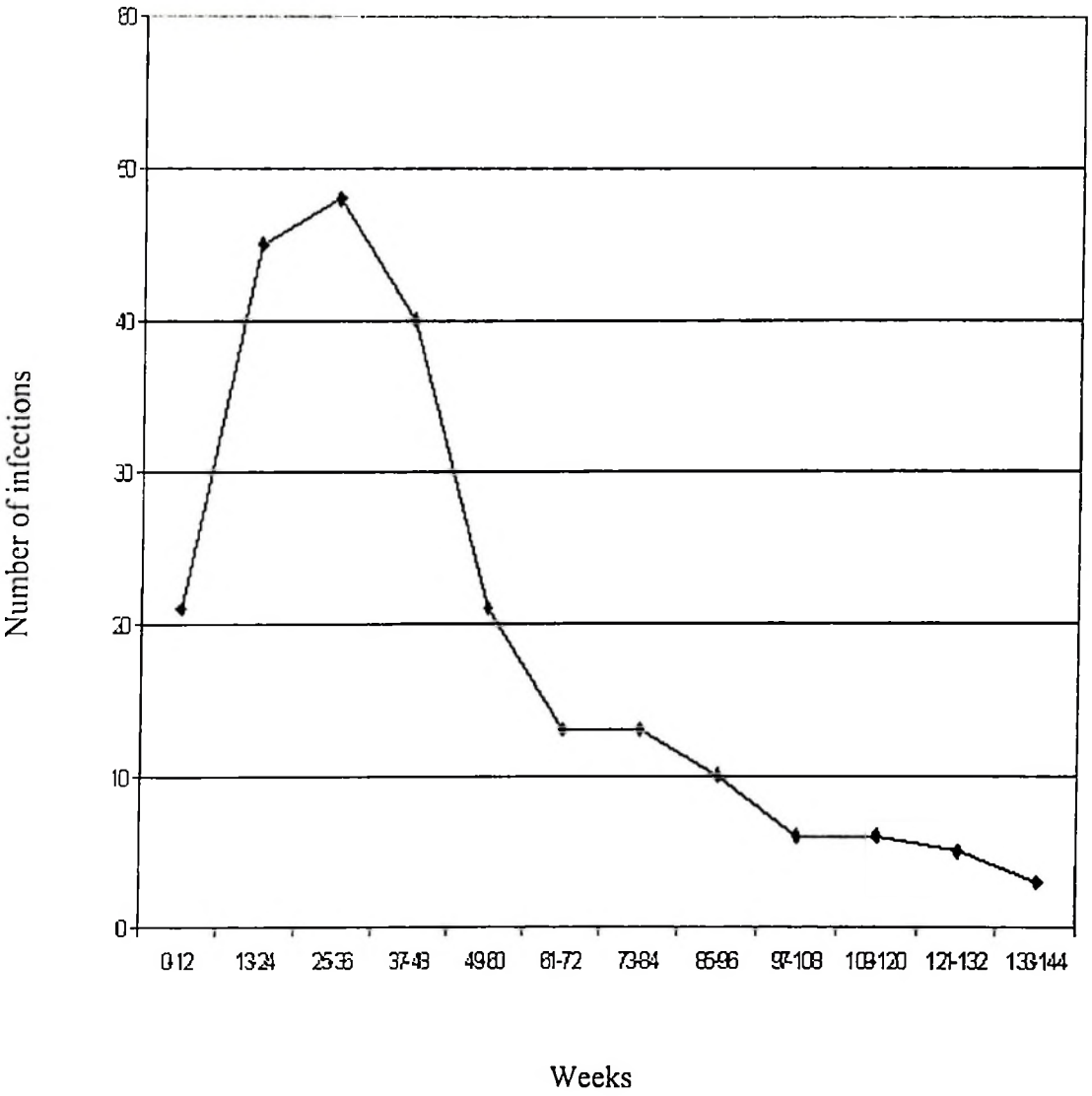


Figure 4: Effect of cord malaria in infant's life

X-axis referring to the incidence of infection number in an individual child

4.2 PCR amplification results

From the study, the samples were selected for analysis on the basis of *Plasmodium falciparum* thick blood smear positive both in the placental and peripheral first infants' parasitemia below three month of age. The presence of more than one band in the samples after PCR products indicates occurrence of multiple infections. Results from PCR genotyping showed that some of placental and infants first *P.falciparum* infection examined contained more than one strain of MSP 2 gene.

Results from placental and cord blood pair and placental blood sample and infants blood that were diagnosed with malaria below three month of age in agarose gel, indicated that pairs that shares the same band size were genetically related and these results were further analyzed by RFLP followed by sequencing.

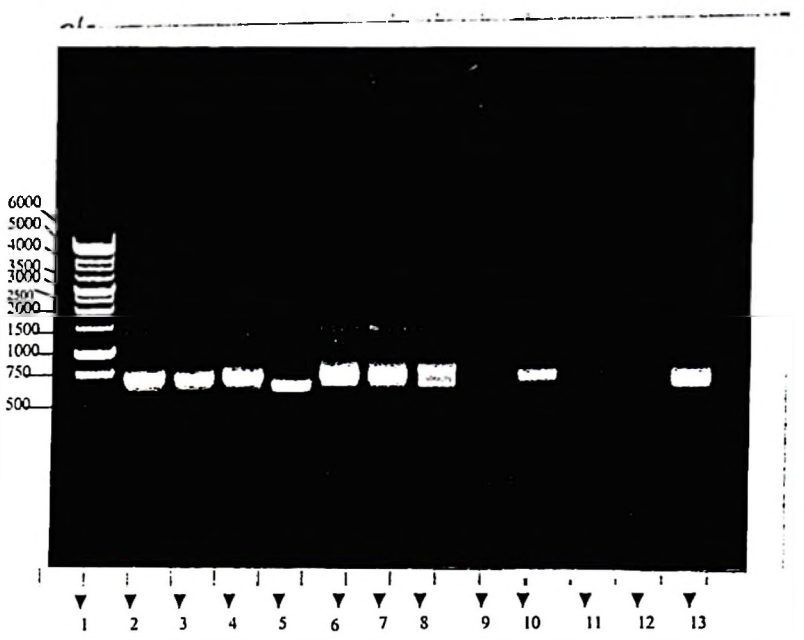


Figure 5: PCR products of placental blood and cord blood samples

PCR products were separated on 2% agarose gel, stained with ethidium bromide and visualized under ultra violet light. Lane 1: DNA size marker. Lanes 2, 4, 6,8,10 and 12: Placental samples, Lanes 3, 5, 7,9,11 and 13: Cord samples and the last lane: negative control.

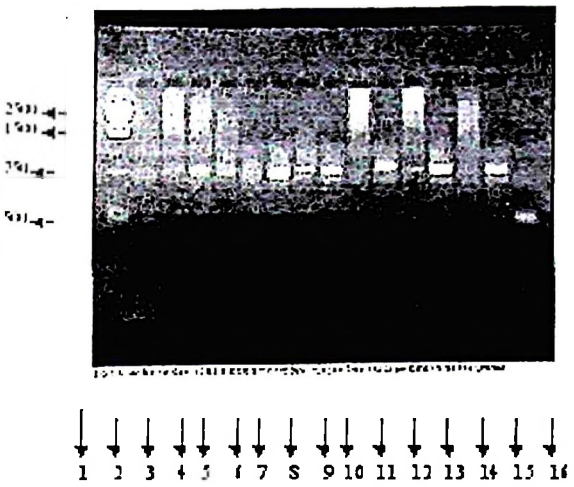


Figure 6: PCR products of infants first parasitaemia and placental blood samples

PCR products were separated on 2% agarose gel, stained with ethidium bromide and visualized under ultra violet. Lane 1: DNA size marker. Lanes 2,4,6,10,12 and 14: Infants samples (Below three month of age), Lanes 3, 5, 7, 9, 11, 13, and 15: Placental samples and Lane 16: Negative control

4.3 Genetic relatedness between mothers and infants

PCR- RFLP genotyping was used to determine the number of concurrent infections per blood sample (multiplicity of infection) and to discriminate whether the *Plasmodium falciparum* parasite found in the placental is genetically related with that found in the cord and infants that were diagnosed with malaria parasite below three month of age. Only a single restriction digest (*Hinf I*) was performed on the nested PCR products of the MSP2 gene.

The dimorphic region of MSP2 showed family specific *Hinf I* restriction sites which are located in the variable non-repetitive region and which are shared by most malaria parasite. Few samples showed appearance of a different restriction site that causes the deviations from MSP2 gene of malaria parasite specific restriction fragments. The restriction sizes obtained from *Hinf I* digests are two fragments with lengths 154bp and fragments with length 350bp, 400bp and 115bp were also recorded. Majority of the samples were not digested though the procedure was repeatedly done and, the reason for this was not clearly understood. The use of 3% agarose gel was not very productive in the separation of the fragments hence difficulties in the interpretation. Results from PCR and RFLP were compared with the sequencing results.

4.4 Sequencing results

Sequencing was performed in order to confirm the results that were obtained in PCR amplification. The sequencing results showed that PCR results which were

determined by the size of the band were not enough to confirm congenital malaria This was determined from samples that were sequenced, where by 14 pairs of placental blood samples and cord blood samples were sequenced and analyzed using DNASTAR program. Megalign program align paired sequenced and eight pairs (60%) out of 14 pairs were genetically related by 100% while six pairs (40%) were genetically unrelated by 10%. However, seven pairs of infants samples first parasitemia and placental were sequenced, after megalignment, one pair of placental blood sample and first infants parasitemia (14.3%) were genetically related by 100% while six pairs (85.7%) were genetically unrelated. The samples that were genetically related, 60% and 14.3% indicates that sharing the same PCR band size and RFLP fragments size is not an indication that the parasites are genetically related. Also 60% and 14.3% results are confirmation that *P. falciparum* do cross from the placental to the infants through the cord.

In this study, comparisons of the *P.falciparum* genotype between and within pairs were also determined. The comparison was between PCR and RFLP results by looking at the bands which shared the same sizes as well as sequencing results through alignment of the sequences that were arranged in the phylogenetic tree. Phylogenetic tree is used to determine the estimated closest and diversity of the DNA sequence of a given data set.

Some of the samples showed to share the band size in PCR, RFLP fragments size but for the few sequenced samples indicate that MSP2 gene observed to be sharing the band size and fragments size, their sequence were not closely align in the tree. However, pairs that were genetically related or closely genetically related after

alignment were closely arranged in the tree leaves. Analysis from phylogenetic tree indicates that all samples originated from the MSP 2 gene of *P. falciparum*.

CHAPTER FIVE

5 DISCUSSION

Malaria during pregnancy may results in fetal exposure to malaria when parasites are transmitted across the placenta. This study was aimed at determining that infants diagnosed with malaria parasites in the age below three month acquire the infection congenitally from the mother through placenta. Usually infants below 3 month have been observed to have lesser incidence, severity, parasitemia and death from malaria. (Morley *et al.*, 1964). The mechanism of this resistance is explained, to be due to an immunologic perspective. Attention has been focused on the possible contribution of maternal Immunoglobulin G (IgG), antibody acquired by the fetus while in- utero (Wagner *et al.*, 1998 and Riley *et al.*, 2000).

In this study more primigravidae were infected with malaria parasites than multigravidae, indicating the former to be at a higher risk than the later group. These observations are consistent with the findings of previous studies in malaria- endemic regions where, among several factors, gravidity independently influenced the occurrence of placental malaria (Okolo *et al.*, 1992; McGregor *et al.*, 1983). It is not yet clear why primigravidae are more susceptible to placental malaria and suffer from its consequences more than multigravidae. The general concept of pregnancy induced immunosuppression does not satisfactorily explain this. Fried *et al.* (1998) have suggested possible explanations. They showed that multigravid mothers develop malaria antibodies which block adhesion of parasites to chondroitin sulphate A receptors in the placenta in subsequent pregnancies. However, the influence of young maternal age on the prevalence of placental malaria infection was nullified

when gravid was controlled for, suggesting that young age and primiparity are correlated.

The presence of malaria parasites in the cord was observed to have a significant impact in the future life of the infants was determined. Infants diagnosed with cord malaria during delivery were observed to be more frequently re-infected with *Plasmodium falciparum* and the infection was observed to decrease as the infants grow older, presumably when the infants started to develop antimalaria immunity. Several authors (McGregory *et al.*, 1983; McGregor, 1984; Snow *et al.*, 1999; Steketee *et al.*, 2000) reported that the vast majority of severe malaria and deaths in young children occurs before immunity against malaria develops. Few studies have directly examined the effects of pregnancy malaria which can lead to infection of cord with malaria parasites that is detrimental on infant's health. A study conducted in southern Cameroon found no significant difference in the frequency of malaria between infants born to placental malaria- positive mothers and infants born to placental- negative mothers (38.5%) during the first two years of life (Le Hesran *et al.*, 1997). However the study recorded the age- specific prevalence of *Plasmodium falciparum* parasitemia was consistently higher between 11 and 64 weeks of age among infants born to mothers with placental malaria. Findings from this study and others suggest that placental malaria especially for primigravid may increase malaria risk in offspring. However, there is a likelihood that this was attributed by the complication from malaria during pregnancy especially the first pregnancy which is more frequently infected because of low immunity against malaria (McGregory *et al.*, 1983; McGregor 1984; Snow *et al.*, 1999; Steketee *et al.*, 2001). Further investigation on this area is recommended.

Overall, offspring of placental malaria positive mothers and infants diagnosed with cord malaria experienced their first parasitemia at a significantly younger age. The risk of early first parasitemia is highest among offspring of placental malaria positive multigravid. This concur with study carried in Muheza district in Tanzania where it was reported that infants from multigravidae mother diagnosed with placental malaria were in a highest risk of early parasitemia than infants from primigravidae mothers (Mutabingwa *et al.*, 2005). In Malawi, high placental parasite densities were associated with increased risk of cord blood parasitemia (Redd *et al.*, 1996), supporting the conjuncture that congenital malaria could account for the relationship between placental malaria and susceptibility of infants (Cot *et al.*, 2003). Although the expectation was to see increase of susceptibility of early clinical malaria to the offspring of primigravid women detected with placental malaria to be in the greater degree, results from this study prove the opposite pattern. Findings form this study suggests that placental malaria decreases susceptibility in the offspring of primigravidae and increases susceptibility in the offspring of multigravidae. The reason for this may be that among primigravidae, the pronounced inflammatory response to placental malaria could confer protection by reducing parasitemia (Stevenso *et al.*, 2004).

The mean gestation age in this study, was observed to be 36.3 weeks with the range from 32-40 weeks which is below the normal gestation age of >37 weeks (Dubowitz *et al.*, 1970). During the study period, most of the newborn infants born from mothers with placental *Plasmodium falciparum* were below gestation age. These findings may suggest that women who had pre-term delivery could have been due to

parasitization of placenta. The observation is consistent with the findings of previous study (Stekettee *et al.*, 1996 and McGregor *et al.*, 1983.

In contrast, McGregor *et al.*, (1983) did not find a significant association between placental malaria and premature delivery in their study. The mean weights of newborn infants were 2.9kg. The low birthweight was associated with parasitisation of the placenta. A similar association was observed elsewhere in sub-saharan Africa, endemic for malaria (Verhoeff *et al.*, 2001; Stekettee *et al.*, 2001 and Menendez *et al.*, 2000). The mean birthweight (2.9kg) and low birthweight in this study was higher than those reported previously by (Okolo *et al.*, 1992; Bulmer *et al.*, 1993 and McGregor *et al.*, 1983). Differences in these reports could be due to several factors, such as intensity of infection, proportion of primigravidae, and/or severity of infection. It is difficult to quantify the exact contribution of placental malaria to the high incidence of low birth weight reported annually. This is because, social and environmental factors such as maternal malnutrition, work overload, poor weight gain in pregnancy and intercurrent infections among other things are also important causes of low birthweight (Lawoyin, 1991)

Prevalence of *P. falciparum* in cord blood was observed where by 6% of infants born from placental malaria were diagnosed with cord malaria. This result concur with findings by Kassberger *et al.* (2002) who reported 17 of the 37 cord blood samples (46%) to be positive by PCR, while Motoi *et al.* (2000) diagnosed cord malaria in one baby out of 298 (0.33%) by PCR but not detected by blood smear. Studies in Africa suggested that 7-10% of more newborn may have malaria in their cord blood though may not show evidence of active infection. Using conventional Giemsa

staining method, in Malawi, (Kamwendo *et al.*, 2002.) detected malaria parasites in half of babies with cord blood and different genotype of parasites than their mothers at the time of delivery. However, it is difficult to detect scant levels of parasitemia with Giemsa stain method (blood smear) because it requires highly skilled technicians. On the other hand, polymerase-chain reaction (PCR) technique has been shown to be more sensitive than Giemsa staining method (Roper *et al.*, 1996; Vu *et al.*, 1995). Each of these findings suggested that at least a significant part of the transmission of parasite from mother to child must occur well before the time of delivery. This explains the findings from this study where by one infant developed clinical malaria below three month of age. A strong association between congenital malaria and both fever and death during the first days of life was reported by Nyirjesy *et al.* (1993), while Santhanakrishman *et al.,* 1985) reported two cases of congenital malaria diagnosed in the first four months of life in a study in Madras, India that involved 221 infants with malaria which was diagnosed by blood smear.

Different reports of congenital malaria that has been published varied according to the sample materials involved, such as peripheral blood of neonates or cord blood. Previsous reports indicated an incidence of peripheral prasitaemia between 0.3 and 20% (Akindele *et al.*, 1993; Covell, 1950).

Since it has been observed that mother- to fetal transmission of *P. falciparum* is more frequent than reported, the immediate question to ask is why parasites are not more commonly detected in cord blood smears. In order to account for negative thick blood smears (Giemsa stain) and positive PCR samples, we can only speculate that parasite numbers are too low to be detected.

Results from PCR genotyping showed that some of placenta and infants first The pre term delivery rate of 79.8% in this study is higher than previous findings in Gambia (Greenwood *et al.*, 1989). *P.falciparum* infection examined contained more than one strain of MSP 2 gene. These results may be explained by the extensive daily dynamics, whereby parasites present in the blood can differ from day to day (Färnert *et al.*, 1997; Magesa, 1999). In this study, it was observed that 0.5% (5/1017) of placental samples which were positive by blood smear were negative by PCR. Possible explanations for insensitive of the MSP 2 marker, include low concentrations of DNA in the samples or the presence of point mutations at the positions where the MSP 2 specific primers anneal as well as the presence of hemoglobin in the DNA extracts as proposed by (Kassberger *et al.*, 2002).

PCR results and fragments size from RFLP suggest that, MSP 2 gene of *P.falciparum* parasites can share bands size and fragments sizes but can not always be genetically related. This was confirmed by sequencing results that indicate differences in the bands size is an indication of the existence of clonal diversity in the study population. The differences between MSP 2 gene of *P. falciparum* parasites in the population of these analyzed samples (observed between and within placental, cords blood and infants first parasitemia) may be explained by the structural diversity in the MSP 2 gene which is due to the central domain containing repeats that vary in number, length and sequence as proposed (Smythe *et al.*, 1990a; Fenton *et al.*, 1991 and Marshall *et al.*, 1992). Felger *et al.*, (1994), also found extensive polymorphism in MSP 2 in malaria endemic area of Papua New Guinea. On the other hand, differences between parasites populations observed in this study may be explained by the fact that more than *P. falciparum* parasite strains (multiple infections) do sequent

in the placenta and one strain cross the placenta and detected in the cord blood. Extensive infection by parasite that differs from one infection to the other occurs during the time of gestation and may have resulted in the detected differences.

Congenital malaria detected in the 14.3% where by the *P. falciparum* that did cross from the placental to the infants through the cord caused clinical malaria to the infant. On, the other hand, 40% of placental and cord pair that were genetically related indicates that, there are multiple infections in the placenta which may infect the mother during gestation period; this was observed also by Färnert *et al.* (1997). Also, 40% rule out the possibility of contamination during sample collection but it is an indication that at least one strain from the presented complex infection in the placental crossed to the cord.

There is no research finding that shows at what trimester (first, second or third) of gestation period do the parasite crosses the placenta. During gestation period one strain from the population of *P. falciparum* in the placenta cross from the placenta to the cord and causes clinical malaria. Therefore, 85.7% pairs (placental and infants) which were generically unrelated may partly of wholly be attributed to *P.falciparum* population from the placenta to the cord which causes the clinical malaria in infants. The findings from this study which showed 18 (14%) infants below three month of age develop clinical malaria and one (14%) out of those had the parasite which was genetically related to that found in the placenta confirm that the parasite were congenitally acquired from the placental. This result suggests that although neonates below 3 month of age were thought to be resistant to malaria, a proportional of them

do get the disease as a result of congenital transmission. This agrees with the findings by Pasvol *et al.*, (1976).

Some arguments to explain the absence of parasites in the new-born include the failure of parasite to grow in cord blood and fast elimination of parasite from the fetal circulation. Other explanations of this phenomenon include immunity acquired from the mother (Pasvol *et al.*, 1976; Shear *et al.*, 1998). *P*-aminobenzoic acid deficiency due to milk diet, and high concentration of fetal hemoglobin in the erythrocytes during the first few months of life, have also been explained to contribute to the resistance of infants to malaria (Weatherall and Clegg, 1972; Pasvol *et al.*, 1976,) However, the result shows that transplacental transmission of *P.falciparum* parasite to the cord occurs and causes clinical congenital malaria though in few infants.

Development of the *P. falciparum* in these infants that causes clinical manifestation can be due to the presence of heavy *P. falciparum* infection sequestered in the placenta which reduce the transmission of maternal immunity and leave them susceptible to malaria. In the absence of evidence for other mechanisms, maternal malaria has been attributed to the immunosuppression of pregnancy (Weinberg, 1984), which is thought to lead to decrease in transmission of maternal immunity to the child.

In conclusion, findings from this study suggest that transplacental transmission is a common occurrence as previously reported and in turn results in congenital malaria which is likely to have important consequences for fetal and newborn development.

Genetic relatedness of the *P. falciparum* which was detected in the placental and that found in the infants below three months of age signify congenital malaria. Malaria parasitemia of the placenta has strong likelihood to result in congenital malaria infection. Direct infection to the fetus could contribute to premature, low birthweight babies or could increase the likelihood of early infants' infection as well as frequent infections of the infants. Although parasitemia is more frequent in primigravid women, the converse is true in their offspring, especially in offspring of multigravid placental malaria positive women. Placental malaria in multigravid, on the other hand, is a significant risk factor for parasitemia to resulting infant. Preventive antimalarial chemotherapy administered to multigravid women close to term may reduce the frequency of parasitemia in their offspring. There are hypothesis that a preventive dose of antimalarial chemotherapy to pregnant women in their third trimester before term may reduce *Plasmodium falciparum* parasitemia in their offspring. The present findings indicate that pregnancy malaria may also have prolonged effects on immunity and susceptibility to malaria re-infection in infants, and that these effects are strongly influenced by maternal gravidity. Further investigation of these relationships with assessment of other potential confounders, as well as their impact on infant's mortality and morbidity need to be done. Similarly further research has to be done to determine if treatment of asymptomatic newborn with anti-malaria agents will be cost effective in reducing the consequences of congenital malaria infections.

Preventing malaria is essential to ensure favorable pregnancy outcomes. For women without immunity, the need for prophylaxis is apparent, as malaria can precipitate severe syndromes. In areas of stable malaria transmission, though, most malaria

infections are asymptomatic, while causing adverse effects on both mother and fetus.. The problems of pregnancy malaria in this area are often unrecognized or recognized too late. Therefore, in the absence of thorough surveillance programs, case management alone does not effectively prevent the adverse effects of malaria during pregnancy. Active control measure like, intermittent preventive treatment, the use of treated bed nets are required to reduce or prevent malaria infections during pregnancy and it is outcome.

Intermittent preventive treatment has been observed to be effective and protective against LBW through its effect on reducing placental and umbilical cord blood malaria infection. When evaluating antenatal care programs, health policy makers must consider providing an effective preventive drug as a means to prevent low birth weight, anemia, intrauterine growth retardation, premature delivery and neonatal malaria.

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APPENDICES

Appendix 1: DNA extraction protocol

DNA Extraction

DNA was extracted from blood dried on filter papers using Generation® capture card kit.

Preparation

A dry block heater (containing Aluminium heat equalizing block) was set to 50°C. About 3mm (1/8 inch) disk from the sample collection card was punched and placed into a 0.2or 0.6ml tube or into a well of a 96-well plate (polypropylene U-bottom plate from mash Biomedical Products catalog number N29069).

DNA purification

200µl of DNA purification solution (solution 1) was added to the disk and incubated for 15 minutes at 50°C. The solution was pipetted up and down one time and then discarded. 200µl of DNA elution solution (solution 2) was added to the disk and incubated for 15 minutes at 60°C.The solution was then pipette up and down one time and as much solution as possible were removed. The purified DNA remains bound to the disk.

DNA elution

50-100µl DNA elution solution (solution 2) was added to the tube. The solution was pipette 15 times to mix, and the eluted DNA was transferred to a clean eppendof tube.

Appendix 2: Part of questionnaire for mother and child records

Place form barcode here (start with R)

Form R (Recruitment)

Date recruited: ☐☐/☐☐/☐☐☐☐ (dd/mm/yyyy)

Inclusion/exclusion criteria:

If the participant is above age of consent, does the individual understand study explanation and give consent to participate? ☐ Yes ☐ No

If the participant is below age of consent, does the guardian/parent understand study explanation and give consent to participate? ☐ Yes ☐ No

Outcome

If consent is No, the individual can not participate. Thank them for their time.

If consent is Yes, and informed consent form is signed, continue to fill the rest of the recruitment form and give study ID.

Study ID # : 02☐☐☐☐☐☐

Age: _____ (check one) ☐ years ☐ months ☐ days old

Gender (check one): ☐ Male ☐ Female

Village _____

Mother Tribe _____

Father Tribe _____

Ten Cell Leader _____

Participant sleeps under an Insecticide-Treated Bednet (ITN): ☐ Yes ☐ No

Others in home sleep under an ITN: ☐ Yes ☐ No

If the participant is an adult female,

Number of pregnancies before this study: _____

Number of fetal losses before this study: _____

Number of fetal losses with gestational age greater than 3 months: _____

Gestation in weeks (if pregnant):_____

Date of last Delivery/Miscarriage: ☐☐/☐☐☐☐ (mm/yyyy)

Initials _____

Form NB (Delivery Hospitalization - Newborn)

Place form barcode here (start with I)

Study ID: 02☐☐☐☐☐

Date of visit (dd/mm/yyyy): ☐☐/☐☐/☐☐☐☐

Number delivered: (check one)

☐ Singleton ☐ Triplets
☐ Twins ☐ Quadruplets

Pregnancy Outcome:

Infant 1
☐ Viable ☐ Still Birth
☐ Abortion ☐ Neonatal Death

Infant 2 (if twins)
☐ Viable ☐ Still Birth
☐ Abortion ☐ Neonatal Death

Infant 3 (if triplets)
☐ Viable ☐ Still Birth
☐ Abortion ☐ Neonatal Death

Zygosity : (check one)

☐ Monozygotic ☐ Trizygotic
☐ Dizygotic ☐ Quadrazygotic

After delivery:

Mother Height_____ (cm) Mother Weight_____ (kg)

Newborn Physical Exam

Infant ID	*Sex*	Date delivered (dd/mm/yyyy)	Time delivered	Dubowitz	Height (cm)	Weight (kg)	Temp (°C)	Head Circ. (cm)	Score (APGAR) 1'	Score (APGAR) 5'
1										
2										
3										
4										

Comments:

Initials _____

Data entry 1 code: ☐☐
Data entry 2 code: ☐☐

Form NB (Version 1.1.0)
11/30/2005

Signature:
Date:

Form SC (Sample Collection)

Place form barcode here (start with S)

Study ID: 02☐☐☐☐☐☐

Sample Collection Date (dd/mm/yyyy): ☐☐/☐☐/☐☐☐☐

!!!Important: this form must accompany samples to the lab

Visiting type:

- ☐ Delivery hospitalization
- ☐ Non-Delivery hospitalization
- ☐ 2-week/ 4-week/Walk-in visit

Patient is:

- ☐ Participant
- ☐ Infant 1
- ☐ Infant 2 (if twins)
- ☐ Infant 3 (if triplets)

Instruction: Peripheral or HS/FS (for newborns) blood samples should be collected at all visits. During delivery hospitalization, all samples listed below are collected; HS/FS is collected from newborn and recorded on infant's SC Form(s).

	Sample Type	Sample Type barcode	Collection Date	Collection Time	Volume of blood (ml)	Anti coagulant volume (ml)	Initials	Blood Group
☐	Peripheral Blood	ST001						
☐	HS/FS	ST002			N/A	N/A		N/A
☐	Placental Blood	ST003						N/A
☐	Cord Blood	ST004						
☐	Tissue Frozen	ST005			N/A	N/A		N/A
☐	Tissue RNA Later	ST006			N/A	N/A		N/A

If patient is an infant, was Placenta sample collected? ☐Yes ☐No

If yes, fill in the following: Placenta Weight _____ Kg

Cord Length _____ cm