Effect of *Plasmodium falciparum* Malaria on Epstein Barr Virus (EBV) Reactivation during Pregnancy and Subsequent Shedding of EBV in Breast milk Postpartum

Ibrahim Ismail Daud

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university or for any other award.

Signature..... Date.....

Ibrahim Ismail Daud

This thesis has been submitted with our approval as University supervisors.

1. Signature..... Date.....

Prof. Rosemary Rochford

SUNY Upstate Medical University, NY, USA

2. Signature..... Date.....

Prof. Elizabeth A. Bukusi

Kenya Medical Research Institute (KEMRI), Kenya

3. Signature..... Date.....

Prof. Zipporah W. Ng'ang'a

JKUAT, Kenya

DEDICATION

I dedicate this work to my mother, Maryam Hish, and father, Ismail Daud, who were instrumental and source of inspiration throughout my studies. This goes to my lovely wife Jowhara H. Ahmed who was very supportive and patient during my studies. My sons Ayman, Zait, Zubeir and Zakir. My siblings Ruqiya, Abdul-Rashid, Mohamed, Halima, Abdul-latif, Ramla and Sumaiyah.

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ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immunodeficiency Syndrome
ANC	Antenatal Care
BCR	B cell receptor
BL	Burkitt's lymphoma
CIDR1α	Cysteine-rich interdomain region 1α
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
DNA	Deoxyribonucleic acid
EA	Early Antigen
EAd	EBV Early Antigen diffuse
EBER	EBV encoded mRNA
eBL	endemic Burkitt's Lymphoma
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
ELISA	Enzyme Linked Immunosorbent Assay
FAM	6-carboxy-fluorescein
HIV	Human immunodeficiency virus
HSV	Herpes Simplex Virus
IFNγ	Interféron-gamma
Ig	Immunoglobulin

IgG	immunoglobulin G
IL	interleukin
IL-1 β	Interleukin - 1β
IM	Infectious Mononucleosis
LMP	Latent membrane protein
MFI	Mean Fluorescent Intensity
MHC	Major histocompatibility complex
NIH	National Institute of Health
NK	Natural Killer
OHL	Oral Hairy Leukoplakia
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
Q-PCR	Real-time quantitative PCR
SES	Socio-economic Status
TAMRA	6-carboxy-tetramethylrhodamine
TGFβ	Transforming growth-factor β
TNF- α	Tumor Necrosis Factor α
VCA	Viral Capsid Antigen

ABSTRACT

Epstein–Barr virus (EBV) is an oncogenic virus that has been implicated in the etiology of endemic Burkitt's lymphoma (eBL). Infection with EBV early in life and repeated *Plasmodium falciparum* malaria exposures have been linked to the etiology of eBL. Previous study reported that 35% of children in Kisumu were infected before 6 months of age. However, the underlying mechanism that predisposes infants to EBV infection before 6 months of age and the role of *P. falciparum* malaria in EBV transmission is a significant gap in the etiology of eBL. The objective of this study was to determine the effect of *P. falciparum* malaria infection on EBV reactivation during pregnancy, and subsequent EBV shedding in breast milk postpartum among pregnant women. The study enrolled 175 HIV negative pregnant women attending the antenatal clinic and followed them prospectively from antenatal to delivery through to postpartum. Data on demographic, obstetrics and socioeconomic status were collected. Blood and breast milk samples were collected at various visits. Malaria diagnosis and EBV load were measured by quantitative polymerase chain reaction assay (Q-PCR). EBV serology was measured using ELISA. DNAse I based assay was used to assess whether there was encapsidated virus in breast-milk. EBV DNA positive breast-milk supernatant was exposed to 10^6 cells/ml Peripheral Blood Mononuclear Cells (PBMC) and observed for evidence of transformation. Results show that pregnant women who had malaria during pregnancy were more likely to have a detectable EBV DNA than pregnant women who had no evidence of malaria during pregnancy (64% vs. 36%, p=0.01). EBV load, as quantified using area under the longitudinal observation curve (AUC), was significantly higher in women with *P. falciparum* malaria than in women without malaria (p =0.01). Increase in EBV load correlated with that of malaria load (p <0.0001). Independent of malaria infection, EBV load was significantly higher at third trimester (p =0.04) than first and second trimester of pregnancy. EBV DNA and EBV load in breast-milk was significantly higher at 6 weeks and decreased sequentially in subsequent visits (p < .0001). Virus in breast milk supernatant was found to be DNAse I resistant in 24/40

(60%) of samples, and showed evidence of lymphocyte transformation. Being infected with *P. falciparum* infection at delivery was significantly associated with increased EBV shedding in early breast milk ($p = 0.02$), whereas, levels of EBV load in maternal blood at delivery was positively correlated with that of EBV load at 6 weeks postpartum ($p = 0.002$). The findings suggest that malaria during pregnancy causes EBV reactivation leading to high EBV load in maternal circulation, which subsequently increases shedding of infectious EBV in breast milk, a possible conduit of EBV transmission in infants at an early age. This study recommends sustained long-term efforts in malaria control programs such as, use of insecticide treated bed nets, intermittent preventive therapy, and indoor vector control, with a view to reducing EBV reactivation during pregnancy and subsequent EBV shedding in breast milk, consequently stemming eBL.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Epstein–Barr virus (EBV) is a human gamma-herpes virus that infects and persists in >90% of the world adult population clinically manifesting either as an asymptomatic infection or as acute infectious mononucleosis (Silins et al., 2001; Young & Rickinson, 2004). However, EBV infection is associated with malignant transformation of infected cells and is etiologically linked to endemic Burkitt's lymphoma (eBL), the most common childhood cancer in equatorial Africa (Kutok & Wang, 2006; I. Magrath, 1990). Previous epidemiologic studies (Dalldorf, 1963; Morrow, 1985; Rainey et al., 2007) as well as recent study done in Kenya (Rainey et al., 2007) reported a striking overlap between an elevated incidence of malaria transmission and eBL, and confirmed that holoendemic malaria was associated with an increased risk for eBL. EBV is also implicated in nasopharyngeal carcinoma, a common cancer that occurs in China (W. Henle, Henle, & Ho, 1978), T-cell lymphomas commonly found in Taiwan and Japan (Su et al., 1990) and a subset of Hodgkin's lymphoma and gastric carcinoma (Shibata et al., 1991). In Kenya eBL is more frequent around Lake Victoria and along the costal region, which are malaria holoendemic regions (Mwanda, Rochford, Moormann, et al., 2004; Rainey et al., 2007). The average annual incidence rate of eBL in Kenya is 0.83 per 100,000 children (Mwanda, Rochford, Moormann, et al., 2004).

Geographical differences in age of primary EBV infections exist with infants in sub-Saharan Africa getting infected early in life than their counter parts in the developed world where infections occur in adulthood (Biggar, Henle, Bocker, et al., 1978). A previous study in Kenya reported that there is a significantly earlier age of primary EBV infection in infants from Kisumu – a malaria holoendemic region of Kenya – compared to infants from Nandi, an area with little malaria transmission (Erwan Piriou et al., 2012), suggesting a role for malaria in increasing EBV risk in early infancy. Strikingly, 35% of children in Kisumu were infected before 6 months of age (Erwan Piriou et al.,

2012), supporting the long-held hypothesis that EBV infection early in life and holoendemic malaria are risk factors for eBL (de-The, 1977)

Cellular immunity, particularly $CD8^+$ cytotoxic T lymphocytes (CTL) play a pivotal role in immunosurveillance of EBV infections (Callan, 2003; Moormann et al., 2007; Njie et al., 2009). As pregnancy is thought to be a state of repressed cellular immune responses (Luppi, 2003; Motran et al., 2002), it is possible that the immunological control of EBV infections may be naturally altered. Using serology as a marker of EBV reactivation, early studies showed an increased incidence of EBV reactivation during pregnancy (Haeri, Baker, & Boggess, 2010; Purtilo & Sakamoto, 1982; Sakamoto et al., 1982). However, Meyohas et al (Meyohas et al., 1996) measured EBV DNA in pregnant women and found no significant differences in the prevalence of EBV DNA in pregnant women compared to non-pregnant women. Moreover, EBV has rarely been implicated in *in utero i*nfection and few studies reported the risk of Mother-to-child transmission of EBV (Baschat, Towbin, Bowles, Harman, & Weiner, 2003; Meyohas et al., 1996). Pregnant women are at a greater risk of infection with *P. falciparum* with substantial adverse effects on both maternal and fetal health (Desai et al., 2007; Rogerson, Hviid, Duffy, Leke, & Taylor, 2007). *P. falciparum* malaria is known to interfere with EBV biology and EBV-specific immunity (Moormann et al., 2005; Moormann et al., 2007; E. Piriou et al., 2009) and can induce EBV reactivation (D. Donati et al., 2006; Rochford, Cannon, & Moormann, 2005). To the best of our knowledge, no study has been done on impact of *P. falciparum* malaria on EBV reactivation in pregnant women.

EBV is transmitted primarily through contact with saliva and viral DNA can be detected in saliva in adults (S. M. Mbulaiteye et al., 2006) and children (D. Donati et al., 2006; S. M. Mbulaiteye et al., 2006) by PCR. An alternative source of infectious virus for infants is breast milk, where EBV DNA was detected in breast milk of nearly half of the mothers (Gantt et al., 2008; Junker et al., 1991). Recent studies done in Kenya documented EBV infections that occurred before 6 months of age (Erwan Piriou et al.,

2012), suggesting that perinatal transmission of EBV may occur as a result of breast-feeding. Perinatal transmission through breast milk has been suggested previously for cytomegalovirus (CMV) (Hamprecht, Witzel, Maschmann, Speer, & Jahn, 2000), and Herpes Simplex Virus (HSV) (Dunkle, Schmidt, & O'Connor, 1979), which are members of the herpesviridae. Though previous cross sectional studies reported detection of EBV DNA in breast milk (Gantt et al., 2008; Junker et al., 1991), there are significant gaps in our understanding of the dynamics of EBV load over time and whether EBV in breast milk is a cell-free and infectious virus.

Though both EBV infection early in life and holoendemic malaria are known risk factors for eBL, why children are infected early in life with EBV, and what role malaria plays in EBV transmission remain elusive. This study hypothesized that infection with malaria during pregnancy could cause EBV reactivation leading to high EBV load in circulation, which subsequently lead to increased EBV shedding in breast milk, a possible conduit of EBV transmission. In this prospective study in Western Kenya, where malaria is holoendemic (Moormann et al., 2005; Ndenga et al., 2006) and eBL risk is high (Rainey et al., 2007), pregnant women were recruited and actively followed through delivery and postpartum, to determine the impact of *P. falciparum* malaria on EBV reactivation during pregnancy and the subsequent shedding of EBV in breast milk postpartum.

1.2 Statement of the Problem

In Kenya eBL is more frequent around Lake Victoria and along the costal region, which are malaria holoendemic regions (Mwanda, Rochford, Moormann, et al., 2004; Rainey et al., 2007). The average annual incidence rate of eBL in Kenya is 0.83 per 100 000 children (Mwanda, Rochford, Moormann, et al., 2004). It is well established that EBV infection early in life and repeated malaria infection are the key risk factors for the development of eBL (D. P. Burkitt, 1972; I. T. Magrath, 1991; Mwanda, 2004). However, why children are getting infected with EBV at an early age and how malaria modulates EBV infection early in life is unknown. In addition, although previous cross sectional studies detected EBV DNA in breast milk (Gantt et al., 2008; Junker et al., 1991), there are significant gaps in our understanding of the impact of *P. falciparum* malaria on EBV shedding in breast milk over time and whether the EBV virus in breast milk is infectious. Therefore, this prospective study aimed to evaluate the impact of *P. falciparum* malaria infection on EBV reactivation during pregnancy and subsequent shedding of infectious/transforming EBV in breast milk postpartum.

1.3 Justification

The concept of polymicrobial diseases – diseases aetiologically linked to infection by more than one pathogen – is a novel and rapidly emerging concept that has gained an increased attention in the recent past. Endemic Burkitt's lymphoma (BL) is often described as a polymicrobial disease because of its close association with infection by more than one pathogen, EBV and *P. falciparum* malaria (D. P. Burkitt, 1972; I. T. Magrath, 1991; Rochford et al., 2005). Endemic BL is a monoclonal B cell non-Hodgkin's lymphoma characterized by a high proliferative index and is the most common childhood cancer in Equatorial Africa with a peak age of 6 years (D. P. Burkitt, 1972; I. T. Magrath, 1991; Mwanda, 2004); indicating that there is a very short time frame between exposure to cancer promoting events and the emergence of malignancy. It is well established that EBV infection early in life and repeated malaria infection are the key risk factors for the development of eBL (D. P. Burkitt, 1972; I. T. Magrath,

1991; Mwanda, 2004). However, why children are getting infected with EBV at an early age and how malaria modulates EBV infection early in life is unknown. Moreover, although previous cross sectional studies detected EBV DNA in breast milk (Gantt et al., 2008; Junker et al., 1991), there are significant gaps in our understanding of the impact of *P. falciparum* malaria on breast milk EBV load over time. Therefore, this prospective study aimed to evaluate the impact of *P. falciparum* malaria infection on EBV reactivation during pregnancy and subsequent shedding of infectious/transforming EBV in breast milk postpartum.

It is difficult to eradicate the prevalence of EBV-associated malignancies without preventing viral transmission. Thus, understanding the impact of malaria in EBV reactivation and subsequent shedding dynamics in breast milk over time, are critical in establishing effective interventions. Furthermore, although treatment of eBL can be effective in developed countries, there are severe limitations in the delivery of anti-cancer drugs in Africa (Mwanda, Rochford, Rainey, & Wilson, 2004), therefore, prevention of eBL remains' as a viable and important goal.

1.4 Research Questions

1. What is the prevalence of malaria and the anthropometric measures of birth outcomes among the pregnant women participating in the cohort study?
2. Does the EBV DNA and EBV load during pregnancy differ between pregnant women with and without *P. falciparum* malaria?
3. What is the association between EBV load and *P. falciparum* malaria load among pregnant women with *P. falciparum* malaria?
4. Does the level of EBV load differ with gestational age of pregnancy among pregnant women with and without *P. falciparum* malaria?
5. What are the dynamics of EBV load relative to gestational age of pregnancy among pregnant women without malaria infection?

6. What is the EBV DNA and EBV load in breast milk overtime (6, 10, 14 and 18 weeks postpartum)?
7. Does breast milk contain encapsidated and infectious EBV?
8. What are the risk factors for EBV shedding in early breast milk samples (6 weeks) postpartum?
9. What is the correlation of EBV load in maternal blood at delivery and early breast-milk samples (6 weeks) postpartum?

1.5 Hypothesis

1.5.1 Null Hypothesis

Plasmodium falciparum malaria infection has no effect on EBV reactivation during pregnancy and subsequent shedding of EBV in breast-milk.

1.6 Objectives

1.6.1 General Objective

To determine the effect of *P. falciparum* malaria infection on EBV reactivation during pregnancy, and subsequent EBV shedding in breast milk postpartum among pregnant women.

1.6.1.1 Specific Objectives:

1. To determine the prevalence of malaria, helminthes infections and the anthropometric measures of birth outcomes among the pregnant women participating in the cohort study.
2. To determine EBV DNA and EBV load during pregnancy among pregnant women with and without *P. falciparum* malaria.
3. To determine association of EBV load and *P. falciparum* malaria load among pregnant women with *p. falciparum* malaria.
4. To determine the differences in EBV load with gestational age among pregnant women with and without *P. falciparum* malaria.

5. To determine the dynamics EBV load relative to gestational age among pregnant women without malaria infection.
6. To determine the EBV DNA and EBV load in breast milk overtime (6, 10, 14 and 18 weeks postpartum).
7. To determine whether EBV in breast milk is encapsidated and infectious.
8. To determine risk factors for EBV shedding in early breast milk samples (6 weeks) postpartum.
9. To determine the correlation of EBV load in maternal blood at delivery and early breast-milk samples (6 weeks) postpartum.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epstein-Barr Virus (EBV)

2.1.1 History of EBV

In the 1950s, a British Surgeon Dennis Burkitt working in equatorial Africa described and isolated cells from an aggressive lymphoma, later named after him. The strict geographical and climatic distribution as well as the increased frequency of Burkitt's lymphoma in children living in equatorial Africa points to specific interactions between environmental, genetic and viral factors in its etiology. It was not until 1964 that Anthony Epstein and his colleagues Yvonne Barr and Bert Achong identified herpesvirus-like particles from Burkitt's lymphoma biopsies using Electron microscope (Epstein, Achong, & Barr, 1964). The virus particle identified from the biopsies was later called the Epstein-Barr virus (EBV).

The etiological link between EBV and Burkitt's lymphoma, a tumor involving mainly the jaw (D. Burkitt, 1958) was documented (G. Henle & Henle, 1966). Thereafter, EBV was further linked to nasopharyngeal carcinoma (G. Henle & Henle, 1966; zur Hausen et al., 1970), and several other malignancies, including gastric carcinomas, Hodgkins lymphoma, and numerous T cell lymphomas (Rickinson, 2001). In Kenya eBL is more frequent around Lake Victoria and along the costal region, which are malaria holoendemic regions (Mwanda, Rochford, Moormann, et al., 2004; Rainey et al., 2007). The average annual incidence rate of eBL in Kenya is 0.83 per 100 000 children (Mwanda, Rochford, Moormann, et al., 2004). A map of Africa and Kenya showing the distribution of Burkitt's lymphoma in Africa and Kenya respectively is shown in figure 2.1 (W. Henle, Henle, & Lennette, 1979) and figure 2.2 (Rainey et al., 2007).

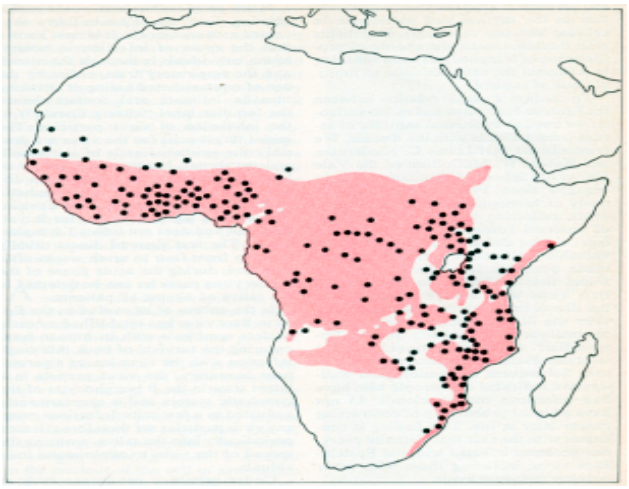


Figure 2.1: Distribution of Burkitt's lymphoma in Africa
"Distribution of Burkitt's lymphoma in Africa reveals that clusters of cases (*back dots*) are found in areas where malaria is endemic because of climatic factors (*light colour*)". (Adapted from (W. Henle et al., 1979).

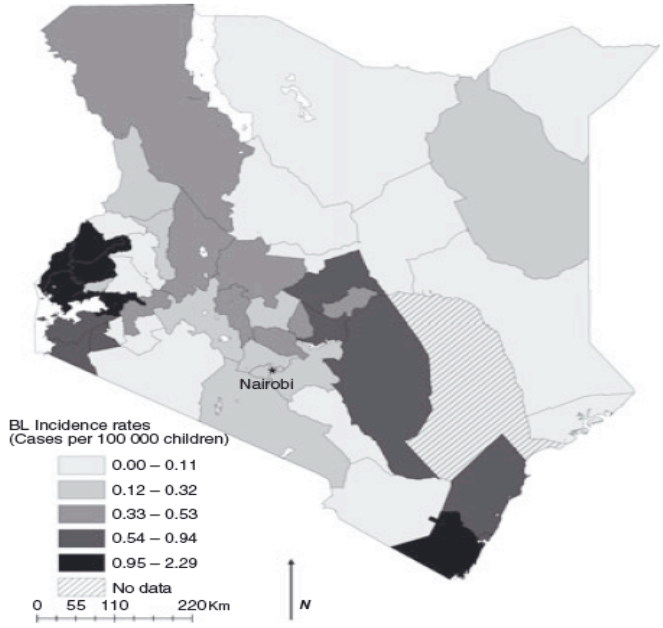


Figure 2.2: Distribution of Burkitt's lymphoma in Kenya.
Endemic Burkitt's lymphoma is more frequent around Lake Victoria and along the costal region, which are malaria holoendemic regions of Kenya.
Adapted from (Rainey et al., 2007)

2.1.2 Classification and Genomic organization of EBV

EBV is about 170 kb, enveloped, and double-stranded DNA virus with a single linear genome, which is circularized after infection and maintained as an extrachromosomal episome (Adams & Lindahl, 1975; Baer et al., 1984). EBV is a human herpes virus classified within the gamma herpesviruses subfamily and establishes a latent life long infection. Based on sequence variation in the EBV nuclear antigens 2 and 3 (EBNA 2 and 3), EBV has been classified into two strains namely, EBV-1 and EBV-2 (also known as types A and B) (Sample, Hummel, Braun, Birkenbach, & Kieff, 1986).

The EBV genome is wrapped around and organized into a series of unique internal and terminal repeat domains (Baer et al., 1984). The genome encodes about 80 different proteins that are involved in both the latent and lytic phases of cycle (Baer et al., 1984). The nomenclature of EBV open-reading frames (ORFs) is based on the size of the *Bam*HI-restriction fragment containing the RNA site start with rightward transcriptional orientation (figure 2.3).

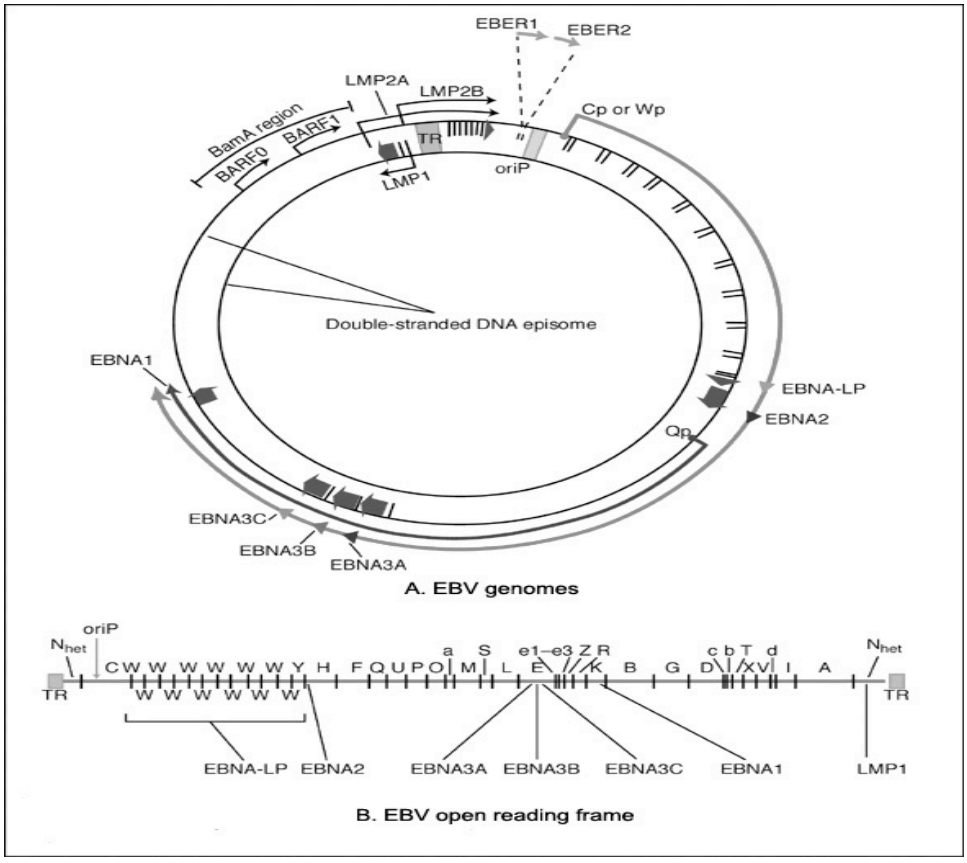


Figure 2.3: EBV genome and open reading frame (ORF) showing all latent genes. Adapted from (Young & Rickinson, 2004).

2.2 Epidemiology of EBV

EBV is ubiquitous in the worlds' population infecting more than 90% of adult population with primary infection presenting as asymptomatic and the virus establishes life-long infections in the host (Black, Woodall, Evans, Liebhaber, & Henle, 1970; Lang, Garruto, & Gajdusek, 1977; Morrow, 1985). The World Health Organization's International Agency for Research on Cancer (IARC) has classified EBV as a group 1 carcinogen and considered to have direct role in the etiology of endemic form of Burkitt lymphoma, gastric carcinomas, nasopharyngeal carcinoma, and a subclass of Hodgkin lymphoma (IARC, 1997). However, the underlying mechanism linking EBV infection of B cells to the development of the malignant B cell is unknown. The ubiquitous nature of

EBV in the population points to the requirement for other co-factors in the etiology of EBV-associated cancers. For eBL, malaria is a clear co-factor (Morrow, 1985), however, the cellular processes leading to malignancy remains elusive.

2.2.1 Transmission of EBV

EBV is a strict human pathogen transmitted primarily via contact with saliva (S. Mbulaiteye et al., 2006). The frequency of EBV DNA in saliva is 100% among adults and children tested by PCR (D. Donati et al., 2006; S. Mbulaiteye et al., 2006). Previous study using transformation assay measured EBV in saliva by evaluating the ability of saliva extracts to transform lymphocytes in culture (Miller et al. 1973).

EBV DNA of cell-associated virus and infectious cell free virus can be detected in cervical secretions of women (Andersson-Ellstrom, Bergstrom, Svennerholm, & Milsom, 1997; Israele, Shirley, & Sixbey, 1991; Rickinson, 2001; Sixbey, Lemon, & Pagano, 1986), implying that sexual transmission is a likely route of EBV transmission as well as raising the possibility of neonatal infection. Previous studies examined and found sexual activity among heterosexual relationships as well as in homosexual partnerships (Higgins et al., 2007; Woodman et al., 2005) as a potential risk factor for EBV transmission. Other potential risk factor for EBV transmission is increased number of sexual partners (Crawford et al., 2002; Woodman et al., 2005).

An alternative source of infectious virus for infants is the breast milk. EBV DNA was detected in breast milk of nearly half of the mothers seen in British Columbia's Children's Hospital Breast Milk Service (Junker et al., 1991). Recent studies done in Kenya documented EBV infections that occurred before 6 months of age (Erwan Piriou et al., 2012), suggesting that perinatal transmission of EBV may occur as a result of breast-feeding. Perinatal transmission through breast milk has been suggested previously for cytomegalovirus (CMV) (Hamprecht et al., 2000), and Herpes simplex virus (HSV) (Dunkle et al., 1979), members of the herpesviridae. Though EBV DNA has been detected in breast milk (Gantt et al., 2008; Junker et al., 1991), there are significant gaps

in our understanding of the dynamics of EBV load over time and whether EBV in breast milk is cell-free and infectious virus.

Previous studies attempted to determine whether mother to child EBV transmission occurs. A study in 1973 detected EBV infected cells in 1 out of 696 samples tested (Chang & Blankenship, 1973), while another study in 1976 identified EBV transformed cells in cord blood of 1 out of 40 samples (Visintine, Gerber, & Nahmias, 1976). On the other hand, additional study reported no EBV transformed cells from blood of neonates (Joncas, Boucher, Granger-Julien, & Filion, 1974). These findings show that it is not well established whether EBV can be transmitted *in utero*.

2.2.2 Age of primary EBV infection

Age of primary EBV infection varies considerably in the world. Using seroconversion, two peaks of EBV infection has been identified, age 2–4 years and 15 years (G. Henle & Henle, 1970). For instance, in developed countries, the majority of the population is not infected with EBV until young adulthood and this later age of infection can cause infectious mononucleosis, a self-limiting disease (Rickinson, 2001). The prevailing concept is that primary EBV infection cannot occur until after the loss of maternal antibodies after 8 months of age (Rickinson, 2001). For example, (K. H. Chan, Tam, Peiris, Seto, & Ng, 2001) studied a longitudinal cohort of 66 infants in Hong Kong and did not see any infection with EBV until after 8 months of age arguing for a protective role of maternal antibodies and delay of infection until they have waned. However, there is limited number of published studies on primary EBV infection in infants (Biggar, Henle, Fleisher, et al., 1978; K. H. Chan et al., 2001) and most studies have utilized only serologic markers as indicators of infection. In the Kenyan malaria holoendemic area, why 35% of infants were infected with EBV by 6 months of age (Erwan Piriou et al., 2012), is significant gap in our understanding of EBV. Could infectious diseases during pregnancy such as malaria lead to reactivation of EBV during pregnancy that subsequently lead to increase of EBV shedding in saliva and breast milk or could it be due to early waning of maternal antibodies?

2.2.3 Sero-epidemiology of EBV Infection

The EBV life cycle comprises both lytic and latent phases that induce different antibody responses (Hadinoto et al., 2008; Rowe, Kelly, Bell, & Rickinson, 2009). Antibodies specific for the EBV antigens—VCA, EAd, Zta and EBNA-1—have been used as clinical indicators of infection. EBV nuclear antigen (EBNA)-1 is a latent antigen that is expressed in latently infected memory B cells and is the only latent antigen consistently expressed in BL tumors (Young & Rickinson, 2004). Viral capsid antigen (VCA) and early antigen (EA) are expressed during lytic phases of replication (Dardari et al., 2008), and elevated antibody titers against VCA and EA have been used clinically as evidence of past infection (e.g. VCA IgG) or viral reactivation (e.g. EAd IgG). More recently, detection of antibodies against the EBV immediate early transactivator protein Zta (also known as ZEBRA) have been used diagnostically to detect individuals at risk for nasopharyngeal carcinoma (NPC), another EBV associated malignancy (K. H. Chan et al., 2003; Dardari et al., 2008; Njie et al., 2009). Taken together, these data indicate the antibody levels or dynamics in serological profiles is a useful serological marker for early detection or prognosis EBV associated malignancies.

2.2.4 EBV Infections

2.2.4.1 Host range and target cells for EBV

Although EBV is virtually ubiquitous in nature, humans serve as the only natural host. EBV preferentially infects and establishes latent infection in lymphocytes through the binding of the viral glycoprotein gp350 and gp42 to CD21 receptor and human leukocyte antigen (HLA) respectively and is able to induce proliferation of the latently infected cells (Tanner, Weis, Fearon, Whang, & Kieff, 1987; Young & Rickinson, 2004).

EBV is not only a B-lymphotropic virus, but it can also infect T lymphocytes and epithelial cells resulting in various clinical conditions including a subset of T-cell lymphomas and epithelial cell tumors such as nasopharyngeal and gastric carcinomas (Thompson & Kurzrock, 2004).

2.2.4.2 Establishment and maintenance of EBV latency

EBV spreads via the salivary tract to infect epithelial cells of the oropharynx, posterior nasopharynx, parotid gland, and possibly tonsilar lymphocytes, where the virus initiate lytic replication and releases progeny virions that in turn infect resting naïve B-cells in the underlying lymphoid tissues. Upon infecting naïve-resting B-cells, EBV switches on the latent proteins: Latent membrane proteins (LMP-1, LMP2-A and LMP2B), EBV-nuclear antigens (EBNA-1, EBNA-2, EBNA-3, EBNA-4, EBNA-5 and EBNA-6), and EBERs and BART (Thorley-Lawson & Allday, 2008; Tomkinson, Robertson, & Kieff, 1993). These latent proteins subsequently induce the resting naïve B-cells into an activated lymphoblast (Abbot et al., 1990), that travel to the follicles where they initiate germinal center reactions and establish the default transcription programme (latency II) (Thorley-Lawson & Mann, 1985). During the latency II program the virus switches on survival signals by expressing LMP1, which drives class switch recombination, an LMP2A that drives somatic hyper-mutation (Belhadj et al., 2003). Then, the B cells exit germinal centers and enter the memory B-cell compartment where EBV latency transcription programme (latency 0) switches on and all viral protein expression is turned off (Thorley-Lawson & Allday, 2008). These B cells are maintained by normal B cell homeostasis where EBV switches to latency 1 program or EBNA-1 alone program that allows the viral genome to be maintained in the nucleus of B-cells (Sun & Thorley-Lawson, 2007). Eventually, the memory B cells return to the tonsil, where they sometimes undergo plasma-cell differentiation, which activates lytic viral replication. The resulting virus is spread via saliva and serves as a source of EBV transmission to B cells or infects other susceptible hosts (Thorley-Lawson & Allday, 2008).

2.2.4.3 Reactivation of EBV from latency

To persist for lifelong period following primary infection, EBV hides in memory B cells and remains in a latent state. However, EBV periodically reactivates resulting in production of infectious virions (Flemington & Speck, 1990). The precise mechanism that triggers EBV reactivation is unknown though it may serve as a means for virus to

spread to uninfected B-cells. EBV protein Z (also known as Zta or ZEBRA) is important in the switch from latent to lytic cycle (Flemington and Speck 1990). Z protein transactivates the viral early promoters by binding to upstream binding sites known as Z response elements. Z protein also acts as a trigger to disrupt latency by transactivating the genes encoding two other regulatory proteins BRLF1 and BMLF1. Together these three proteins initiate a cascade of expression of about 100 replication-associated genes EA and VCA (Cayrol & Flemington, 1995; Farrell, Rowe, Rooney, & Kouzarides, 1989).

2.2.4.4 *In Vitro* EBV Infection

EBV infection of B cells results in lympho-proliferation, also called immortalization, which gives rise to the production of lymphoblastoid cell lines (LCL) *in vitro* (Miller, 1982). This proliferation of infected B cells is analyzed as a representative model of the ability of EBV to establish latency during *in vivo* infection (Thorley-Lawson & Gross, 2004). LCL in culture is dependent on the expression of 6 nuclear antigens including Epstein Barr Nuclear Antigen (EBNA) 1, 2, 3a, 3b, 3c, LP; 3 latent membrane proteins, LMP-1, LMP-2a and 2b; and other transcripts and proteins including untranslated RNAs, EBV encoded mRNA (EBNA) 1, EBNA2, and a family of BamH1A transcripts (Farrell, 1995). Expressions of the above major EBV latency genes activate the resting B cells and sustain their continuous proliferation (Joseph, Babcock, & Thorley-Lawson, 2000; Thorley-Lawson, Miyashita, & Khan, 1996).

2.2.5 EBV and Associated Malignancies

EBV is an oncogenic virus and is associated with the development of a number of malignancies of lymphocytic and epithelial origin (Young & Rickinson, 2004). EBV associated malignancies are thought to have endemic patterns of incidence, develop in situations of immunosuppression or are a distinct subset of more common cancers (Rickinson, 2001). The endemic EBV-associated tumors was originally isolated from samples of African endemic Burkitt's Lymphoma (eBL), a common childhood cancer in equatorial Africa (Epstein et al., 1964). Thereafter, EBV was implicated in

nasopharyngeal carcinoma, a common cancer that occurs in China (W. Henle et al., 1978), T-cell lymphomas commonly found in Taiwan and Japan (Su et al., 1990)and later a subset of Hodgkin's lymphoma and gastric carcinoma (Shibata et al., 1991).

2.2.5.1 Burkitt's lymphoma

Endemic BL is an aggressive monoclonal B cell non-Hodgkin's lymphoma characterized with high proliferative index and occurs at a higher rate in males than females (I. T. Magrath, 1991).There are 3 forms of BL found worldwide: endemic, sporadic, and AIDS-associated and all carry a t8;14 chromosomal translocation resulting in the deregulation of the c-myc oncogene (Bellan, De Falco, Lazzi, & Leoncini, 2003; I. Magrath, 1990). It is likely however that different pathogenic mechanism drive the emergence of these BL subtypes (De Falco et al., 2007). The hallmark of eBL is the occurrence of chromosomal translocation affecting the *c-myc* oncogene on chromosome 8 and possibly one of the immunoglobulin heavy or kappa and lambda light chain loci on chromosome 14, 2 or 22 respectively (Thorley-Lawson and Allday, 2008). The occurrence of the translocation brings the *c-myc* oncogene under the transcriptional control of the immunoglobulin locus (Klein, 1978; Nagy et al., 2009). It is this cytogenetic abnormality that drives unregulated cell growth and proliferation that is the hallmark of eBL (Thorley-Lawson and Allday, 2008). The *c-myc* oncogene is a regulator gene that codes for a transcription factor. The expression of this gene is tightly regulated but when it is deregulated as happens in eBL, thereby resulting in uncontrolled growth and proliferation of cells leading to eBL. These proliferating cells are characterized by being immortal, a defining signature of many cancers (Klein et al., 1976; Thorley-Lawson and Allday, 2008).

Endemic BL is extranodal and tumors are frequently found in the jaw or abdominal region (Mwanda, Whalen, et al., 2004). The peak age of onset is 6 years (Mwanda, Rochford, Moormann, et al., 2004), indicating that there is a very short time frame between exposure to cancer promoting events and the emergence of malignancy. In Kenya, eBL is more frequent around Lake Victoria and along the coastal region, which

are malaria holoendemic areas (Mwanda, Rochford, Moormann, et al., 2004; Rainey et al., 2007). A summary of Burkitt's lymphoma subtypes is shown in table 2.1, as adapted from (Rochford et al., 2005), and a child presenting with Burkitt's lymphoma in the lower maxillary quadrant of the jaw is shown in figure 2.4.

The first study to suggest a link between BL and malaria was by Dalldorf, in 1962 (Dalldorf, 1962). Subsequent epidemiologic studies as well as studies recently performed in Kenya confirmed that holoendemic malaria (e.g. perennial and intense malaria transmission) was associated with an increased risk for BL (Facer & Playfair, 1989; Morrow, 1985; Rainey et al., 2007). Molecular as well as epidemiologic studies have shown that there is an etiologic link between EBV and BL (de-The, Lavoue, & Muenz, 1978; Labrecque, Lampert, Kazembe, Philips, & Griffin, 1994; Rickinson, 2001). Support for a causal relationship came from a large-scale prospective study conducted in Uganda in the 1970s. More than 40,000 children were pre-bled, serum was stored and when tumors appeared, very high antibody titers against the EBV viral capsid antigen (VCA) were found in children that subsequently developed BL. The elevated VCA titers, and the stability of the elevated VCA antibodies over time, led (de-The, 1977) to suggest that infection of infants with EBV early in life could result in an infection that was poorly controlled by the host and thus increased the risk for eBL.

Table 2.1: Characteristics of Burkitt's lymphoma (BL) subtypes

Characteristics	**Endemic**	**Sporadic**	**HIV Associated**
Geographical Association	Equatorial Africa* Papua New Guinea	United States, Europe,	Worldwide
EBV-Association	>90%	<20%	~30%
Incidence Rate	1–20/100,000	0.01/100,000	Variable
Age Range	2–14 years	All ages	All ages
Tumor Site	Extranodal	Lymph nodes	Lymph nodes
c-myc translocation	Yes	Yes	Yes
Cofactors	Malaria, EBV	Unknown	HIV infection

"*There is some evidence shoeing that in Africa all three subtypes can be present". Adapted from (Rochford et al., 2005)

Figure 2.4: A child presenting with Burkitt's lymphoma of the jaw.
(Source: http://www.frtomskids.org/images/Burkitts1.jpg) Accessed 10 December 2012.

2.3 EBV and host immune response

EBV has co-evolved with humans and infects over 90% of the worlds' population and remains asymptomatic, suggesting effective control by the host immune system. EBV-host interaction entails a dynamic balance between host immunosurveillance, viral

replication and proliferation of virus-infected cells.

EBV infection elicits both cellular and humoral immune responses, with $CD8^+$ cytotoxic T lymphocytes (CTL) playing a pivotal role in the control of persistent EBV infections (Moormann et al., 2007; Njie et al., 2009) secondary to production of interferon (IFN)-γ (Callan, 2003; Hislop, Taylor, Sauce, & Rickinson, 2007; Njie et al., 2009). CTLs specific for both EBV latent and lytic proteins are readily detectable in healthy EBV-seropositive individuals and the loss of CTL specific responses leads to increased risk of EBV associated malignancies (Callan, 2003; Lunemann et al., 2008). Acute EBV infection is associated with activation and expansion of EBV-specific CTLs that gradually decline as viremia drops (Odumade et al., 2012).

There is scanty information on the role of CD4 T cells in EBV infections, with no CD4 T cell being detected following acute EBV infections (Maini, Gudgeon, Wedderburn, Rickinson, & Beverley, 2000). Previous studies described the presence of EBV-specific CD4 T cells in circulation of EBV seropositive (Khanna et al., 1995), as well as in eBL patients (Khanna et al., 1997).

Antibodies against various EBV proteins are also essential in EBV infection, diagnosis and characterization of EBV persistence (Nystad & Myrmel, 2007). For instance, detection of IgM antibodies to viral capsid antigen (VCA) signifies acute EBV infection (C. W. Chan, Chiang, Chan, & Lau, 2003; Nystad & Myrmel, 2007), whereas detection of IgG against early antigens (EA) signifies late primary or previous infection (Nystad & Myrmel, 2007). In addition, anti-EBV VCA and Epstein-Barr nuclear antigen (EBNA) 1-specific IgG persist for life in healthy carriers, whereas detection of early antigen diffuse (EAd)-specific and Z trans-activation antigen (Zta) IgG signifies EBV reactivation (Asito et al., 2010; E. Piriou et al., 2009; Tedeschi et al., 1995).

2.4 EBV, Malaria and Pregnancy

2.4.1 Role of malaria in EBV infection and Disease

In the last few years, several studies have pointed to a profound dysregulation of EBV persistence and immunity in children due to malaria (D. Donati et al., 2006; Moormann et al., 2005; Moormann et al., 2007; Njie et al., 2009; Rasti et al., 2005; Yone, Kube, Kremsner, & Luty, 2006). Past studies observed an increased viral load in peripheral blood of children living in a malaria holoendemic area (Moormann et al., 2005), and serological evidence of long term viral reactivation (E. Piriou et al., 2009). Others have found elevation of viral DNA in plasma during an episode of acute malaria (D. Donati et al., 2006; Rasti et al., 2005) with a decline in EBV DNA in the plasma following treatment (Yone et al., 2006), indicative of viral reactivation. Expansion of latently infected cells during acute malaria has also been found (Lam, Syed, Whittle, & Crawford, 1991; Njie et al., 2009). In transplant patients, EBV viral load is monitored closely and elevated viral load is associated with increased risk for post-transplant lymphoproliferative disease (Stevens et al., 2001), but yet these patients do not go on to develop BL. Hence, elevated viral load alone is not sufficient for the emergence of a malignant clone.

Recent clues point to a model where chronic antigenic activation of EBV-infected B cells within the context of repeated *P. falciparum* infections may lead to cytogenetic abnormalities induced by the enzyme activation induced cytidine deaminase (AID) (Okazaki, Kotani, & Honjo, 2007). AID is required for class switch recombination and somatic hypermutation in germinal center B cells (McBride et al., 2008). But AID over-expression in AID deficient B cells was observed to sufficiently to induce IgH-c-myc translocations (Ramiro et al., 2006), characteristic of BL. In addition, AID induces lesions on the c-myc gene (Robbiani et al., 2008). Moreover, when Em-cmyc transgenic mice were crossed with AID deficient mice, only AID+/+ mice developed predominantly mature B cell lymphomas (Kotani et al., 2007). While all these studies were done in mouse models, these data argue for a critical role of AID in the c-myc:IgH translocation characteristic of BL and suggest that aberrant AID expression could be a

risk factor for lymphomagenesis. In support of this hypothesis, two studies found a strong correlation between AID expression in peripheral blood lymphocytes and increased risk for non-Hodgkin's lymphoma (Agopian et al., 2009; Epeldegui et al., 2007).

2.4.2 EBV Reactivation in Pregnancy

Using serology as a marker of EBV reactivation, early studies showed an increased incidence of EBV reactivation during pregnancy among healthy pregnant women, suggesting that EBV reactivation during pregnancy can occur (Haeri et al., 2010; Purtilo & Sakamoto, 1982; Sakamoto et al., 1982). However, Meyohas et al., (1996), measured EBV DNA using PCR in pregnant women and found no significant differences in the prevalence of EBV DNA in pregnant women compared to non-pregnant women (Meyohas et al., 1996).

2.4.3 Malaria in Pregnancy

Infection of pregnant women with *P. falciparum* has significant adverse effects on both maternal health and fetal morbidity and mortality (Desai et al., 2007; Rogerson et al., 2007). In sub-Saharan Africa, an estimated 25% of pregnant women will have placental malaria, which frequently causes low-birth weight infants, stillbirth and fetal growth restriction (Desai et al., 2007). Although the greater burden of placental malaria is on primagravid women, women of all gravidities are at risk for malaria infections (Desai et al., 2007), and the gravidity-based immunity of the mother can affect the outcome for the fetus and infant.

While previous studies on placental malaria have focused on birth outcomes, more recent studies are finding that infants born to mothers with malaria have altered innate and adaptive immune responses (Adegnika et al., 2008; Brustoski et al., 2005; Ismaili et al., 2003; Rachas et al., 2012). Several groups have reported that *in utero* sensitization to parasitic antigens occurs, and that this exposure affects both the development of anti-malaria specific antibodies to prevent infection as well as infant T cell responses at birth

with a shift towards Th2 mediated responses in cord blood mononuclear cells from parasitized placenta (Adegnika et al., 2008; Brustoski et al., 2005; Ismaili et al., 2003; Rachas et al., 2012).

Another mechanism that placental malaria could affect infant immunity was suggested by a recent finding that tolerance to antigens encountered *in utero* develops through establishment of regulatory T cells (Mold et al., 2008). Exposure to malaria during pregnancy was found to induce a tolerant phenotype in the infants who had a greater risk for malaria over time and evidence of T cell anergy (Malhotra et al., 2009). Thus, a consequence of *in utero* exposure to malaria could be a significantly reduced ability of infants to respond to both *Plasmodium* as well as other infectious diseases encountered early in life and their subsequent susceptibility to infectious diseases during infancy.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site

The current study was carried out at Chulaimbo Sub-District Hospital located near the shores of Lake Victoria in Kisumu County of western Kenya. Chulaimbo sub-district hospital serves predominantly rural populations. Transmission of malaria in this area is holoendemic (perennial and intense malaria transmission) with two seasonal peaks, June to August and November to December (Moormann et al., 2005; Ndenga et al., 2006). Annual entomologic inoculation rates in Kisumu may exceed 300 infectious bites per person (John et al., 2005). The area has a high incidence of eBL (Rainey et al., 2007).

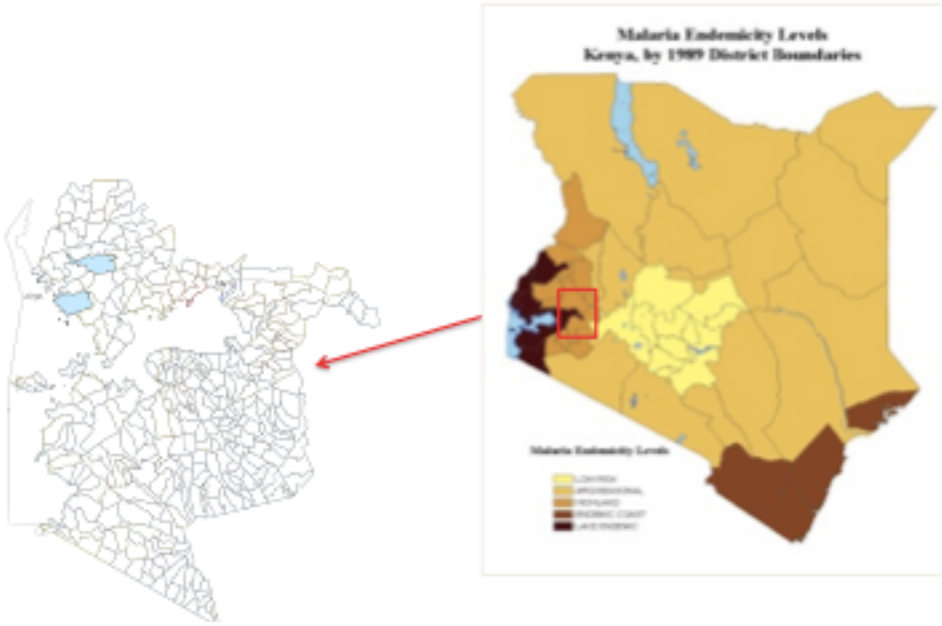


Figure 3.1: Map of Kenya showing the study site.
Adapted from Rainey et al., (2007).

3.2 Study Design

A prospective cohort study was conducted where pregnant women attending the antenatal clinic were recruited and followed them from antenatal (up to 4 visits) to delivery through to postpartum (up to 4 visits). This sub-study was nested within an ongoing prospective study titled *"Effects of Malaria and HIV on EBV Persistence in Infants and their Mothers*: KEMRI SSC Protocol number 1910". The objective of the parent study was to determine the effects of placental malaria on transfer of maternal EBV-specific neutralizing antibodies and in utero sensitization to EBV antigens.

3.3 Sampling Methods

Non-random quota sampling technique was used. Two hundred expectant mothers were interviewed, recruited and followed up till 18 weeks postpartum.

3.4 Study Population

The study population comprised of pregnant women, of all gravidities, residing within 10 km distance of the hospital and attending Chulaimbo Sub-District Hospital antenatal clinic (ANC). The women were recruited into this study after obtaining written informed consent. All pregnant women were tested for HIV as part of Prevention of Maternal-to-Child-Transmission of HIV programs in accordance with the Kenya Ministry of Health national guidelines (MOH, 2012).

3.4.1 Inclusion criteria:

- Pregnant women, aged 18 years and above
- Pregnant women of all gravidities
- Gestational age less than 30 weeks
- Residency within a 10km distance of the hospital
- Able and willing to give informed consent for study participation
- Willing to return to the clinic for follow up clinical procedures and laboratory testing
- Having a normal full blood count
- HIV negative

3.4.2 Exclusion criteria:

- Other apparent infections besides malaria during pregnancy
- Significant antepartum hemorrhage or miscarriage
- Severe anaemia – (haemoglobin [Hb] less than 7.5 g/dL).
- Delivery at home
- Twin deliveries,
- Blood transfusion ≤ 24 hours before delivery

3.5 Sample Size and power calculation

The main outcome of this study is the proportion of EBV reactivation in pregnant women with and without *P. falciparum* malaria infection. For a study in which two proportions are compared with a chi-square test or a *z*-test that is based on the normal approximation to the binomial distribution, the equation for sample size as described by (Eng, 2003) is:

$$N = \frac{2\left(z_{1-\alpha_{/2}}\sqrt{2\bar{p}(1-\bar{p})} + z_{1-\beta}\sqrt{p_c(1-p_c)+p_a(1-p_a)}\right)^2}{(p_c - p_a)^2}$$

Where; p_c and p_a are pre-study estimates of the two proportions to be compared, p_c -p_a (that is, the minimum expected difference). The two groups comprising *N* are assumed to be equal in number, and it is assumed that two-tailed statistical analysis will be used. Now based on the preliminary data from our laboratory, the level of EBV reactivation among pregnant women with *P. falciparum* malaria infection was estimated to be p_C =48% and I assumed a 5-fold reduction (p_a=10%) in the level of EBV reactivation among pregnant women without *P. falciparum* malaria infection. For a significance criterion of α=0.05 and a power of 80%, using the formula above, with: p_c=0.48, p_a = *0.10*, p_c–p_a = 0.38, $\bar{p}$ = (0.48+0.10)/2=0.19, $z_{1-\alpha_{/2}}$=1.960, and $z_{1-\beta}$=0.8, the estimated total sample size N required was = 66 (33 per group)

From our previous study, we estimated a loss to follow-up of 18% over the ~10 months follow-up period and thus a final total sample size of 80 pregnant women (40 per group)

was required. Therefore, to achieve the objectives of this study, we sought to enroll a cohort of 100 – 150 pregnant women and follow them up to 18 weeks postpartum.

3.6 Enrolment of Study participants and follow-up visit procedures

At the initial visit, each pregnant woman meeting the inclusion criteria and willing to participate in study was consented. Secondly, enrolment Participant Identification Number (PTID) was assigned to each participant at this visit and detailed demographic, obstetrics and clinical questionnaires administered (Appendix 1), followed by a baseline general physical examination. Gestational age was evaluated by measurement of fundal height and history of the last menstrual period. All pregnant women were enrolled in a six-month period from June to November 2011. As part of the study participant's health care, the pregnant women were closely monitored for any illness and all cases were reported to the study clinicians for treatment as per Kenya Ministry of Health (MOH) guidelines. A total of 200 pregnant women were screened for inclusion into the study. Of these 25 were HIV positive and were excluded. The remaining 175 pregnant women were longitudinally evaluated in an active monthly antenatal follow-up visits (up to 4 per mother), through delivery and postpartum (up to 4 per mother). Of these, there were 93 pregnant women that delivered at Chulaimbo sub-district hospital and had samples collected at delivery. Of these, there were 91 mothers that were further followed and where possible breast milk samples collected at approximately, 6, 10, 14 and 18 weeks postpartum (figure 3.2).

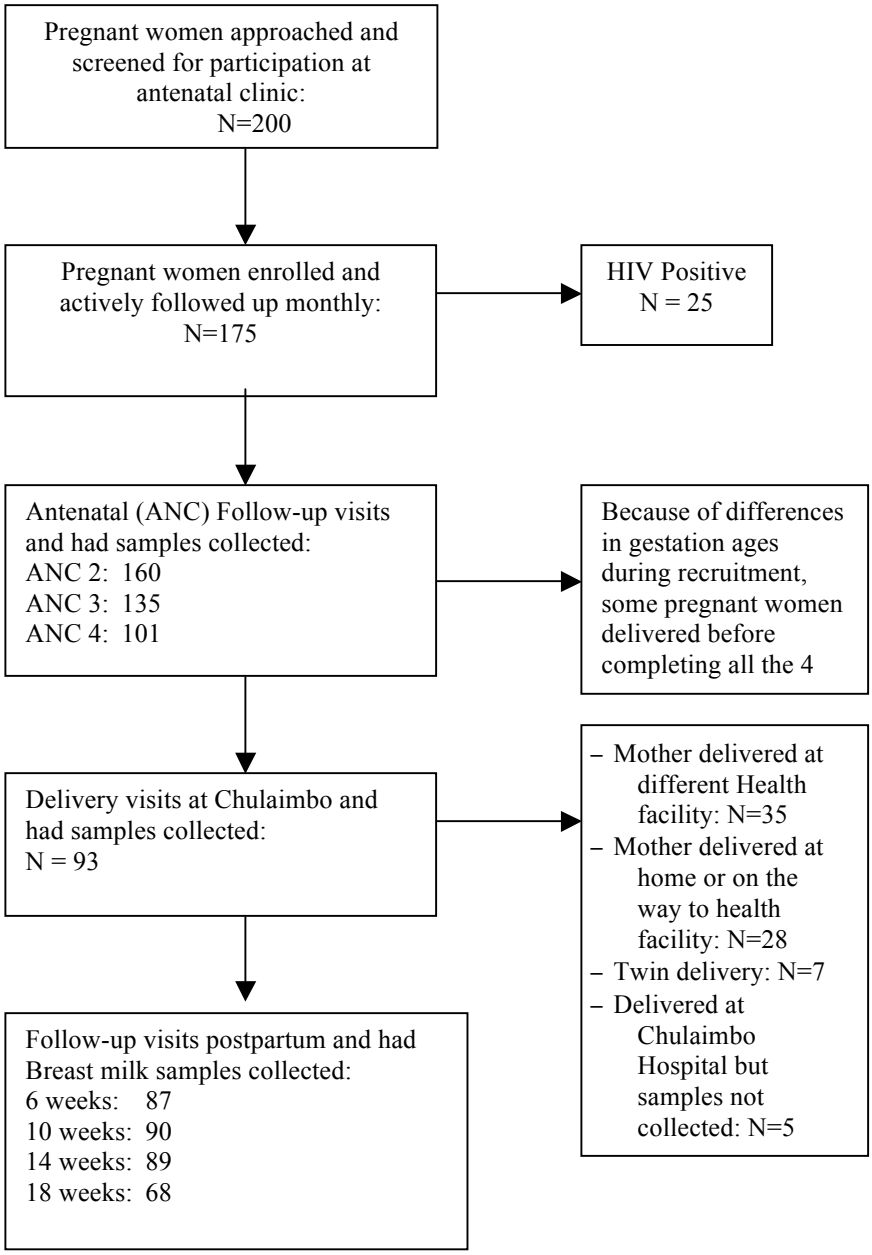


Figure 3.2: Study participants' enrolment and follow-up schema.

3.7 Blood and Breast Milk Specimens collection and Processing

At enrolment visit, participants provided about 2 –4mL of venous blood collected by venipuncture in EDTA tubes for *P. falciparum* malaria diagnosis and EBV load. During follow-up antenatal visits, finger-prick blood, about 0.5 ml, was collected in EDTA tubes for malaria diagnosis and EBV load. Less than 24 hours after delivery, 2–4 ml of peripheral blood sample was collected from the mother for malaria diagnosis, and EBV-load. All samples were transported to the SUNY Upstate research laboratory located at the Center for Global Health Research, KEMRI in Kisumu for processing within 2 – 3 hours of blood collection. The EDTA anti-coagulated blood was centrifuged at 1500 rpm for 5 min to separate plasma from the blood pellet. An equivalent vol/vol of sterile 1x PBS was used to replace the aliquoted plasma. The aliquots were then stored at -80^{0}C until analysis.

Breast milk (3-5ml) was collected using an aseptic technique. Mothers were first assisted to wash their hands using soap. Milk was then manually expressed into sterile tubes, after discarding the first several drops. Breast milk samples were then refrigerated and transported back to the laboratory at the Center for Global Health Research, KEMRI Kisumu for processing within 2 – 3 hours of collection. The whole milk was aspirated into a 1ml sterile tube and aliquots were stored at −80°C until analysis.

3.8 DNA Extraction from Whole Blood and Breast Milk

DNA was extracted from 200µL of whole blood collected in EDTA tubes by use of a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's protocol (Appendix 2). DNA purity and quantity was determined by assaying 2 µl of total DNA extract on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Aliquots (1ml) of previously frozen whole milk was centrifuged at 5000 rpm for 5 minutes at 4°C to separate the lipid (top creamy layer), supernatant (whey), and cell

pellet. The creamy top layer was carefully picked and discarded, and the clear supernatant (whey) was aspirated into a separate tube and stored at −80°C. The remaining volume of cell pellet was adjusted to 200 µl with PBS and DNA was extracted by use of a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's protocol (Appendix 3). DNA purity and quantity was determined by assaying 2µl of total DNA extract on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

3.9 DNA Amplification of *P. falciparum* and EBV using Real-time quantitative (RTQ) PCR

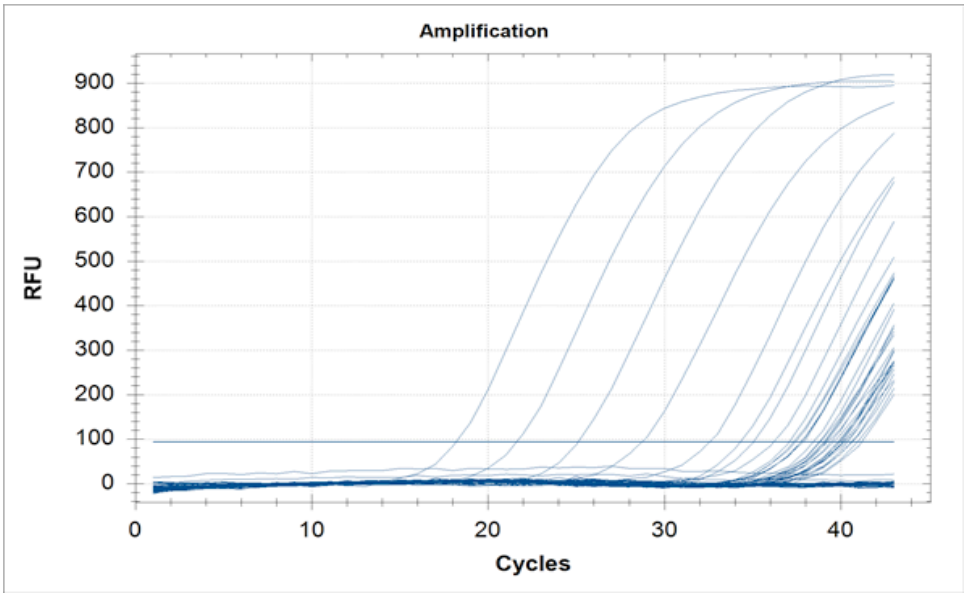
3.9.1 Diagnosis of *P. falciparum* malaria

Plasmodium falciparum malaria parasite was detected by RTQ –PCR using primers and probe that amplify the multi-copy 18S (small subunit) ribosomal RNA genes of the *P. falciparum* malaria parasite, which are stable and highly conserved as described previously (Hermsen et al., 2001). The following primers and probe sequences were used: forward primer 5'–GTA ATT GGA ATG ATA GGA ATT TAC AAG GT–3'_ (position 507-535), reverse primer 5' – TCA ACT ACG AAC GTT TTA ACT GCA AC–3'_ (position 614–639); and probe TGC CAG CAG CCG CGG TAA TTC (position 567–587). The probe was labeled with 6-carboxy-fluorescein (FAM) as a reporter and 6-carboxy-tetramethylrhodamine (TAMRA) as a quencher. Each specimen was assayed in 25 µl reactions containing 5µl of whole blood DNA, 6 µM of each primer and 4 µM probe, and 1X BioRad super mix (BioRad Laboratories, Hercules, CA). The real-time quantitative (RTQ) PCR cycle used was as follows: 10 min at 95^{o}C, and 40 cycles of 15 s at 95^{o}C and 1 min at 60^{o}C using BioRad CFX Manager platform (BioRad Laboratories, Hercules, CA). Each RTQ –PCR plate reaction contained no template and positive controls for all amplicons studied.

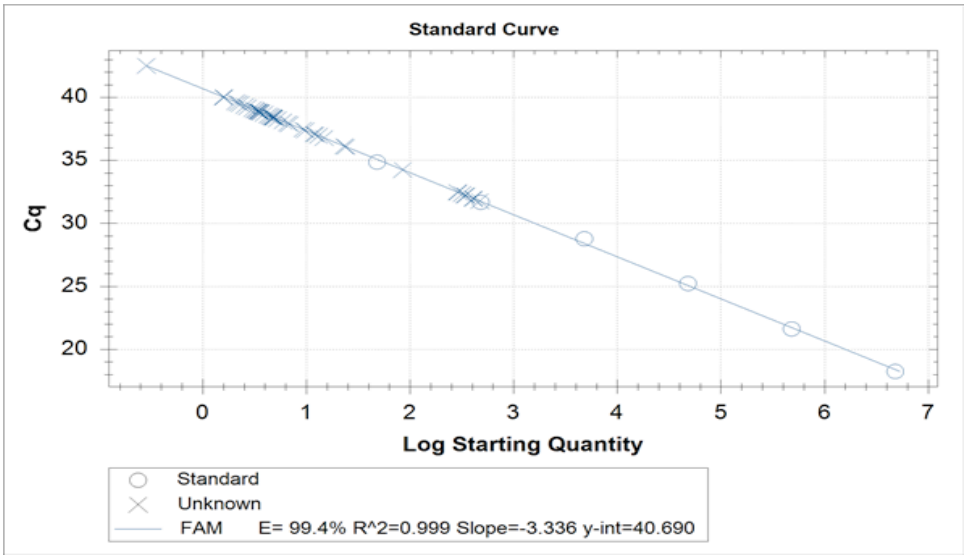
3.9.2 Measurement of EBV load in blood and Breast Milk

EBV DNA was detected by RTQ –PCR using primers and probe that amplify a 70-bp region of the EBV BALF5 gene. The forward primer: 5' –CGG AAG CCC TCT GGA CTT C- 3'; and reverse: 5' –CCC TGT TTA TCC GAT GGA ATG- 3'. The fluorescence probe was 5' –TGT ACA CGC ACG AGA AAT GCG CCT BHQ-1- 3'. Each specimen was assayed in 25 µl reactions containing 5µl of whole blood or breast milk pellet DNA, 6 µM of each primer and 4 µM probe, and 1X BioRad super mix (BioRad Laboratories, Hercules, CA). The real-time quantitative (RTQ) PCR cycle used was as follows: 2 min at 50^{o}C, 10 min at 95^{o}C, and 40 cycles of 15 s at 95^{o}C and 1 min at 60^{o}C using BioRad CFX Manager platform (BioRad Laboratories, Hercules, CA). To generate a standard curve, DNA extracted from the Namalwa BL cell line (ATCCCRl-1432), which contains 2 integrated copies of the EBV genome per cell. Samples were also analyzed for β-actin as a positive PCR control, using commercially available probes and primers (PE Applied Biosystems). Each RTQ –PCR plate contained no template and positive controls for all amplicons studied. EBV load was normalized to the number of β-actin DNA multiplexed and then calculated on the basis of copies of EBV genome per microgram of DNA. Only samples with a positive β-actin signal were used for further analysis. Figure 3.3, A and B, illustrates the amplification and the standard curve that were used for extrapolation of values for each samples run.

A



B



KEY:

RFU – Relative Fluorescent Unit

Cq – Quantification Cycle

Figure 3.3: A representative Quantitative Real Time –PCR amplification Curve (A) and the standard curve (B), generated from the Namalwa BL cell line and was used for the extrapolation of values for the samples tested.

3.10 Measurement of EBV-VCA antibodies by ELISA

Plasma samples were analyzed for the presence and titers of antibodies to EBV-specific IgG Viral capsid antigen (VCA) using a synthetic peptide based enzyme-linked immune-sorbent assay (ELISA) for immune-dominant epitopes derived from VCA-p18 as described previously (Fachiroh et al., 2006; E. Piriou et al., 2009). Ninety-six-well ELISA plates (Immulon 4HBX, Thermo Labsystems, MA, USA) were coated with 1 µg/ml VCA-p18 peptides and incubated overnight at 4ºC. After overnight incubation at 4°C, antigen was discarded and 200 ml blocking buffer (3% bovine serum albumin (BSA; Roche Diagnostic, Germany) in PBS was added to each well. After a 1 hour incubation at 37°C, the wells were emptied and washed three times with PBS-T (PBS containing 0.05% Tween 20). Then, 100 ml of 1:100 PBS diluted plasma sample was added and incubated for 1 h at 37°C. The wells were emptied and washed four times with PBS-T (PBS containing 0.05% Tween 20). Subsequently, rabbit anti-human IgA-horseradish peroxidase (Dako, Dk 2600 Glostrup, Denmark) conjugate (diluted 1:4,000 in PBS), was added and incubated for 1 h at 37°C. The wells were emptied and washed four times with PBS-T (PBS containing 0.05% Tween 20). Then, 100 ml/well of tetramethylbenzidine substrate solution (Bioscience, USA) was added and kept in the dark for 20 min for IgG detection. Stop solution, 100 ml of 1 M H_2SO_4 was added to stop the reaction. The optical density was determined at 450 nm (OD450) using an ELISA reader (DYNEX Technologies, Inc. West Sussex UK). The cut-off value was defined as a mean of OD from negative control, plus three standard deviations.

3.11 DNase I Treatment Procedure

To confirm the presence of infectious virus in the breast-milk, a strategy for separating lytic virions from naked viral DNA was established based on differential degradation by DNase I, given that the capsid would protect encapsidated lytic virions from digestion. A sub set of previously frozen breast milk (n=40) was DNase I treated as per manufactures instructions. Briefly, aliquot (1ml) of previously stored breast-milk was centrifuged at 5000 rpm for 5 minutes at 4°C**.** The creamy top layer was carefully picked and

discarded, and 100 µl of the breast-milk supernatant (whey) was digested with 2.5 µl of DNase I (Invitrogen, USA) at room temperature; then stop solution, 10 µl of 10X buffer (Invitrogen, USA) was added and incubated at 65°C for 10 min. The volume was adjusted to 200 µl by adding 85 µl of sterile PBS, and proceeded with DNA extraction using QiaAmp DNA Mini kit protocol (Qiagen, Valencia, CA) in accordance with the manufacturer's protocol (Appendix 3). DNA purity and quantity was determined by assaying 2 µl of total DNA extract on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

3.12 PBMC Isolation and Proliferation Assay

PBMCs were separated from sodium heparin anticoagulated whole blood by standard Ficoll-Hypaque density gradient centrifugation. In this procedure, the anti-coagulated 5-8 ml of blood was layered carefully onto 5 ml of Ficoll-paque (GE Healthcare, Sweden) in a 15ml tube and then centrifuged at 1500 rpm for 30 minutes. Plasma was transferred into sterile tubes and stored at -80^{0}C while the PBMCs was collected using a sterile 10ml pipette and transferred into a 15 ml tube. The cells were washed by adding sterile 1× PBS, pH 7.0, to bring a total volume in the tube to 12 ml followed by centrifugation for 15 min. at 1200 rpm at room temperature. The supernatant was aspirated off, the pellet broken by gentle flicking of the tube and then washed again as described above and centrifuged for 10 min at 1200 rpm. The supernatant was aspirated, the pellets broken by gently flicking the tubes and the cells re-suspended in 1 mL of sterile 1 × PBS, pH 7.0. To count cells, 10µL of 0.4% Turk's solution was used to dilute the cells in a 1:1 ratio and placed in a Haemocytometer and examined under a microscope. The yield was calculated using the formula (cell count in 1ml = (# cells counted in 4 squares)×2×10^4). Cells were subsequently washed at least twice in complete RPMI 1640 culture medium containing 1% Penicillin/Streptomycin, 1% L-glutamine, and 10% fetal bovine serum.

Aliquot (1ml) of previously stored breast-milk was centrifuged at 5000 rpm for 5 minutes at 4°C**.** The creamy top layer was carefully picked and discarded and 500µl of

breast-milk supernatant was exposed to PBMC and plated at 10^6 cells/ml in complete RPMI 1640 containing 1 μg/ml cyclosporine-A. Cell cultures were maintained at 37°C and supplemented with 5% CO_2. Cultures were checked and examined at 3 days, 1 week, 2 weeks and 1 month while observing for features of transformation (e.g. blast formation, clumping and proliferation). Cell aggregation was visualized microscopically at 40x magnification.

3.13 Data Management

Data obtained were entered into a computer with a password-protected access database with login information known only by the study team. The computer was stored in a locked office with restricted access in the KEMRI research facility in Kisian, Kenya. The data was in the form of an encrypted access database file with user-level security. All computers and databases are password protected. Study participant data are coded and no identifiable information was obtainable from this code.

For sample inventory, before processing samples were keyed into laboratory sample reception book with PTID number, sample type/appearance and date of collection. After processing, the samples wererecoded into the sample storage inventory in the order of enrollment in an ascending order and stored appropriately. Individual specimens were labeled with PTID, sample type and stored in 10x10 cryoboxes labeled with a serial cryobox number for easy tracking of individual samples.

3.14 Data Analysis

All statistical analyses were performed using Stata, IC software (13.1, StataCorp LP, College Station, TX) and Graphpad Prism version 5.01 Software (Graphpad Software, Inc., La Jolla, CA.); and setting 2-tailed alpha to reject the null hypothesis at 0.05. The data are expressed as percentages for categorical data while continuous data are presented using median with range. Differences in proportions were evaluated using chi-square test or Fisher exact test. Pairwise comparisons were done using Mann-Whitney U

test. Malaria and EBV viral load data were measured longitudinally during pregnancy, with each observation contribution to an overall area under the curve (AUC), (by trapezoidal methods) for statistical analysis. AUC analysis is a method for evaluating the overall response of a continuously scaled outcome that can increase and decrease over time. Repeated observations of detectable EBV load were recorded throughout the course of study during pregnancy, and these values for any given individual may increase or decrease given their exposure and immune response. In order to "capture" the effect of their exposure and immune response in an overall sense, we literally calculate the areas under the curve that would be drawn for each individual given all of their recorded viral loads, and used this one number (their area under the curve) to represent the entirety of their viral challenge (Newson, 2002). These AUC data were also correlated using the Somer's D non-parametric measure of association for primary hypothesis testing. Somer's D was also used for comparing EBV AUC between women with and without any positive malaria test results. Subsequent comparisons of repeated EBV viral load observations during the first and second *versus* third trimesters of pregnancy were log-transformed and compared via unpaired t-tests. Frequency of EBV DNA in breast milk overtime was evaluated using chi-square test. Levels of EBV load in breast milk overtime were evaluated by 1-way analysis of variance (ANOVA) after log transformation. Regression analysis was used in which the level of EBV load in breast milk at 6 weeks postpartum was log transformed and regressed with, parity, bed net use, evidence of *P. falciparum* malaria infection during pregnancy, evidence of malaria infection at delivery, and EBV DNA detection at delivery. In adjusted analysis, EBV load (log transformed copies/ml) was regressed with evidence of malaria infection at delivery, while accounting for a set of covariates (parity, and maternal age).

3.15 Ethical Considerations

Approval of this study was obtained from the Kenya Medical Research Institute (KEMRI SCC No. 2323; Appendix 4 and 5). Written informed consent was obtained from the study participants before any sample collection (Appendix 6 and 7).

Participants were provided with an explanation of the purpose, risks and benefits of the study to the community members before consenting for their participation in the study. Participants were also assured that the information they were giving was going to be treated with confidentiality. One of the benefits to the participants included treatment for fever, malaria, diarrhea and anemia according to the Kenya Ministry of Health Guidlines. Also, the information garnered from this study will be important for prevention programs for Burkitt's Lymphoma. Participants were also informed of their right to withdraw from the study at any time and for any reason without fear of any negative penalty or loss of benefits. All the samples collected were stripped of personal identity and coded using unique study identification numbers to protect study participants' privacy and only the principal investigator and a few core members of staff had access to personal details of the study participants, which was kept under key and lock. To ensure that we got a more representative population and to encourage mothers to return to the hospital for delivery the study offered to pay the birth cost.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic, obstetric, clinical characteristics and of study participants at enrolment

A total of 175 HIV negative pregnant women were enrolled in this study. The mean age of the pregnant women was 22.3 years (SD [5.6]) and 39% of the women were primagravidae. The majority (58%) of the women had at least upper primary level of education, with 82% being of the Luo ethnic group. Sixty five percent of the women were married, while 61% of them were enrolled at second trimester of pregnancy.

All (100%) participants were EBV seropositive with EBV DNA detectable in blood in 44% of study participants at enrolment. Thirty three percent of the pregnant women were RTQ-PCR malaria positive with a mean parasite load of 112, 230 [range, 0.7465-2160000] at enrolment. The demographic, obstetric, clinical and laboratory characteristics of the study participants are presented in Table 4.1.

Table 4.1: Demographic, obstetric and clinical characteristics of study participants at enrolment.

Characteristics	**Summary***
Mean age in years (± S.D.) at enrolment	22.3 (5.6)
Education	
Lower Primary	20%(35)
Upper Primary	58%(101)
Secondary	16%(28)
Village Polytechnic	1% (1)
Other	5%(10)
Ethnic group	
Luo	82% (144)
Luhya	15% (26)
Kalenjin	1% (1)
Other	2% (4)
Marital status	
Single	31% (55)
Married	65% (114)
Separated	1% (1)
Widowed	2% (3)
Widowed, inherited	1% (1)
Parity	
Multiparous	60%(105)
Primiparous	40%(70)
Gestation age at enrolment	
1st trimester (<14 weeks)	15%(27)
2nd trimester (14-27 weeks)	61%(107)
3rd trimester (≥28 weeks)	24%(41)
Mean hemoglobin (g/dl) (± S.D) at enrolment	10.9(1.7)
EBV seroprevalence by ELISA VCA positive at enrolment	100%(175)
EBV DNA by q-PCR at enrolment	44%(77)
P. falciparum malaria prevalence at enrolment by RTQ-PCR	33% (57)
Mean *P. falciparum* parasite load by RTQ-PCR, [range] at enrolment	112, 230 [0.7465-2160000]

* Categorical data was summarized by percentages and continuous data by standard deviation (S.D).

4.2 Malaria Status of Pregnant Women

Ninety-four, (54%) of the pregnant women had malaria, that is, at least one positive RTQ-PCR malaria test, during follow-up. One hundred and fifty-three of 617 (25%) total follow-up visits had *P. falciparum* malaria. No statistically significant associations were found between pregnant women with and without *P. falciparum* malaria infection relative to their age, hemoglobin level, parity, bed net use and gestational age at enrolment ($p > 0.05$) (Table 4.2).

4.3 The anthropometric measures of birth outcomes among the pregnant women participating in the cohort study.

Of the 175 pregnant women enrolled and actively followed monthly, 65%(105) delivered at Chulaimbo hospital. The mean birth weight of the newborn infants was 3202 g (range, 2000 g – 4000 g). Only 5% (n=5) of the infants weighed less than 2,500 g (low birth weight). The mean head circumference was 34 cm (range, 30 cm – 39 cm) with mean Apgar score (at 10 minutes) ± SD of 9.8 ± 0.97. Of the 5 infants that were LBW, two infants were born to mothers that were malaria positive by both Q-PCR and microscopy at enrolment (Table 4.2).

Table 4.2: Anthropometric measures of birth outcomes among the participants that delivered at Chulaimbo hospital.

Characteristics	Summary*
Sex	
Male	53% (50)
Female	47% (45)
Mean Axillary temperature (^{o}C) (± S.D)	35.7 (0.78)
Mean Weight (g) (range)	3202 (2000 – 4000)
Mean Length (cm) (range)	48.5 (40 – 55)
Mean Head circumference (cm) (range)	34.9 (30 – 39)
Mean APGAR score (at 1 minutes) (± S.D)	8.9 (1.2)
Mean APGAR score (at 10 minutes) (± S.D)	9.8 (0.97)
Low birth weight**	
Yes (<2500g)	5.3% (5)
No (≥2500g)	94.7% (90)

* Categorical data was summarized by percentages and continuous data by standard deviation (S.D).
** Low birth weight as defined by the World Health Organization (WHO) (WHO, 2011).

Table 4.3: Malaria Status of Pregnant Women *versus* Demographic, obstetric, clinical and laboratory history at follow-up.

Variable	**Had malaria during pregnancy, 94(54%)**	**No malaria during pregnancy, 81(46%)**	***P* value***
Mean Gestation Age, weeks (±SD) at enrolment	21.3 ± 6.4	21.4± 6.3	0.93
Parity			
Primiparous	41(58.6%)	29(41.4%)	0.35
Multiparous	53(50.4%)	52(49.5%)	
Hemoglobin (Hb) level at enrolment**			
Moderately Anemic (Hb >7.5, <11g/dl)	49(56.3%)	38(43.7%)	0.54
None-Anemic (Hb ≥11g/dl)	45(51.1%)	43(48.9%)	
Age at enrolment			
<20 years	46(57.8%)	36(43.9%)	0.66
20 – 30 years	38(50.0%)	38(50.0%)	
>30 years	10(58.8%)	7(41.2%)	
Bed net use among pregnant women at enrolment			
Yes	71(53%)	64(47%)	0.71
No	23(57%)	17(43%)	

* All estimated using Fisher's exact test except gestation age, which was estimated by unpaired t-test. All tests of statistical significance are two sided.
** Cut-off as defined by the World Health Organization (WHO) for pregnant women(WHO, 2011).

4.4 EBV DNA in pregnant women with and without *P. falciparum* infection

Pregnant women that had evidence of *P. falciparum* malaria infection during pregnancy were more likely to have a detectable EBV DNA than pregnant women that had no evidence of malaria infection during pregnancy (64% vs. 36%, p=0.01) (Figure 4.1).

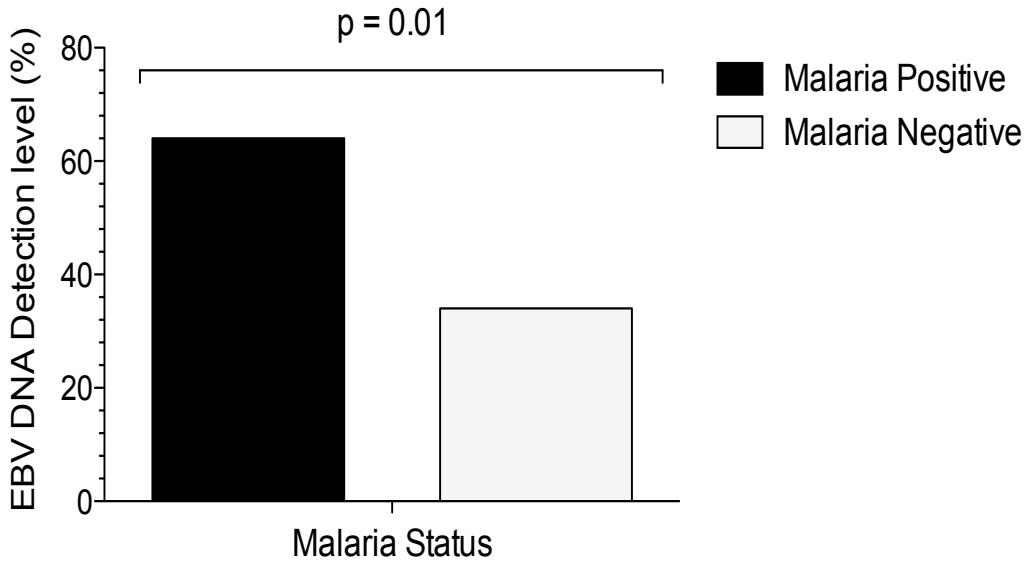


Figure 4.1: Comparison of EBV DNA detection among pregnant women with and without malaria infection.

EBV DNA detection level in women that had malaria detected during pregnancy versus those that had no evidence of malaria infection during pregnancy were compared using Fisher's exact test was used to estimate significance.

4.5 Dynamics of EBV Load in pregnancy

Significantly higher EBV load was found at third trimester (unpaired t-tests, p= 0.04) relative to first and second trimester of pregnancy (Figure 4.2).

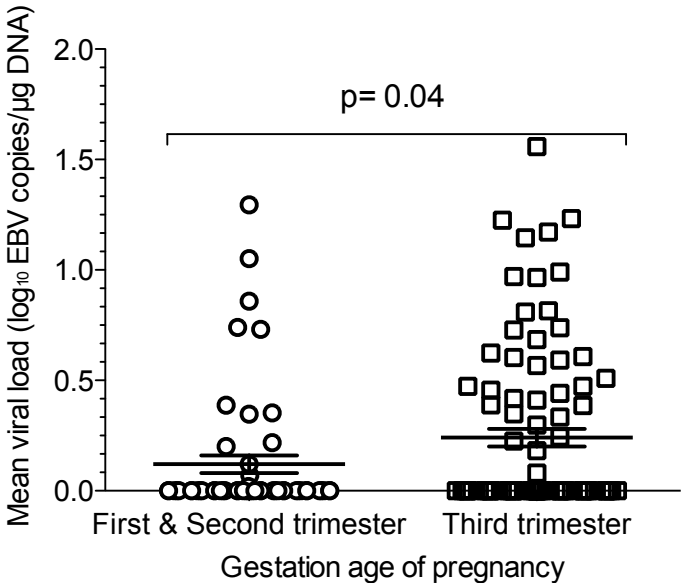


Figure 4.2: Comparison of levels of EBV load in first and second trimesters *versus* third trimester of pregnancy independent of malaria infection.

Only pregnant women with no malaria infection were included in the analysis. Because the resultant data had few counts at first semester, the data for first and second trimester was combined. The data was then categorized according to gestational age (first and second, *versus* third trimesters) and compared the log transformed EBV load data among groups using unpaired t-tests.

4.6 EBV viral load in pregnant women with and without *P. falciparum* malaria

EBV load as quantified by area under the longitudinal observation curve (AUC) was significantly higher in women that had malaria detected than in women that had no evidence of malaria infection during pregnancy (p= 0.01) (Figure 4.3).

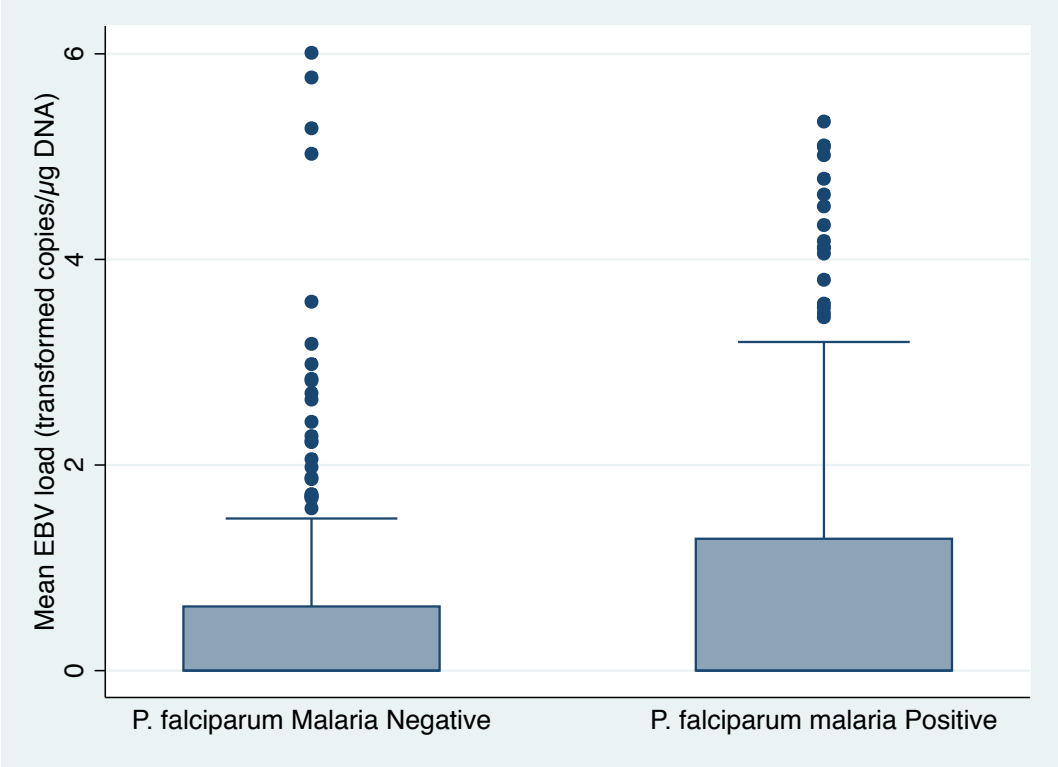


Figure 4.3: Comparison of levels of EBV load among pregnant women with and without *P. falciparum* malaria infection.

EBV load in women that had malaria detected during pregnancy versus those that had no evidence of malaria infection during pregnancy were compared using EBV load area under the longitudinal observation curve (AUC).

4.7 Association between malaria load and EBV load during pregnancy

A highly significant positive association was found between malaria load *versus* EBV load among the pregnant women that had detectable *P. falciparum* infection ($p < 0.0001$).

4.8 EBV load in pregnant women with *P. falciparum* malaria relative to gestation age of pregnancy

Median EBV load was significantly higher both at first and second ($p = 0.02$) and third ($p = 0.05$) trimesters of pregnancy in pregnant women with malaria in comparison to women without malaria (Mann Whitney U test, Figure 4.4).

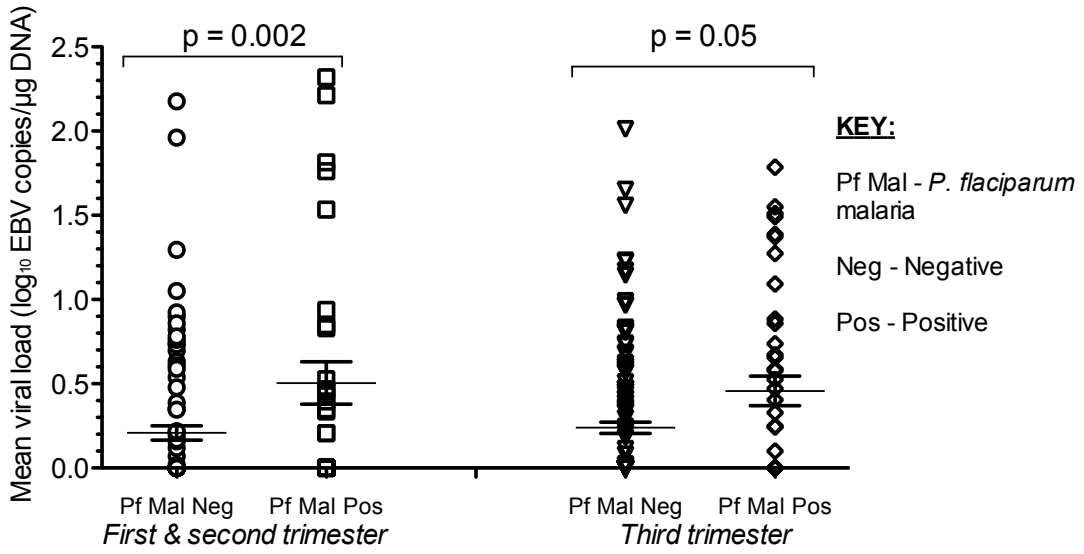


Figure 4.4: Comparison of EBV load in pregnant women with and without *P. falciparum* malaria infection relative to gestational age of pregnancy.

Gestational age-related difference in EBV load between pregnant women with and without *P. falciparum* malaria was determined. Because the resultant data had few counts at first semester, the data for first and second trimester was combined. The data was then categorized according to gestational age (first and second *versus* third trimesters) relative to malaria status.

4.9 EBV DNA in breast milk at 6, 10, 14 and 18 weeks weeks Postpartum

EBV DNA detected in breast-milk was significantly higher at 6 weeks and decreased sequentially in subsequent visits (6 weeks 77% [66/87]; 10 weeks 58% [52/90]; 14 weeks 31% [28/89]; 18 weeks 24% [16/68]; p <0.0001) (Figure 4.5).

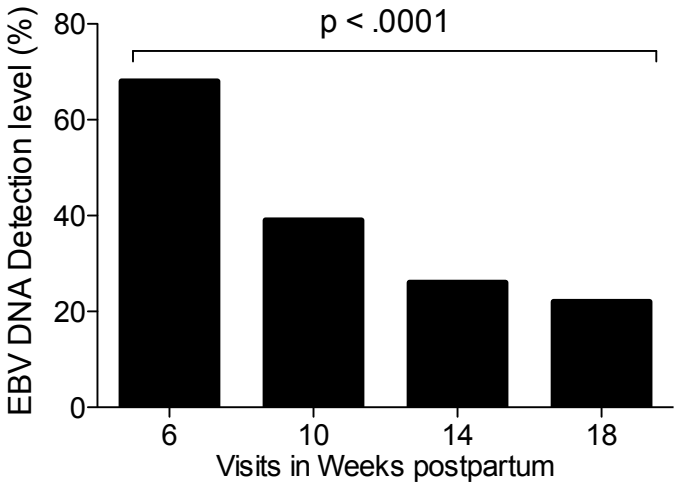


Figure 4.5: EBV DNA in breast-milk at 6, 10, 14 and 18 weeks postpartum. The difference in proportions over the four time points was determined using Chi Square test. The prevalence of EBV DNA was a significantly higher at 6 weeks and decreased sequentially in subsequent visits postpartum (*P* < .0001).

4.10 EBV load in breast milk at 6, 10, 14 and 18 weeks weeks Postpartum

EBV load was higher, mean ± SE, at 6 weeks and decrease sequentially in subsequent visits (6 weeks, 3.66 (±0.109); 10 weeks, 2.85 (±0.090); 14 weeks, 2.79 (±0.086); 18 weeks, 2.53 (±0.075); unpaired t-test, p <0.0001) (Figure 4.6).

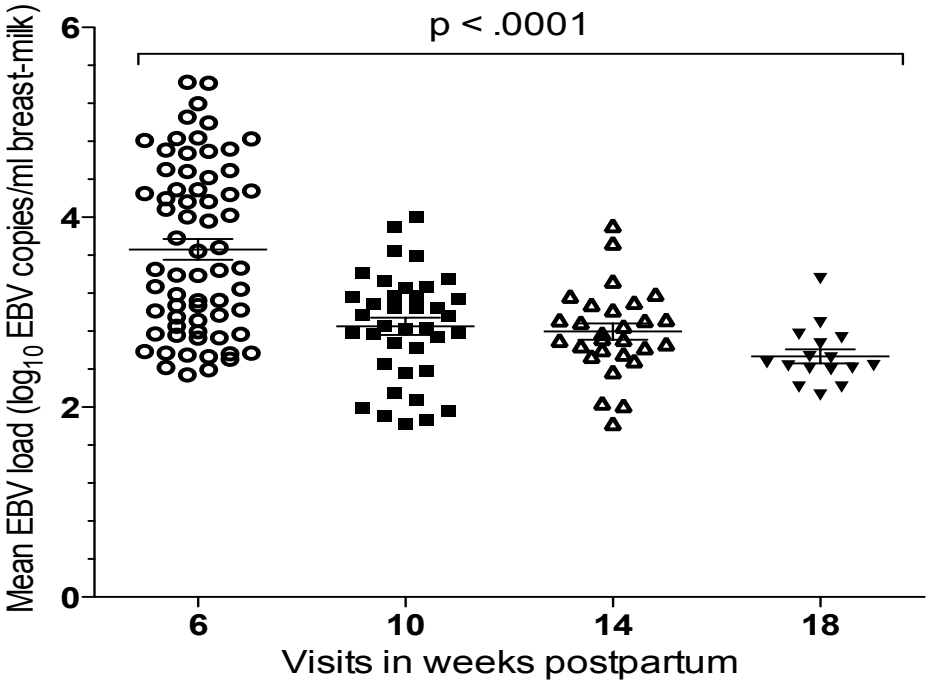


Figure 4.6: EBV loads in breast-milk at 6, 10, 14, and 18 weeks postpartum.
EBV load was determined by real-time quantitative polymerase chain reaction. The log-transformed EBV load data was submitted to a 1-way analysis of variance (ANOVA). The mean EBV load was a significantly higher at 6 weeks and decreased sequentially in subsequent visits postpartum (*P* < .0001).

4.11 EBV in breast-milk is encapsidated

DNase I resistant EBV was observed in 24/40 (60%) of the samples, suggesting that the virus DNA in breast milk supernatant (whey) was encapsidated (Figure 4.7).

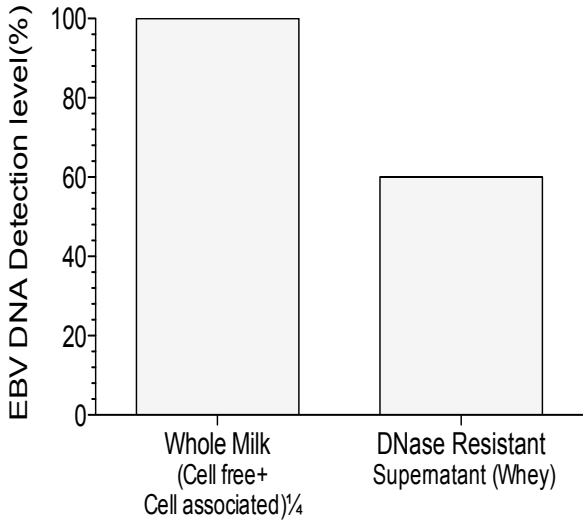
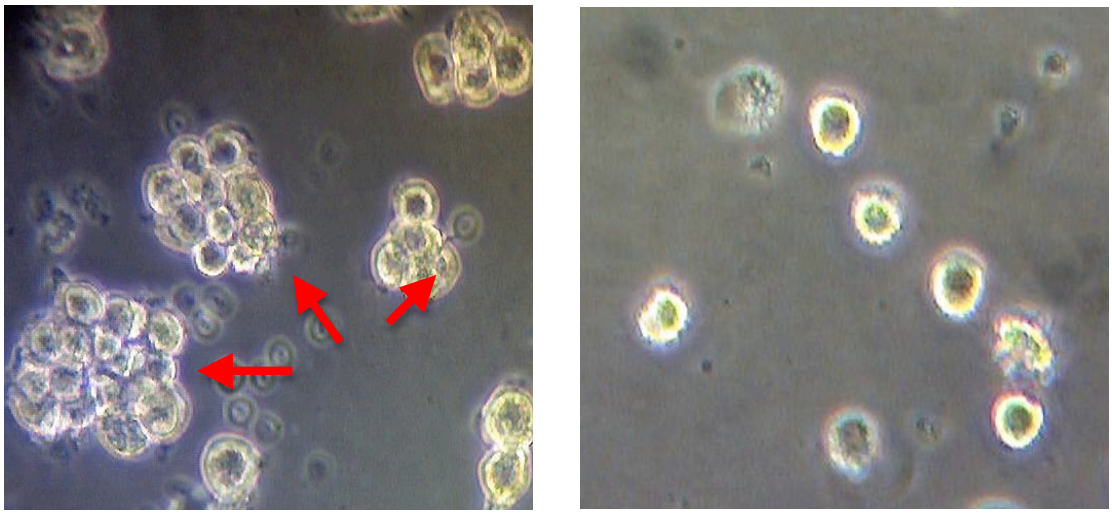


Figure 4.7: Comparison of EBV DNA in whole (unfractionated) breast-milk, *versus* DNase I treated breast-milk supernatant (whey).

A subset of breast-milk samples (n=40) was treated with DNAse I prior to DNA extraction. The strategy was to segregate encapsidated virions from naked viral DNA, based on differential degradation by DNase I, given that the capsid would protect intact virions from digestion. RTQ-PCR was then used to measure the levels of EBV after DNase I treatment.

4.12 Infection and transformation of PBMC by EBV in breast-milk

Evidence of viral transformation (blast formation, clumping and proliferation) was observed after one week of incubation. In contrast, PBMC treated with media alone, cells had not undergone proliferation (Figure 4.8).



A B

Figure 4.8: Exposed cell clusters (blasts) in breast-milk exposed (A) and unexposed [control] (B) cells at one-week post infection.

To assess whether EBV in breast milk is infectious, 20 breast milk samples that were EBV PCR positive were assessed for their capacity to induce B cell proliferation. About 500µl of EBV DNA positive breast-milk supernatant or media was exposed to PBMC and examined for evidence of transformation.

4.13 Association between *P. falciparum* infection at delivery versus EBV shedding in breast milk at 6 weeks postpartum.

In unadjusted analysis, *P. falciparum* infection at delivery was associated with higher viral load in early (6 weeks postpartum) breast milk ($p = 0.02$). After adjusting for maternal age and parity, *P. falciparum* malaria infection at delivery remained positively associated with elevated EBV load at 6 weeks postpartum ($p = 0.01$) (table 4.4).

Table 4.4: Unadjusted and adjusted regression analysis of association of EBV load in breast milk at 6 weeks postpartum and maternal characteristics during pregnancy

Variable	N=85*	Unadjusted		Adjusted**	
		Coef. [95% CI]	*P* value	Coef. [95% CI]	*P* value
Had *P. falciparum* malaria Infection during pregnancy					
No	35	Ref			
Yes	43	-0.9 [-2.89 – 0.98]	0.32		
***P. falciparum* infection Status at delivery**					
Negative	57	Ref			
Positive	10	3.2 [0.50 – 6.0]	**0.02**	3.3 [0.56 – 6.0]	**0.01**
EBV DNA Status at delivery					
Undetectable	32	Ref			
Detectable	35	0.2 [-1.84 – 2.19]	0.86		
Parity					
Primigravida	24	Ref			
Multigravida	51	-1.4 [-3.4 – 0.65]	0.17		
Current Bed net use at enrolment					
No	18	Ref			
Yes	60	0.6 [-1.70 – 2.88]	0.61		
Helminthes Infection at enrolment					
No	59	Ref			
Yes	16	-0.2 [-.68 – 0.30]	0.45		

Coef. – Coefficient, CI – Confidence Interval
*Total number may not add up to 85 in all categories due to missing values.
**Adjusted for maternal age and parity.

4.14 Correlation between EBV load in the maternal blood at delivery versus breast milk at 6 weeks postpartum

Level of EBV load in maternal blood at delivery was positively correlated with that of breast milk at 6 weeks postpartum (Pearson $r^2 = 0.559$, $p = 0.002$), (Figure 4.9).

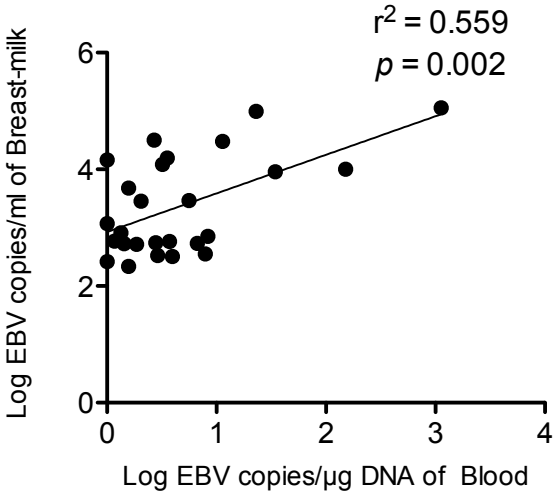


Figure 4.9: Correlation between EBV loads in maternal blood at delivery, and that of breast milk at 6 weeks postpartum.

EBV load in maternal blood at delivery was assessed to determine whether there is correlation with EBV load in breast milk at 6 weeks postpartum. The log-transformed EBV load data was subjected to Pearson correlation test. Level of EBV load in maternal blood at delivery was positively correlated with the level of EBV load in breast milk at 6 weeks postpartum (Pearson $r^2 = 0.559$, $p = 0.002$).

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

Despite the well-established link and our recent support of the long-held hypothesis that EBV infection early in life and holoendemic malaria being the risk factors for Burkitt's lymphoma (eBL) (de-The, 1977; Erwan Piriou et al., 2012), little is known about why children are infected early in life with EBV, and the role of malaria in EBV transmission. This study hypothesized that infection with malaria during pregnancy could cause EBV reactivation leading to high EBV load in circulation and into breast milk, which could subsequently enhance early age of EBV infection.

5.1.1 Cohort characteristics and birth coutcomes among study population

All participants in the current study were EBV seropositive, confirming results of previous studies that showed a high rate of EBV seroprevalence among adults in Africa (Moormann et al., 2005). *P. falciparum* malaria PCR prevalence was 33% at enrolment, which is consistent with previous reported malaria PCR prevalence of 33.1% among pregnant women from Western Kenya (Perrault et al., 2009). No significant association between parity, bed net use and gestational age of pregnancy was observed among pregnant women with and without evidence of malaria infection. However, previous studies reported pregnancy associated malaria morbidity being dependent on covariates such as, parity, bed net use and gestational age of pregnancy (Ibhanesebhor & Okolo, 1992; Okoko, Enwere, & Ota, 2003). This lack of finding is not surprising as the study was investigating the impact of malaria on EBV reactivation and, therefore, accordingly selected for participants with normal blood count and no apparent infections besides malaria. The rather stringent inclusion criteria employed may have consequently circumvented the prospects of finding any significant association with the foregoing variables.

In this study, a low prevalence of adverse pregnancy outcomes was observed where only a 5% prevalence of low birth weight (LBW) among the infants that were born to mothers that delivered at Chulaimbo sub-district hospital. Other studies in similar sites have observed LBW outcomes of about 18% (Menendez et al., 2000; Yatich et al., 2010). Risk factors associated with LBW include malaria (Gebremariam, 2005), anemia (Tako et al., 2005; Yatich et al., 2010; Yazdani, Tadbiri, & Shakeri, 2004) and late commencement of antenatal care (Gebremariam, 2005) or a combination thereof. Because the majority (61%) of the participants were recruited when they were in their second trimester of pregnancy (Daud et al., 2014), it is likely that they have had more ANC visits (average of 4 visits) and therefore more medical evaluation, which could have subsequently lead to an improved birth outcomes.

5.1.2 EBV DNA and EBV load during pregnancy among pregnant women with and without *P. falciparum* malaria

This is the first study to report significantly higher EBV DNA levels and elevated EBV loads in pregnant women with malaria compared to women without malaria during pregnancy independent of gestational age of pregnancy. Consistent with these findings, previous studies (D. Donati et al., 2006; Lam et al., 1991; Yone et al., 2006), albeit in children, reported significantly elevated peripheral blood EBV DNA loads – indicative of viral reactivation – following malaria infection, suggesting that malaria has inherent effects on EBV–host balance.

The possible mechanisms through which *P. falciparum* malaria induce EBV reactivation is thought to be via a multiplicity of pathways: an interaction between a specific malaria antigen, the cysteine rich interdomain 1 alpha (CIDR1α) of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), and B cells (Daria Donati et al., 2004); leading to activation of memory B cells, which in turn results in increased production of EBV in virus-positive cell line (Chene et al., 2007). Secondly, expansion of latently infected B cells during acute malaria has also been postulated (Lam et al., 1991; Njie et al., 2009).

Thirdly, malaria parasite is also known to possess unmethylated CpG DNA, a ligand for Toll like receptor (TLR) 9 (Wu, Gowda, Kumar, & Gowda, 2010), which can potentially enhance EBV induced proliferation and activation of B cells (Iskra, Kalla, Delecluse, Hammerschmidt, & Moosmann, 2010).

Significantly higher EBV load was observed among pregnant women with evidence of malaria infection independent of the gestation age of pregnancy. The hallmark of a successful pregnancy is the delicate modulation of immune network particularly the Th1/Th2 cytokine equilibrium (Marzi et al., 1996; Raghupathy, 1997), and *P. falciparum* infection during pregnancy profoundly disturbs this balance (Fievet et al., 2001), and may therefore impair immunosurveillance of EBV during pregnancy. In addition, because pregnancy itself could modulate immune function especially T-cell functions, which appear to be down-regulated during pregnancy (Luppi, 2003; Motran et al., 2002), it is plausible that the synergistic effect of *P. falciparum* malaria and pregnancy could significantly alter and shift the dynamic balance between EBV and the host immunity in favor of the virus.

Moreover, several studies have pointed to a profound dysregulation of EBV persistence and immunity in children due to malaria (D. Donati et al., 2006; Moormann et al., 2005; Moormann et al., 2007; Njie et al., 2009; Rasti et al., 2005; Yone et al., 2006). Besides, pregnancy associated *P. falciparum* malaria not only influences pregnancy outcomes but also the development of neonatal immune responses (Adegnika et al., 2008; Brustoski et al., 2005; Ismaili et al., 2003; Rachas et al., 2012), suggesting that the ability of infants – born to mothers that had malaria during pregnancy – to effectively control EBV and other infections early in life may be impaired.

Furthermore, earlier studies to examine the effects of pregnancy on EBV persistence only focused on healthy pregnant women from the developed world, often correlating EBV reactivation with adverse pregnancy outcomes (Avgil et al., 2008; Eskild, Bruu,

Stray-Pedersen, & Jenum, 2005), seldom examining in the context of infectious diseases such as malaria. While using different approach in determining EBV reactivation, some studies (Haeri et al., 2010; Purtilo & Sakamoto, 1982; Sakamoto et al., 1982) but not others (Eskild et al., 2005; Meyohas et al., 1996) reported increased EBV reactivation during pregnancy among pregnant women.

5.1.3 Association of EBV load and *P. falciparum* malaria load among pregnant women with *P. falciparum* malaria

In this study, increase in malaria load was associated with increase in EBV load, suggesting that the level of EBV load is linearly proportional to the parasite density. This findings are in consistent with previous findings of increased viral load in peripheral blood of children living in a malaria holoendemic area, an area characterized by high parasite densities, compared to children living in malaria sporadic area (Moormann et al., 2005).

5.1.4 The dynamics EBV load relative to gestational age of pregnancy among pregnant women without malaria infections

Higher EBV load observed during the third trimester of pregnancy independent of malaria infection is novel and suggests that the dynamics of EBV load during pregnancy is affected by gestational age of pregnancy. The underlying mechanism that modulates EBV reactivation during pregnancy is unknown. However, a previous seminal finding on increase of $CD4^+$ $CD25^+$ T regulatory cells (Tregs) at early pregnancy, dramatically peaking during the second and third trimesters, with subsequent decline postpartum (Somerset, Zheng, Kilby, Sansom, & Drayson, 2004), may perhaps explain the observed phenomenon in this study. Tregs are known potent suppressors of T-cell functions (Sakaguchi, 2004), and T cells particularly $CD8^+$ cytotoxic T lymphocytes (CTL) play a pivotal role in maintenance of persistent EBV infections (Callan, 2003; Moormann et al., 2007; Njie et al., 2009). Thus, elevated EBV load observed during the last phase of pregnancy have been secondary to increased levels of Tregs, which in turn compromised

host control of EBV persistence.

5.1.5 EBV DNA and EBV load in breast milk at 6, 10, 14 and 18 weeks weeks Postpartum

The present study is the first to examine longitudinal breast-milk samples obtained from EBV seropositive mothers from 6 weeks to 18 weeks postpartum in an area where malaria is endemic and the eBL risk is high. Level of EBV load was significantly higher at 6 weeks postpartum with declining trends over time. The detectable EBV DNA at 6 weeks postpartum was 77% with declining trends over time. Previous studies albeit cross sectional reported EBV DNA prevalence of 45% in Zimbabwe (Gantt et al., 2008), and 46% in British Columbia women (Junker et al., 1991), confirming that EBV shedding in breast milk can occur.

The increased shedding of EBV in early breast milk samples suggest that EBV shedding in breast milk is greatest during the first few weeks postpartum and that breast-fed infants are exposed to a higher EBV load soon after delivery. The high viral load in early milk could be because of higher cell-associated virus in early milk than in mature milk, or an increased EBV load in early milk. Besides, the observed declining trend of both levels and EBV load overtime could be as a result of a rebound of immunity especially cell mediated immunity during postpartum (Shimaoka et al., 2000; Watanabe et al., 1997); thus, the mothers were able to control EBV postpartum, following reactivation during pregnancy.

5.1.6 EBV in breast milk is encapsidated and infectious

Still largely unexplored was whether the breast milk EBV DNA is encapsidated or it represents naked DNA. To assess this, an assay that relies on DNase I treatment, which selectively degrade naked viral DNA was used in this study. EBV in breast milk supernatant was found to resist degradation by DNase I, suggesting that the virus was encapsidated. The origin of cell free virus in breast milk is largely unknown. However,

EBV is known to infect and utilize monocytes, macrophages, and epithelial cells, as carriers for successful dissemination to the sites of shedding (Walling, Flaitz, Hosein, Montes-Walters, & Nichols, 2004; Walling, Ray, Nichols, Flaitz, & Nichols, 2007). And these cells are abundant in the cellular components of early milk (Goldman, 1993; Michie, 1998; Rivas, el-Mohandes, & Katona, 1994), suggesting that the virus found in breast milk might have been carried and homed to the mammary gland and released into the breast milk. In addition, during the lytic replication of virus following reactivation, host cells could lyse and release infectious virions into the circulation (Adler et al., 2002).

5.1.7 Infection and transformation of PBMC by EBV in breast-milk

This is the first study to report evidence of viral transformation (blast formation, clumping and proliferation) following exposure of EBV DNA positive breast milk supernatant to PBMC. This finding suggests that the virus in breast milk is an infectious virus capable of inducing proliferation/transformation in lymphocytes. The proliferation of PBMCs implied that either the cells could have been activated following recognition of viral particles, or could have been directly infected by the virus. EBV is known to efficiently immortalize primary B cells, which gives rise to indefinite growing lymphoblastoid cell lines *in vitro* (Miller, 1982). This proliferation of infected B cells mimics a representative model of the ability of EBV to establish latency during *in vivo* infection (Thorley-Lawson & Gross, 2004).

5.1.8 Risk factors for EBV shedding in early breast milk samples (6 weeks) postpartum

In this study, maternal malaria infection at delivery was significantly associated with elevated EBV load in early breast-milk. This supports the hypothesis that significant increase of EBV load in the maternal circulation following reactivation caused by maternal malaria could in turn lead to increased shedding of EBV in breast milk. A possible ramification of this is the transmission of EBV to infants early in life. The

consequences of early age of infection on the establishment and maintenance of EBV persistence are not known. However, because the neonatal immune system is not well developed, lack immunologic memory, and skewed towards a Th2 phenotype (Adkins, Leclerc, & Marshall-Clarke, 2004; Morein, Blomqvist, & Hu, 2007; Siegrist, 2007); EBV infection early in life could lead to ineffective or minimal control of the virus.

5.1.9 The correlation of EBV load in maternal blood at delivery and early breast-milk samples (6 weeks) postpartum.

EBV load in maternal blood at delivery correlated with that of breast milk at 6 weeks postpartum. This finding further corroborate the hypothesis that the level of reactivation of EBV during pregnancy is linearly proportional to the shedding of virus in breast milk. In addition, this suggests that breast-feeding infants – born to women with high viral load in the maternal circulation during delivery – could be exposed to a higher EBV load soon after delivery.

A study conducted in Japan found comparable rates of EBV transmission in breast versus formula-fed children at the age of 12–23 months, indicating that breastfeeding does not add any potential risk for mother-to-child transmission of EBV (Kusuhara et al., 1997). However, geographical differences in age of primary EBV infections exist with infants in sub-Saharan Africa getting infected early in life than their counterparts in the developed world where infections occur in adolescent and adulthood (Biggar, Henle, Bocker, et al., 1978). Recent study carried out in Kenya documented EBV infections that occurred before 6 months of age among children living in malaria holoendemic area (Erwan Piriou et al., 2012). Thus, the findings from Japan (Kusuhara et al., 1997) might not account for the differences in age of primary infection and could not be extrapolated to our cohort.

Because of the enormous benefit of breast-feeding to the child survival, especially in the low-income settings, cessation of breast-feeding is not feasible. Therefore, strategies that will minimize EBV shedding in breast milk should be urgently developed. For instance,

a sustained long-term effort in malaria control programs such as, use of insecticide treated nets, intermittent preventive therapy among pregnant women, and indoor vector control are a needed to stem EBV reactivation during pregnancy and subsequent shedding in breast milk. Secondly, active and passive immunization such as vaccine could be considered.

The potential strength of this study is that pregnant women were actively followed up monthly during pregnancy through to delivery and postpartum, and categorized according to their malaria history. Therefore, an accurate malaria infection history for each pregnant woman was documented. Moreover, because pregnancy itself could modulate immune functions, one group of pregnant women with malaria was compared with another without malaria, with a view to control for the possibility of pregnancy itself playing a confounding role and having an influence on the results. Furthermore, RTQ-PCR was used for malaria detection, which in addition to detecting submicroscopic parasitaemia, also allows for accurate quantification as well as speciation of malaria parasites. Finally, examined longitudinal breast-milk samples obtained from EBV seropositive mothers from 6 weeks to 18 weeks postpartum and observed that virus in breast milk is a free and infectious.

5.2 SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

5.2.1 Summary of findings

In summary, pregnant women with malaria were more likely to have a detectable EBV DNA and elevated EBV loads than in women without malaria regardless of gestational age of pregnancy. Significantly higher EBV DNA and elevated EBV loads were found in early than mature breast milk. EBV in breast milk is encapsidated and infectious. Being infected with malaria at delivery was associated with increased EBV shedding in early breast milk. Finally, level of EBV load in maternal blood at delivery correlated with that of breast milk at 6 weeks postpartum.

5.2.2 Conclusions

1. Significantly higher EBV DNA was found in pregnant women with *P. falciparum* malaria infection compared to women without evidence of *P. falciparum* malaria infection.
2. Significantly elevated EBV load was found in pregnant women with *P. falciparum* malaria compared to women without evidence of *P. falciparum* malaria infection.
3. Significantly higher EBV load was found at third trimester relative to first and second trimester of pregnancy.
4. Significantly elevated EBV load was found in pregnant women with *P. falciparum* malaria compared to women without evidence of *P. falciparum* malaria infection independent of gestation age of pregnancy.
5. Significantly higher level of EBV DNA was found in early breast milk (6 weeks postpartum) with declining trends over time.
6. Significantly elevated EBV load was observed in early breast milk (6 weeks postpartum) with declining trends over time.
7. The breast-milk contains free and infectious EBV capable of inducing transformation in lymphocytes.

8. Being infected with *P. falciparum* malaria at delivery was significantly associated with increased EBV shedding in early breast milk.
9. EBV load in maternal blood at delivery positively correlated with the levels of EBV load in early breast milk (at 6 weeks) postpartum.

5.2.3 Recommendations

1. The findings that pregnant women with *P. falciparum* malaria have elevated EBV load suggest that a sustained long-term effort in malaria control programs such as, bed net use, ITN, IPT, and indoor vector control are needed to stem EBV reactivation during pregnancy and subsequent shedding of infectious EBV in breast milk.
2. The observation that pregnant women with *P. falciparum* malaria have elevated EBV load in circulation and in breast milk suggest that vaccine strategies for prevention of postnatal transmission of EBV early in life could be considered by immunizing the infants in the first few weeks of postpartum. In addition, identify vaccine candidates targeting the pregnant women with a view to confer passive immunity to their infants is needed.
3. Further studies on whether EBV-specific neutralizing antibodies develop in the breast milk of EBV-infected mothers, and how such antibodies protect the infants is urgently needed.

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APPENDICES

Appendix 1: Questionnaires

Form Version: PCB19Feb2011

SUNY CHAP Infant Cohort Study

Participant Identification Form

Participant ID: ECH-|__|__|__|__|__|-M

*Please record carefully and confirm. This ID will be PERMANENTLY assigned to the participant for the entirety of the study!

Recruitment Date: ___/_____/_____(dd/mmm/yyyy)

Demographic Information:

1. Tribe Luo☐ Luhya☐ Kalenjin☐ Kikuyu☐ Other☐
   a. If other, specify:_____
2. Mother's Date of Birth ___/_____/_____(dd/mmm/yyyy)
3. Age of Mother: _____
4. Marital Status: Single☐ Married☐ Divorced☐ Separated☐
   Widowed☐ Widowed, Inherited☐ Cohabiting☐ Other☐
   If other, specify:_____
5. Highest level of education attained by mother:
   None☐ Lower Primary School☐ Upper Primary School☐
   Secondary School☐ Adult Education☐ Village Polytechnic☐
   Other☐ If other, specify: _____
6. Highest level of education attained by spouse:
   None☐ Lower Primary School☐ Upper Primary School☐
   Secondary School☐ Adult Education☐ Village Polytechnic☐
   Other☐ If other, specify: _____
7. Occupation of mother: _____
8. Occupation of spouse:_____

Personal Details of Mother

9. Mother's Muslim-Christian/Middle/Family name:

   _____ / _____ / _____
   Muslim-Christian Middle Family

10. ANC Number: _____
11. Child's Estimated Date of Birth: ___/_____/____ (dd/mmm/yyyy)

[Page 1 of 2]

Location of Residence of Mother:

12. Residence Location: _____(12)
13. Residence Sublocation: _____(13)
14. Village Name: _____(14)
15. Nearest common point to residence: _____(15)
16. Mother's telephone number: _____ (16)
    a. Secondary Contact Person: _____(16a)
    b. Secondary Contact Telephone Number: _____(16b)
    c. Relationship to Secondary Contact: _____(16c)
17. Household owner's name: _____(17)
18. Main identifying feature near the house: _____(18)
19. Nearest shop: _____(19)
20. Nearest School: _____(20)
21. Nearest Matatu stage: _____(21)

Is this participant's residence greater than 10km from Chulaimbo Sub-district hospital?

No ☐ **Yes** ☐

Form Completed By: _____ Date: ____/_____/_____
(dd/mmm/yyyy)

For Data Entry Use Only

Entered By: _____ Date (dd/mmm/yyyy): ____/_____/_____
Reviewed By: _____ Date (dd/mmm/yyyy): ____/_____/_____

CHAP Infant Cohort Study

MATERNAL SICK VISIT FORM

PARTICIPANTS ID ECH-__|__|__|__|-M Visit Date: ____/____/_____
(dd/mmm/yyyy)

To be completed by Clinician/Nurse;

Symptoms	**NO**	**YES**
1. Is the patient sick today?	☐	☐
2. Does the patient have a fever today?	☐	☐
3. Fever in the past two days?	☐	☐
4. Cough in the past two days?	☐	☐
5. Headache in the past two days?	☐	☐
6. Chills in the past two days?	☐	☐
7. Diarrhea in the past two days?	☐	☐
8. Stomach ache in the past two days?	☐	☐
9. Is a bed net being used?	☐	☐
10. Other symptoms in the past two days?	☐	☐

If yes, please describe _____

_____

Physical Examination

11. Current level of consciousness Full ☐ Drowsy ☐ Comatose ☐

12. Axilliary Temperature (degrees C) ☐

13. Heartbeat Rate (beats per minute) ☐

14. Respiratory Rate (breath per minute) ☐

15. Weight (kg) ☐

16. Blood pressure **Systolic** ☐ **Diastolic** ☐

17. Spleen palpable? NO ☐ YES ☐

If yes, number of cm below left costophrenic margin ☐

18. Other abnormalities present NO ☐ YES ☐

If yes please describe _____

_____

Did you receive any of the following malaria medications in the past month?

	Yes	**No**
19. Amodiaquine	☐	☐
20. Fansidar	☐	☐
21. Coartem	☐	☐
22. Quinine	☐	☐
23. Artusenate	☐	☐
24. Other Medication	☐	☐

If yes please specify: _____

_____

To be completed by the Laboratory Personnel

25. Was blood collected? (1 green top tube) Yes ☐ No ☐

26. Was blood collected? (Capillary tube) Yes ☐ No ☐
    26.1. Blood Code ☐

27. Hb (gms/dl) ☐

Malaria (BS) Make 2 slides, perform one quick read.

28. Asexual parasite/microliter ☐

29. Gametocytes parasites/microliter ☐

Laboratory completed by: _____ Date: _____

30. Final Diagnosis: _____
    _____
    _____

31. Was medication received today? No ☐ Yes ☐
    If yes specify _____

32. Was a urine dipstick analysis performed? No ☐ Yes ☐
    If yes, include results below.

Clinical completed by:_____ **Date**:_____/_____/_____
(dd/mmm/yyyy)

KEMRI Urinalysis Result

Blood	Negative ☐	Positive ☐	Not Performed ☐
Bilirubin	Negative ☐	Positive ☐	Not Performed ☐
Glucose	Negative ☐	Positive ☐	Not Performed ☐
Leucocyte	Negative ☐	Positive ☐	Not Performed ☐

Specific Gravity: _____
Ph: _____

Nitrite	Negative ☐	Positive ☐	Not Performed ☐
Protein	Negative ☐	Positive ☐	Not Performed ☐
Ketones	Negative ☐	Positive ☐	Not Performed ☐
Urobilinogen	Negative ☐	Positive ☐	Not Performed ☐

Deposits	Negative ☐		Positive ☐		
Pus Cells	Negative ☐		Positive ☐	Value	
Epith Cells	Negative ☐		Positive ☐	Value	
T. Vaginalis	Negative ☐		Positive ☐	Value	
Yeast Cells	Negative ☐		Positive ☐	Value	
RBC	Negative ☐		Positive ☐	Value	
Granular Casts	Negative ☐		Positive ☐	Value	
Cellular Casts	Negative ☐		Positive ☐	Value	

For Data Entry Use Only

Entered By:_____ **Date** (dd/mmm/yyyy):_____/_____/_____

Reviewed By:_____ **Date** (dd/mmm/yyyy):_____/_____/_____

CHAP Infant Cohort Study

Pregnancy History form

Participant ID: ECH-|__|__|__|__|-M

Form completed by: _____ Date: ___/_____/_____ (dd/mmm/yyyy)

1. Have you ever had any of the following conditions? **No** ☐ **Yes** ☐

If yes, which? TB ☐ Diabetes ☐ STI ☐ Asthma/Allergies ☐ UTI ☐

IN THE PAST 3 MONTHS, HAVE YOU HAD ANY OF THE FOLLOWING?

2. Iron tables No ☐ Yes ☐ Don't Know ☐
3. Folic acid tables No ☐ Yes ☐ Don't Know ☐
4. Blood transfusion No ☐ Yes ☐ Don't Know ☐
5. Herbal medicine No ☐ Yes ☐ Don't Know ☐
6. Medication for malaria No ☐ Yes ☐ Don't Know ☐

If Yes, specify *Chloroquine* ☐ Fansidar ☐ Co-Artem ☐ other ☐

*If other, specify:*_____

7. Medication for worms None ☐ Mebendazole ☐ Other ☐ Do not know ☐

*If other, specify:*_____

8. Other medications No ☐ Yes ☐ Do not know ☐

If Yes, specify _____

9. Date of last menstrual period ____/_____/_____ (dd/mmm/yyyy)

Pregnancy and fertility

10. Have you ever given birth? (if No; skip to 18) No ☐ Yes ☐
11. If yes, how old were you when you ever given birth to? _____(11)
12. How many children have ever given birth to? _____(12)
13. How many are living with you? _____(13)
14. How many are living elsewhere? _____(14)
15. How many are deceased? _____(15)

16. Have you ever had a caesarian section? No ☐ Yes ☐

If yes, why? Fetal distress ☐ Malposition ☐ Cephalopelvis disproportion ☐

17. Details of pregnancies

1st pregnancy.

Date of outcome ____/____/____(dd/mmm/yyyy)

General outcome Stillbirth ☐ Live birth ☐

Specific outcome; Singleton ☐ Twins ☐ Triplets ☐ Other (specify)_____

2nd pregnancy.

Date of outcome ____/____/____(dd/mmm/yyyy)

General outcome Stillbirth ☐ Live birth ☐

Specific outcome; Singleton ☐ Twins ☐ Triplets ☐ Other (specify)_____

3rd pregnancy.

Date of outcome ____/____/____(dd/mmm/yyyy)

General outcome Stillbirth ☐ Live birth ☐

Specific outcome; Singleton ☐ Twins ☐ Triplets ☐ Other (specify)_____

4th pregnancy.

Date of outcome ____/____/____(dd/mmm/yyyy)

General outcome Stillbirth ☐ Live birth ☐

Specific outcome; Singleton ☐ Twins ☐ Triplets ☐ Other (specify)_____

5th pregnancy.

Date of outcome ____/____/____(dd/mmm/yyyy)

General outcome Stillbirth ☐ Live birth ☐

Specific outcome; Singleton ☐ Twins ☐ Triplets ☐ Other (specify)_____

6th pregnancy.

Date of outcome ____/____/____(dd/mmm/yyyy)

General outcome Stillbirth ☐ Live birth ☐

Specific outcome; Singleton ☐ Twins ☐ Triplets ☐ Other (specify)_____

7th pregnancy.

Date of outcome ____/_____/_____(dd/mmm/yyyy)

General outcome Stillbirth ☐ Live birth ☐

Specific outcome; Singleton ☐ Twins ☐ Triplets ☐ Other (specify)_____

8th pregnancy.

Date of outcome ____/_____/_____(dd/mmm/yyyy)

General outcome Stillbirth ☐ Live birth ☐

Specific outcome; Singleton ☐ Twins ☐ Triplets ☐ Other (specify)_____

9th pregnancy.

Date of outcome ____/_____/_____(dd/mmm/yyyy)

General outcome Stillbirth ☐ Live birth ☐

Specific outcome; Singleton ☐ Twins ☐ Triplets ☐ Other (specify)_____

18. Have you had any problem(s) with this pregnancy? No ☐ Yes ☐

19.If yes, describe the problem(s):_____

_____

_____

20. Was the current pregnancy planned? No ☐ Yes ☐

21. Do you use a bed net? No ☐ Yes ☐

For Data Entry Use Only

By:_____ Date (dd/mmm/yyyy):____/_____/_____

d By:_____ Date (dd/mmm/yyyy):____/_____/_____

CHAP Infant Cohort Study

Maternal Prenatal (ANC) Visit Form

PARTICIPANT ID: ECH-__ | __ | __ | __ | - M

Completed By: _____ Date: ____/_____/_____ (dd/mmm/yyyy)

Visit Number ANC☐ ANC2☐ ANC3☐ ANC4☐

1. Estimated gestational age (in weeks): _____
2. Fundal Height (cm): _____

Symptoms	No	Yes
3. Is the patient sick today?	☐	☐
4. Does the patient have a fever today?	☐	☐
5. Fever in the past two days?	☐	☐
6. Cough in the past two days?	☐	☐
7. Headache in the past two days?	☐	☐
8. Chills in the past two days?	☐	☐
9. Diarrhea in the past two days?	☐	☐
10. Stomach ache in the past two days?	☐	☐
11. Is a bed net being used?	☐	☐
12. Other symptoms in the past two days?	☐	☐

If yes, please describe: _____

_____

Physical Exam

13. Weight (kg): _____(13)
14. Height (cm): _____(14)
15. Mid-Upper Arm Circumference [MUAC] (cm): _____(15)
16. Heartbeat Rate (beats per minute) _____(16)
17. Respiratory Rate (breaths per minute) _____(17)
18. Blood Pressure _____ / _____ (18)
    Systolic / Diastolic
19. Axillary Temperature (degrees C): _____(19)

Medications Given at Visit	No	Yes
20. Iron Tables (N=30)	☐	☐
21. Folic Acid (N=30)	☐	☐
22. Fansider Tables (Malaria prophylaxis) (N=3)	☐	☐
23. Tetnus booster given?	☐	☐
24. Deworming (Membendazole500gms)	☐	☐
25. ITN (INSECTS treated net)	☐	☐
26. ARV (prophylaxis) AZT+NVP	☐	☐
27. Other medications	☐	☐

If yes, please specify: _____

_____

28. Clinical Diagnosis: _____

_____

_____

For Data Entry Use Only

d By:_____ Date (dd/mmm/yyyy):____/_____/_____

ed By:_____ Date (dd/mmm/yyyy):____/_____/_____

Form Version: PCB10Feb2013

CHAP Infant Cohort Study

PRENATAL LAB RESULTS FORM
(CHULAIMBO HEALTH CENTRE LAB)

Participant ID ECH-| __|__|__|__|-M

Completed by: _____ Date: ___/____/_____ (dd/mmm/yyyy)

Visit Number: ANC1☐ ANC2☐ ANC3☐ ANC4☐

1. Hemoglobin results (gms)/dL) : ☐

2. VDRL results: Positive☐ Negative☐

Blood group: A☐ B☐ AB☐ O☐ Other☐

If other, specify: _____

3. Malaria (BS) results: Positive☐ Negative☐

Asexual parasites per microliter: ☐

Gametocytes parasites per microliter: ☐

For Data Entry Use Only

tered By:_____ Date (dd/mmm/yyyy):_____/_____/_____

viewed By:_____ Date (dd/mmm/yyyy):_____/_____/_____

Form Version: PCB10Feb2013

CHAP Infant Cohort Study

Prenatal Lab Results (KEMRI)

PARTICIPANT ID: ECH-__ | __ | __ | __ | - M Visit Date: ____/_____/_____ (dd/mmm/yyyy)

Visit Number ANC1 ☐ ANC2 ☐ ANC3 ☐ ANC4 ☐

1. Malaria (BS) Result
   a. Asexual parasites/microliter ☐
   b. Gametocytes parasites/microliter ☐
2. Proteinuria Negative ☐ Positive ☐
3. Glucose Negative ☐ Positive ☐
4. Ketones Negative ☐ Positive ☐
5. Deposits Negative ☐ Positive ☐
   - Pus Cells Negative ☐ Positive ☐ Value ☐
   - Epith Cells Negative ☐ Positive ☐ Value ☐
   - T. Vaginalis Negative ☐ Positive ☐ Value ☐
   - Yeast Cells Negative ☐ Positive ☐ Value ☐
   - RBC Negative ☐ Positive ☐ Value ☐
   - Granular Casts Negative ☐ Positive ☐ Value ☐
   - Cellular Casts Negative ☐ Positive ☐ Value ☐
6. Urine Filtration Yes ☐ No ☐ Not Performed ☐
7. Urine positive for Schistosomiasis? Yes ☐ No ☐ Not Performed ☐
   8. If yes, how many Ova/10mL?
      Filter A: ☐ Filter B: ☐

Stool Results

9. Hookworm Present ☐ Absent ☐ Not Performed ☐
   10. Hookworm (eggs/gm): ☐
11. Trichuris Trichura Present ☐ Absent ☐ Not Performed ☐
    12. Trichuris Trichura (eggs/gm): ☐
13. Ascaris Lumbricodes Present ☐ Absent ☐ Not Performed ☐
    14. Ascaris Lumbricodes (eggs/gm) ☐
15. E. histolytica Present ☐ Absent ☐ Not Performed ☐
16. Giardia lamblia Present ☐ Absent ☐ Not Performed ☐
17. Strongyloides Present ☐ Absent ☐ Not Performed ☐
18. Sch. Mansoni Present ☐ Absent ☐ Not Performed ☐

For Data Entry Use Only

Entered By:_____ Date (dd/mmm/yyyy):____/_____/_____

Reviewed By:_____ Date (dd/mmm/yyyy):____/_____/_____

Appendix 2: Protocol for DNA extraction from Human Blood Samples

Protocol for DNA extraction from human blood samples

1. **Reagents and Supplies:**

- **RNase A**: purchased from Qiagen, catalog # 19101; 1.25ml (100mg/ml).
- **Laminar flow hood**: use flow hood for the first part of the extraction. First, clean hood with 10% bleach and 70% ethanol. Second, place under the hood vortex instrument, pipettes and pipette tips, tube rack, 1.5ml tubes, a 200ml biker full with 10% bleach solution to discard the tips, Rnase A, Buffer AL and protienase K reagents, PBS solution, and 100% (absolute) ethanol.
- **Water bath**: set water bath to 56°C before beginning extraction.
- **Starting volume of blood**: this procedure may be used for a starting volume of 50 to 200µl blood.
- **Thawing blood**: allow blood to thaw at room temperature and under Laminar flow hood in tube rack (**do not place in water bath**).
- **Reagents**: the reagents from this kit are from the Qiagen, excluding sterile filtered PBS and 100% ethanol. If Buffer AL has a precipitate, heat at 55°C until dissolved. Then elution buffer (Buffer AE) contains the following: 10mM Tris, pH 9 and 0.5 mM EDTA.
- **Pipette tips**: all pipette tips are filtered art tips to prevent PCR contamination. Also, fresh tips are used for the addition of all reagents to prevent cross-contamination of samples.
- **Centrifuge**: all centrifugation steps are carried out with a micro centrifuge at room temperature.
- **Labeling of tubes**: the samples will be labeled with CryoTags printed on the laser printer that contain the sample ID designation and extraction date.

2. **Procedure**

2.1 Before starting DNA extraction:

a. Put on lab coat and gloves
b. Turn on Laminar flow hood fan for at least 10 minutes
c. Wipe Laminar flow hood with 10% bleach and then with 70% Ethanol
d. Place bench guard on work surface under the hood
e. Set the water bath to 56°C
f. Place vortexer under hood
g. Place a large 10% bleach waste container under the hood
h. Place sterile filter tips (size 10, 20, 200, and 1000 µl) and pipettors under hood
i. Place under the hood Buffer AL, 100% ethanol, RNase A, Proteinase K, and DNeasy spin column sitting in a 2 ml collection tube

2.2 Getting samples from -80 Freezer:

a. Print cryo labels and assign DNA extraction codes and extraction date
b. Print out a DNA extraction worksheet with the next available DNA extraction codes listed (to record data during the experiment)
c. Remove blood samples (0.5ml purple top tubes) from the -80 freezer and check that the ID number on the DNA extraction worksheet along with the freezer box number and space in the box so that the tube can be replaced if there is blood sample left in the tube
d. Return box to -80 freezer
e. Draw a circle on the top of the purple top blood sample tubes that are going to be used for DNA extraction with a sharpie marker
f. Take tubes to the Laminar flow hood to thaw at room temperature, do not let sit any longer than necessary
g. While thawing, label tubes and columns with the DNA extraction code

2.3 DNA Extraction Procedure (Perform in the Biological Safety Cabinet (i.e. Laminar Flow Hood)

1. Add 4 µl of RNase A (100mg/ml) into the bottom of a sterile 1.5ml micro centrifuge tube.

2. Mix blood (by flicking bottom of tube) and add 200µl of anticoagulated blood to the tube with RNase (if less, bring to 200µl with sterile PBS solution and note on DNA extraction worksheet the amount of sterile PBS added).
3. Mix (by flicking bottom) and Incubate at room temperature for 2 minutes.
   While incubating take any remaining blood sample (purple top tube) to the -80 freezer and place tubes exactly where they had been.
4. Add 20 µl Proteinase K to each tube.
5. Add 200 µl Buffer AL to each tube. Mix thoroughly by vortexing until the solution becomes homogenous (usually about 10 seconds at high speed/sample).
6. Incubate for 10 minutes at 56°C in the water bath.
7. After the 10 minutes incubation, add 200µl 100% room temperature ethanol to each sample. Mix thoroughly by vortexing until the solution becomes homogenous (usually about 10 seconds at high speed/sample).
8. Set P1000 pipette to 630 ml and pipette the mixture, including any precipitate, into the center of the DNeasy spin column sitting in a 2-ml collection tube
9. Centrifuge in the Eppendorf centrifuge at 6000 X *g* (8000 rpm) for 1 minute at room temperature.
   Discard flow-through and collection tube in the large 10% bleach waste container.
10. Place DNeasy spin column into a new 2-ml collection tube. **Add 500µl Buffer AW1**; incubate 5 minutes at room temperature and centrifuge at 6000 X *g* (8000 rpm) for 1 minute at room temperature.
    Discard flow-through and collection tube as above.
11. Place DNeasy spin column into a new 2-ml collection tube. **Add 500µl Buffer AW2**; incubate 5 minutes at room temperature (while incubating label clean 1.5 ml micro centrifuge tubes with DNA extraction code for each tube), and centrifuge at full speed **13200 rpm for 3 minute**s at room temperature to dry the DNeasy membrane. Discard flow-through and collection tube as above.
    When taking the tube out of the centrifuge, be careful not to knock tubes against any surface because the filter in the column might get wet. In the case this happened re-centrifuge the tube(s).
12. Place DNeasy spin column into the clean labeled 1.5ml micro centrifuge tube. Pipette **100µl buffer AE** (half of the original blood volume) directly onto DNeasy membrane. Incubate at room temperature for 5 minutes, and then centrifuge at 6000 X *g* (8000rpm) for 1 minute at room temperature to elute DNA.
13. Repeat elution from step 12 into the same tube in order to combine elutes. Total volume should now be 200µl.
14. Stick sample ID label on each tube.

Measuring DNA Concentration: Please refer to the SOP for *"NanoDrop 2000/2000c Spectrophotometer"* on use and handling.

- 260/280 Ratio: 260/280 ratio should be equal to or greater than 1.75 in acceptable specimens.
- 260/230 Ratio: 260/230 ratio should be equal to or greater than 1.85 in acceptable specimens.
- 200µl of whole blood yields 3-12µg of DNA, and the DNA concentration 34.0ng/µl.

15. Check ID against extraction code and ID on worksheet. Record sample spot. Place samples in -20°C freezer till analysis.

Reference:
Adopted and modified from Rochford SUNY Laboratory
Qiagen Kit

Appendix 3: Protocol For DNA Extraction from Human Breast Milk

PROTOCOL FOR DNA EXTRACTION FROM HUMAN BREAST MILK

(Using QIAamp® DNA Blood Mini Kit)

1. **Reagents and Supplies:**

- **RNase A**: purchased from Qiagen, catalog # 19101; 1.25ml (100mg/ml).
- **Laminar flow hood**: First, clean hood with 10% bleach and 70% ethanol. Second, place under the hood vortex instrument, pipettes and pipette tips, tube rack, 1.5ml tubes, a 200ml container full with 10% bleach solution to discard the tips, Rnase A, Buffer AL and protienase K reagents, 1X PBS solution, and 100% (absolute) ethanol.
- **Water bath**: set water bath to 30°C before beginning extraction.
- **Thawing breast milk**: allow breast milk to thaw at room temperature and under Laminar flow hood in tube rack (**do not place in water bath**).
- **Reagents**: the reagents from this kit are from the Qiagen, excluding sterile filtered PBS and 100% ethanol. If Buffer AL has a precipitate, heat at 55°C until dissolved. Then elution buffer (Buffer AE) contains the following: 10mM Tris, pH 9 and 0.5 mM EDTA.
- **Pipette tips**: all pipette tips are filtered art tips to prevent PCR contamination. Also, fresh tips are used for the addition of all reagents to prevent cross-contamination of samples.
- **Centrifuge**: all centrifugation steps are carried out with a micro centrifuge at room temperature.
- **Labeling of tubes**: the samples will be labeled with CryoTags printed on the laser printer that contain the sample ID designation and extraction date.

2. **Procedure**

2.1 Before starting DNA extraction:

a. Put on lab coat and gloves
b. Turn on Laminar flow hood fan for at least 10 minutes
c. Wipe Laminar flow hood with 10% bleach and then with 70% Ethanol
d. Place bench guard on work surface under the hood
e. Set the water bath to 30°C
f. Place vortexer under hood
g. Place a large 10% bleach waste container under the hood
h. Place sterile filter tips (size 10, 20, 200, and 1000 µl) and pipettors under hood
i. Place under the hood Buffer AL, 100% ethanol, RNase A, Proteinase K, and DNeasy spin column sitting in a 2 ml collection tube

2.2 Getting samples from -80 Freezer:

a. Print cryo labels and assign DNA extraction codes and extraction date
b. Print out a DNA extraction worksheet with the next available DNA extraction codes listed (to record data during the experiment)
c. Remove one vial of breast milk samples from the -80 freezer and check that the ID number on the DNA extraction worksheet along with the freezer box number and space in the box so that the tube can be replaced with the sample left.

d. Return box to -80 freezer
e. Draw a circle on the top of the sample tubes that are going to be used for DNA extraction with a sharpie marker
f. Take tubes to the Laminar flow hood to thaw at room temperature, do not let sit any longer than necessary
g. While thawing, label tubes and columns with the DNA extraction code.

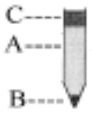
2.3 DNA Extraction Procedure

1. Transfer the breast milk sample into 1.5ml sterile tube
2. Centrifuge the breast milk samples at 400xg (5000rpm) for 10 minutes at room temperature.

Note:

✓ As shown in the diagram below, three layers results; **A**: clear layer and **B**: pellet, **C**: creamy uppermost layer.

✓ Taq functioning in RT-PCR is inhibited by lipoproteins contained in breast milk. Therefore, the resulting creamy top layer is discarded.



3. Carefully pick the creamy top layer **(C)**, and discard.
4. Pick the cloudy supernatant **(A)** and put in another labeled sterile sardet tube.
5. Resuspend the pellet **(B)** in 0.5ml 1X PBS.
6. Wash twice with 1X PBS by centrifuging at 1300rpm for 5 min and decanting the supernatant.
7. Resuspend pellet in **180µl** of 1X PBS.
8. Add **4µl** of RNase
9. Mix (by flicking bottom) and Incubate at room temperature for 2 minutes.
   Note: While incubating take any remaining breast milk sample to the -80 freezer and place tubes exactly where they had been.
10. Add 20 µl Proteinase K to each tube.
11. Add 200 µl Buffer AL to each tube. Mix thoroughly by vortexing until the solution becomes homogenous (usually about 10 -15 seconds at high speed/sample).
12. Incubate at **37°C** for an **1 hour** followed by **70°C for 30** min in a water bath
13. Add **200 µl** of absolute ethanol to the sample and mix by vortexing
14. Set P1000 pipette to 630 ml and pipette the mixture, including any precipitate, into the center of the DNeasy spin column sitting in a 2-ml collection tube
15. Centrifuge in the Eppendorf centrifuge IEC/MicroMax (851 rotor) at 6000 X *g* (8000 rpm) for 1 minute at room temperature. Discard flow-through and collection tube in the large 10% bleach waste container.
16. Place DNeasy spin column into a new 2-ml collection tube. Add **500µl Buffer AW1**; **incubate 5 minutes at room temperature** to better rinse away protein and centrifuge at 6000 X *g* (8000 rpm) for 1 minute at room temperature. Discard flow-through and collection tube as above.
17. Place DNeasy spin column into a new 2-ml collection tube. Add **500µl Buffer AW2**; **incubate 5 minutes at room temperature** (while incubating label clean 1.5 ml micro centrifuge tubes with DNA extraction code for each tube), and centrifuge at full speed (13200 rpm) for 3 minutes at room temperature to dry the DNeasy membrane. Discard flow-through and collection tube as above. When taking the tube out of the centrifuge be careful not to knock tubes against any surface because the filter in the column might get wet. In the case this happened re-centrifuge the tube/s.

18. Place DNeasy spin column into the clean labeled 1.5ml micro centrifuge tube. Pipette **100µl buffer AE** (half of the original breast milk volume) directly onto DNeasy membrane. **Incubate at room temperature for 5 minutes** and then centrifuge at 6000 X *g* (8000rpm) for 1 minute at room temperature to elute DNA.
19. Repeat elution from step 15 into the same tube in order to combine elutes. Total volume should now be 200µl.
20. Stick sample ID label on each tube.

Measuring DNA Concentration: Please refer to the SOP for *"NanoDrop 2000/2000c Spectrophotometer"* on use and handling.

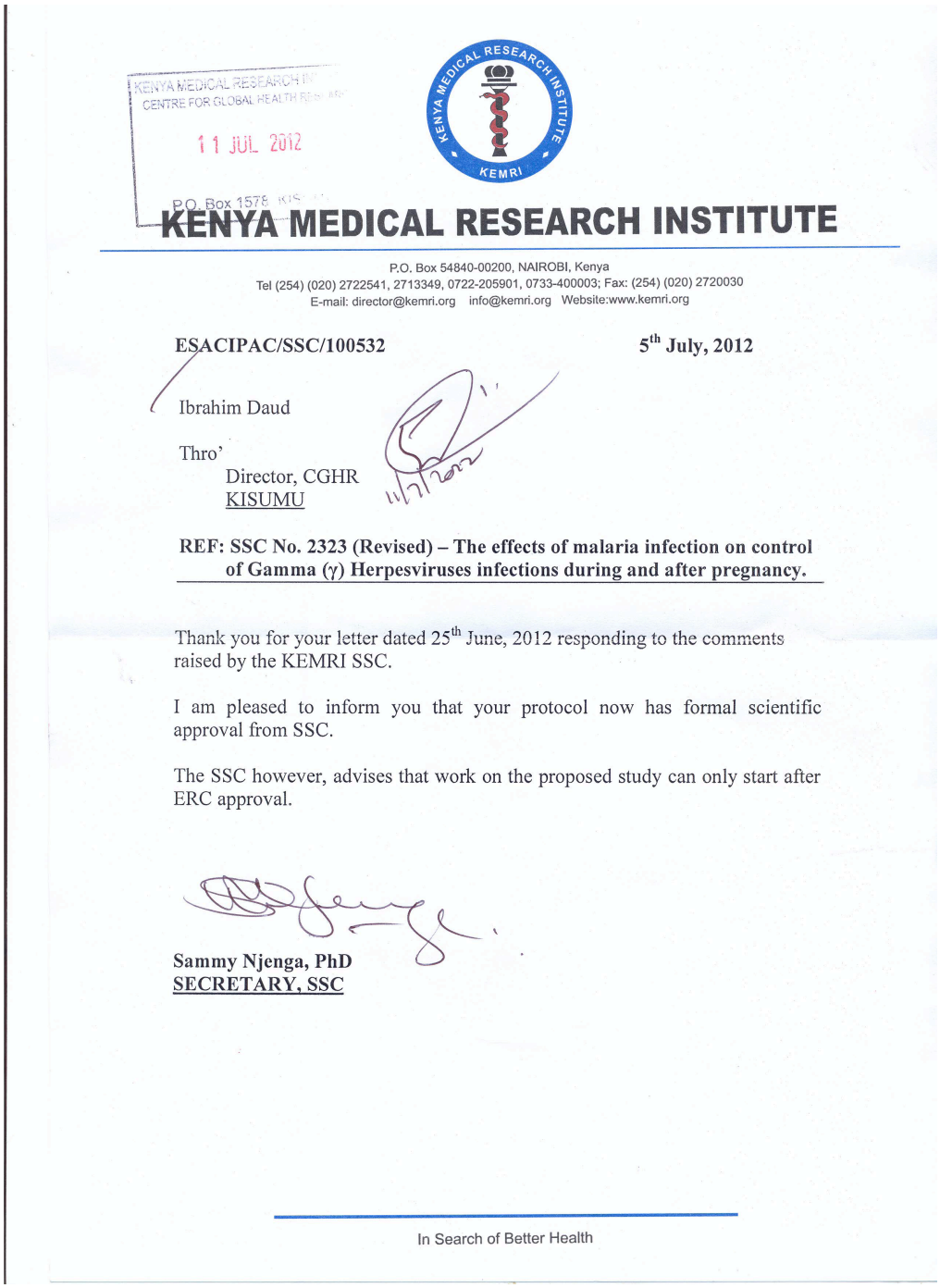
- 260/280 Ratio: 260/280 ratio should be equal to or greater than 1.75 in acceptable specimens.
- 260/230 Ratio: 260/230 ratio should be equal to or greater than 1.85 in acceptable specimens.

Check ID against extraction code and ID on worksheet. Record sample spot. Place samples in -20°C freezers till analysis.

References

- Hamprecht *et al.*, 1998. Detection of cytomegaloviral DNA in human milk cells and cell free milk whey by nested PCR. *Journal of Virological Methods* 70, 167–176.
- SUNY Upstate: Kenya project, 2007. Protocol for DNA extraction from human blood samples (edited 05/24/2007-SUNY LAB)

Appendix 4: KEMRI Scientific Steering Committee Approval



KENYA MEDICAL RESEARCH INSTITUTE
CENTRE FOR GLOBAL HEALTH RESEARCH
11 JUL 2012
P.O. Box 1578 KIS

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

ESACIPAC/SSC/100532 **5th July, 2012**

Ibrahim Daud

Thro'
Director, CGHR
KISUMU

REF: SSC No. 2323 (Revised) – The effects of malaria infection on control of Gamma (γ) Herpesviruses infections during and after pregnancy.

Thank you for your letter dated 25th June, 2012 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval.

Sammy Njenga, PhD
SECRETARY, SSC

In Search of Better Health

Appendix 5: KEMRI Ethical Review Committee Approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **September 24, 2012**

TO: **DR. IBRAHIM I DAUD (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. JOHN VULULE,**
THE DIRECTOR, CGHR,
KISUMU

Dear Sir,

RE: **SSC PROTOOL No. 2323 – 2ND REVISION (*RE-SUBMISSION*): THE EFFECTS OF MALARIA INFECTION ON CONTROL OF GAMMA (γ) HERPES VIRUSES INFECTION DURING AND AFTER PREGNANCY (*VERSION DATED 3 SEPTEMBER 2012*)**

Reference is made to your letter dated September 3, 2012. The ERC Secretariat acknowledges receipt of the revised proposal on September 6, 2012.

This is to inform you that the Committee determines that the issues raised at the 206th meeting of 21st August 2012 are adequately addressed. Consequently, the study is granted approval for implementation effective this **24th day of September 2012** for a period of one year.

Please note that authorization to conduct this study will automatically expire on **September 23, 2013**. If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **August 13, 2013**. The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

You are required to submit any proposed changes to this study to the SSC and ERC for review and the changes should not be initiated until written approval from the ERC is received.

Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the ERC and you should advise the ERC when the study is completed or discontinued.

Work on this project may begin.

Sincerely,

DR. CHRISTINE WASUNNA,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

Appendix 6: Consent form (English Version)

SUNY Upstate Medical University-Kenya Medical Research Institute
Consent for Human Investigational Studies
Effects of malaria on EBV persistence in infants and their mothers

Study Participant Number: _____.

Background and Purpose:

Burkitt's lymphoma is a cancer that affects children and is thought to be caused by the presence of malaria and a virus called the Epstein Barr Virus (EBV). We know that children who live in malaria areas get EBV earlier in life than children that live in areas without malaria. These children are also likely to carry high levels of the virus. In this study we will look at the reasons why this is so.

Women who live in areas with high malaria such as Chulaimbo, when they become pregnant can get malaria parasites in their placenta. The presence of the parasite in the placenta has been known to cause many complications in babies after they are born. We think that mothers who suffer from malaria when they are pregnant may cause the higher levels of virus in their babies. The purpose of this study is to see if the presence of parasites in the placenta may result in babies getting high levels of EBV earlier in life and if this is also the reason that some children suffer from Burkitt's Lymphoma.

Dr. Rosemary Rochford from SUNY Upstate Medical University (SUNY Upstate) in Syracuse, USA and Dr Peter Odada Sumba at of the Kenya Medical Research Institute (KEMRI) are inviting you and your newborn child to participate in this research study.

We are approaching you at the antenatal clinic so that you may agree to participate in the study before your baby is born. If you agree to be in the study we will ask you to fill a form that will give us information about your pregnancy, where you live and when you are expected to deliver. When you come back to the hospital to deliver we will identify you as a study participant so that we may pay for your delivery costs.

We will ask for consent from you for your participation in the study. We will also ask you for consent for your newborn child to participate in this study. Permission will be required from you for both you and your child. We are hoping to enroll 400 women and 400 infants into this study.

Study Procedures

1. Sample Collection:

In order to understand how strongly your child's body responds to malaria and to EBV, we have to know if you had malaria during your pregnancy and if your placenta has

malaria parasites in it. We also want to know when your child is first infected with malaria and how many infections he/she will get and when he/she is infected with EBV. We will collect a small sample of blood from your finger today to test for malaria and also for some studies that will be carried out in our laboratories in Kisian. We will collect your placenta at delivery and will look for malaria parasites in it. We are also requesting for a teaspoon of blood from you once you are rested after your baby's delivery, we will use a needle attached to a syringe to obtain the blood from your arm. We will also request a small blood sample from your child at delivery, which we will obtain from the umbilical cord.

After the birth of your baby, we will set up appointments for you to return to the clinic with your baby at 6,10,14, and 18 weeks of age. W will request to take blood samples from your baby at these appointments. A small amount of blood (a few drops), will be taken from your child's heel or finger. We will also schedule return visits to the clinic for when your baby is 6 months old, and at every 3 month interval to 24 months (2 years of age). Starting at the 6 month visit, we will request to take from your child a larger blood sample, equal to about 2 teaspoons. This sample will be drawn from a vein in your child's arm using a needle attached to a blood collection tube. It is important if you agree to be in this study that you will be around at least for the next year.

We will also collect from you breast milk and saliva samples to see if the EBV virus is in the milk and saliva and if this is how your baby gets infected. Breast milk and saliva will be collected at 6,10, and 14 week visits. We will require about two teaspoons of breast milk and a teaspoon of saliva.

All samples will be transported to the SUNY Upstate/KEMRI laboratory in Kisumu. Tests done in the laboratory will tell us if you had malaria during pregnancy and when your child is infected with malaria and EBV and how your child's body protects him/her against malaria and against EBV. We will also carry out tests that will inform us of your child's immune system. We will monitor your child's growth every time we visit by measuring his/her height and weight.

2. Genetic studies:

We also would like to use part of your child's blood at the SUNY Upstate/KEMRI laboratory to do genetic studies. Genes are composed of the genetic material called DNA. DNA is the part of the cell that is responsible for providing hereditary characteristics (such as eye color) and is used to build proteins. We would like to test his/her blood for genes including sickle cell trait, G6PD deficiency and HLA type that may protect against malaria. We may also test for other genes that are known to affect malaria and EBV. If we get any results from the lab studies that may affect your child's status with respect to malaria, we will inform you. We will also look at genes belonging to the malaria parasite and to EBV. This is important to see if your child has built defenses against different types of malaria parasites or EBV and similar viruses. Since the significance of the tests for changes in the malaria parasite or EBV is not known to

you, we will not release the results of any genetic tests associated with the EBV and malaria parasite testing.

Do you accept for yours and your child's samples to be used for Genetic Studies?

YES_____ NO_____

3. Sample Storage:
Samples collected will be stored in the SUNY-KEMRI laboratories in Kisumu for the full duration of the study and the period required to analyze results. We may save some of your samples for many years to further study Burkitt's Lymphoma in the SUNY/KEMRI Labs in Kisian or in the labs of Dr Rochford at the SUNY Upstate Medical University. If at any time you wish to withdraw your agreement for us to save your and your child's samples, please contact Dr. Peter Odada Sumbab, the Research Project Manager, at KEMRI Kisumu Tel. 254-57-2022989 or 254-733-746854/254-720-766550 and we will destroy the samples.

Do you accept for yours and your child's samples to be stored and when necessary to be shipped to SUNY Upstate Medical University in the US for further investigations?

YES_____ NO_____

Risks:
There are few risks in having your blood taken from the placenta or your arm after delivery. There are also few risks from taking your breast milk and saliva. Minimal risks are associated with collecting your child's blood. The blood drawings include a little bleeding, pain, bruising and, rarely, infection. All of these are uncommon but may occur in very few people. We have never experienced these problems in our previous studies.

Answering questions during the antenatal enrolment interview will not cause any risk to you or your child.

Benefits:
We will be testing for malaria in your placenta, which is not normally done and is therefore a benefit. It is important to know if you had malaria in your placenta because it will tell us how your baby will fight off malaria and EBV later in life. Also as a benefit to your child, when he or she is seen at scheduled visits for us to collect samples, if your child is sick we will treat your child for fever, malaria, diarrhea and anemia according to the Kenya Ministry of Health Guidlines. Also, the information that we get from this study will be important for prevention programs for Burkitt's Lymphoma.

Alternatives:
You do not need to participate in this study to receive medical care for you and your baby. If you choose not to participate, you may still obtain normal delivery care at the

hospital and medical care as provided by the Kenya Ministry of Health, including free testing and treatment for malaria.

Voluntary Participation:
Your participation in this research study is entirely voluntary. Refusing to participate will not alter your usual health care or involve any penalty or loss of benefits. If you decide to join the study, you may withdraw you and or your child at any time and for any reason.

Costs/ Payments:
We will pay for your delivery costs if you participate in this study. We will meet both bed and delivery fees**.** There are no additonal costs to you or your child for participating in this study.

During your participation in the study, you and your child will receive free medical care and attention from the study assigned Clinical Officer for the duration of the study. If you withdraw from the study before you deliver, the study will not pay for your delivery costs. You will however be able to obtain normal delivery care at the hospital but the costs will be your repsonsibilty. If you withdraw from the study after delivery, you will not receive the free medical care and medication for study participants but you can still attend the clinic and receive services provided by the Ministry of Health. If you withdraw at any OTHER time, after our 1st home visit, your child will contuinue to receive free medical care from the study appointed clinical officer for the duration of the study period.

Neither you or your child will be paid for participating in this study.

Questions:
If you have any questions about this study you may speak to Dr. Peter Odada Sumba, the Research Progeram Manager, at the Center for Global Health Research, Kenya Medical Research Institute, PO Box 1578, Kisumu at 254-57-2022989 / 254-733-746854 / 254-720-766550 or to The Director of CGHR, KEMRI in Kisumu at 254-57-2022924. Queries pertaining to research subjects' rights may be made to the KEMRI/National Ethical Review Committee (ERC), PO Box 54840, Nairobi at 254- 2-02722541 or The Director of KEMRI, PO Box 54840, Nairobi at 254- 2-02722541.

In Case of Injury:
In the event of illness or physical injury resulting from taking part in this research study, please contact The Director of the Center for Global Health and Research (CGHR) at KEMRI in Kisumu at PO Box 1578, Kisumu 40100. Tel: 254-57-2022924 or Dr. Peter Odada Sumba at 254-57-2022989/ 254-733746854/254-720-766550

SUNY Upstate Medical University has no plans to give you money if you are injured. You have not waived any of your legal rights by signing this form.

Confidentiality of Records and Authorization to use/share protected health information for research:

If you agree to participate in the study and if you allow your child to participate in this research, yours and your child's health information will be kept confidential. We will assign a number to you and your child that will appear on all the samples that we collect . Your name WILL NOT appear on any of these samples. This is being done to protect your and your child's medical information. There will be a few people that will have your name and numbers. These will be Dr. Rochford, Dr Odada Sumba and the study assistants. If for any reason your samples need to be shipped to the US for analysis, these samples will have only your study number and no names will be included. When we publish or present any of the findings from this study, your names will never be revealed. All information that we collect from you will be will be stored in locked cabinets at CGHR, KEMRI with only a few people in the Research Project, having access to these cabinets. This is also to ensure privacy and protection of your and your child's medical information.

Consent to participate in Research:

The nature and the purpose of the above research study have been explained to me.
Signing below indicates that I have been informed about the research study in which I voluntarily agree to my participation and my child's participation. I have asked questions about the study and the information given to me has permitted me to make a fully informed and free decision about my and my child's participation in the study. By signing this consent form, I do not waive any legal rights, and the investigators are not relieved of any liability they may have. I can withdraw from this study at any time. A copy of this consent form will be provided to me.

Signature of Parent_____ Date_____

Signature of Person Obtaining Consent/Authorization___ Date ___

Signature of Witness_____ Date_____

Appendix 7: Consent form (Dhluo Version)

SUNY Upstate Medical University-Kenya Medical Research Institute
Kalatas yie mar nonro kuom dhano
Appendix 2: Richo ma tuo mar midusi kod ayaki kelo kuom nyithindo ma kudni mar EBV oniang'go

Namba mar Janonro: ECH___ ___ ___ ___- ___.

Weche matut kod Thoro:
Tuo mar ningu en midekre machando nyithindo kendo iparo ni ikele gi bedo mar tuo mar midusi edel kod kudni ma iluongo ni Epstein Barr Virus (EBV). Wang'eyo ni nyithindo maodak egwenge ma tuo mar Midusu (Malaria) yudo kudni mar EBV chon moloyo nyithindo maodok egwenge ma tuo mar midusi onge. Nyithindogi ni eobadho mar ting'o kute gi mang'eny. Enonroni wabiro ng'iyo gigo maomiyo machalo kama.

Mine maodak egwenge ma tuo mar midusi thothie kaka Chulaimbo, kaobedo mapek to nyalo bedo kod kute makelo tuo mar midusi e biechgi. Bedo mar kute makelo tuo mar midusi ebiero ong'e kaka en gino makelo masira mang'eny kuom nyithindo matindo bang' nyuolgi. Waparo ni mine ma bedo gi chandruok mar tuo mar midusi ekinde magipek nyalo kelo kutegi (EBV) mang'eny ne nyithindgi. Thoro mar nonroni en ni mondo wane ka bedo mar kute makelo tuo mar midusi ebiero nyalo miyo nyithindo matindo yudo kute mag EBV chon kapod gitindo kendo kadipo ni ma nyalo bedoni emaomiyo giyudo tuo mar Ningu.

Laktar Rosemary Rochford maoa embalariany mar SUNY Upstate Medical (SUNY Upstate) epiny mar Syracuse, USA kod Laktar Peter Odada Sumba mawuok ekar timo nonro mar ngima dhano epiny Kenya Kenya Medical Research Institute (KEMRI) kwayi ni in kod nyathini kaiyie to udonji enonroni.

Wabiro iri eod ng'iyo mon mapek (Klinik) ka mondo iyie idonji enonroni kapok nyathini onyuol. Kaiyie donjo enonroni to wabiro kwayi mondo ijasi kalatas mabiro nyisowa weche kaluwore kod pek mari, kumaidake kod kinde maiparo ni inyalo nyuole. Ekinde maiduogo eod thieth mondo inyuol to wabiro fuenyi kaka janonro mondo wachulni chudo mag nyuol.

Wabiro kwayo yie mari mondo ibed enonroni. Kendo wabiro kwayo yie mari mondo nyathini obed enonro. Thuolo ibi dwar kuomi mondo in kod nyathini odonji enonroni. Waparo ni warwak mine mapek 400 kod nyithindo matindo 400 enonroni.

Okange mag Nonro

1. Kao gigo maipimo:

Mondo wang'e kaka roteke mag dend nyathini mageng'o tuo mar midusi kod kute mag EBV otegno, nyaka wang'e kadipo ni ne in kod tuo mar midusi ekinde mane ipek kendo kadibed ni biero mari ne nigi kute makelo tuo mar midusi. Wabiro gombo mondo wang'e seche ma nyathini yudoe tuo mar midusi kendo nyadidi mauyudoe tuoni kendo seche maoyudoe kute mag EBV. Wabiro kawo biero eseche mainyuol kendo wabiro ng'iyo kute mag tuo mar midusi eiye. Wabiro kwayi mondo wagol remo kuomi matin madirom kijiko achiel mar chai eseche maiseyueyo bang' nyuolo nyathini, wabiro tiyo kod riwi matin maotud echupa matin mondo wagol remo eler mar kor badi. Wabiro penji penjo matin maluwore kod dak mari kendo kawo nyiriri mar lwasi mar kar dalani. Wabiro kwayo kendo mondo wagol remo matin kuom nyathini eseche maonyuole, mawabiro golo eratudi mar pel kod biero.

Bang' nyuol nyathini, to wabiro miyi odiochieng' maibiro kelo nyathi ekar thieth ka nyathi oromo jumbe 6, 10, 14, kod 18. ekind limbe gi wa biro kwayo remo matin kuom nyathini. Remo matin ibi kaw epony mar tiend nyathini kata lith luete. Wabiro miyi kendo odiochieng' maubiroye kod nyathini ka nyathini ja dweche 6, kendo bang' dweche 3 ka dweche 3 nyaka ochop dweche 24 (Higni 2). Ka nyathini ochopo dweche 6, wabiro kwayo remo mang'eny matin kuom nyathini madirom kijiko 2 mar chae. Remoni ibi kaw eler mani ekor bad nyathini kaitiyogi riyi maotud kod okobo matin mar kawo remo. En gino makare ni kaiyie donjo enonroni to onego bed ni ibiro dak macheigni kuom higni mabiro go.

Wabiro kwayo chak matin mar thuno kod olawo matin mondo wang'e kadipo ni kute mag EBV nie chak kod olawo kendo kadipo ni ma ekama nyathini yudoe kutegi. Chag thuno ibi kaw kanyathi ochopo jumbe 6, 10 kod 14. Wabiro dwaro chag thuno madirom kijiko ariyo mar chai kod olawo madirom kijiko achiel mar chai.

Gigo maipimo duto ibi ter ekar pimo touché mar SUNY Upstate/KEMRI mani Kisumu. Pim maotim ekar pimo tuocheni biro nyisowa kadipo nine in kod tuo mar midusi ekinde mane ipek kendo seche ma nyathini oyudoe kute mag tuo mar midusi kod kute mag EBV kendo kaka dend nyathini kedo kod tuo mar midusi kod kute mag EBV. Wabiro timo pim mabiro nyisowa kaka roteke makedo kod tuo edend nyathini obedo. Wabiro bedoka wang'iyo kaka nyathini dongo ka wapimo ratil kod bor mare esa ka sa mawalime.

2. Nonro mar ng'iyo tudruok ekind nyodo:

Dewaher kendo mondo wati kod bath remb nyathini mawakawuono ekar ng'iyo touché mar SUNY Upstate / KEMRI mondo watim gononro mar ng'iyo kaka nyodo otudore. Nyodo oting'o ng'ieng'ie matindo edel mailuongo ni DNA. DNA en achiel kuom mbui matindo mag remo maochung' ne miyo del kido maopogore opogore kaoa kuom anyuola (kaka kido mar wang') kendo itiyo go eloso gigo magero del. Dewaher mar pimo gigo kaka gino mamiyo ng'ato bedo jamidekre (Sickle cell), kadendi onge gino mailuongo ni G6PD kod ng'iyo tudruok mar reteke mageng'o touché ikind anyuola mailuongo HLA

erembe magi gin gigo makonyo egeng'o tuo mar midusi. Wanyalo bende pimo nyodo mamoko matudo anyuola manyalo bedo kod richo e tuo mar midusi kod kudni mar EBV. Kadipo ni wayudo duoko eod pimo tuoché manyalo bedo kod richo kaluowore kod tuo mar midusi, to wa biro nyisi. Wabiro ng'iyo kendo ng'ieng'ie mag del mag tudruok ekute makelo tuo mar midusi kod kute mag EBV. Mabiro konyowa ng'eyo ka dend nyathini nigi roteke mageng'o kute makelo tuo mar midusi kata kute mag EBV maopogore opogore kod kute mamoko machalre. Nikuop ber pimo lokruok mar kute mar tuo makelo tuo mar midusi kata kudni mar EBV ok oyangore ne in, duoko maowuok enonroni maing'iyo go kaka ng'ieng'ie mag dend kute makelo tuo mar midusi kod kudni mar EBV otudore ok bi miyi.
Be iyie ni rembi kata mar nyathini otigo enonro mar ng'iyo kaka nyodo otudore?
Eee _____Ohoyo_____

3. Kano gigo maokaw maipimo:
Gigo maokaw maipimo ibi kan eod pimo tuoche mar SUNY-KEMRI mani Kisumu kuom ndalo go tee maitimoe nonro kod kinde madwarore mar ng'iyo matuk duoko. Wanyalo kano gigo maokaw mar pim moko kuom higni mathoth mondo otigo etimo nonro mamoko mar tuo mar ningu. Kadipo ni idwaro weyo winjruok mar yie kano gigo maokaw mar pim mari kata mar nyathini mondo kik kan, to yie itudri kod Laktar Peter Odada Sumba, Minaja mar nonroni e kar timo nonro mani KEMRI Kisumu enamba mar simu 254-733-746854/254-720-766550 kendo gigi ibi kethi.

Bende iyie ni gigo ma okaw kuomi kod nyathini okan kendo kanyalore oter e mbalariany mar SUNY Upstate e piny Amerka mondo otimgo nonro mamoko.

Eee _____ Ohoyo_____

<u>Richo:</u>
Nitie richo manok mabiro wuok kuom kawo remo ebiero kata ekor badi bang' nyuol. Bende nitie richo manok kuom kawo chag thuno kata olawo. Richo manok nyalo mana wuok kaokaw remo kuom nyathini. Richo mar kawo remo oting'o gigo kaka chuer matin ewang' kama ogole, Rem matin, gwarruok, kod manok ahinya en adhola. Magi duto ok hiny timore ahinya katakamano to nyalo timore nejimanok. Katakamano richo pok one enonro moko mawasebedo kawatimo.

Duoko penjo eseche ma waruaki enonro ok bi bedo kod richo moro amora kuomi kata kuom nyathini.

<u>Ber:</u>
Wabiro pimo kute makelo tuo mar midusi e biechi, maok osebedo katimore kendo en ber. En gino maber mokalo mondo ing'e kain kod tuo mar midusi ebiechi kata ohoyo nikech mabiro konyowa ng'eyo kaka roteke mag dend nyathini mageng'o tuo mar midusi kod kute mar midusi biro bedo bang'e. Ber kendo mabiro konyo nyathini, en ni eseche ma inene ekar thieth chieng' mawachike mondo wa wapime, kanyathini oyudni

tou to wabiro thietho nyathini kuom del maohore, tuo mar midusi, diep, kod remo manok kaluwore kod chike mag thieth mag migawo mar thieth epiny Kenya. Weche maoha enonroni biro konyo e loso yore mag geng'o tuo mar ningu endalo mabiro.

Yore mamoko:
Ok ochuno ni nyaka ibedi enonoroni eka iyud thieth mari kod mar nyathini. Kaiyiero ni ok ibed enonroni to pod ibiro yudo rit, kony kod thieth makare ekar thieth kaluwore kod chike mag migawo mar thieth epiny Kenya, magi oting'o pimo kod thieth manono etuo mar midusi.

Bedo enonro maonge achuno moro amora:
Bedoni enonroni en kuom hero mari kendo onge achuno moro amora. Tamruok mari bedo enonroni ok bi loko kit ngimani mapile kendo ok bi miyi kum moro amora kendo ok bi moni yudo ber moro amora. Kaiyiero bedo enonroni, to in kata nyathini nyalo wuok esa asaya.

Chudo:
Wabiro chulo chudo mar nyuol ekar thieth kaibedo enonroni. Wa biro chulo chudo mar ng'ango kod mar nyuol. Onge chudo moro amora maopogore maibi miyi kata nyathini kuom bedo enonroni.

Ekinde main enonroni, in kod nyathini biro yudo thieth manono kod arita kaoa kuom laktar mar nonro kuom ndalo duto main enonro. Kaiwuok enonro kapok inyuol, to nonro ok bi chulo chudo moro amora mar nyuol. Katakamano ibiro yudo thieth kod arita makare eod thieth to chudo biro bedo ewiyi. Kaigolo nyathini enonro bang' nyuol, ok ibi yudo thieth manono kaoa enonro katakamano pod inyalo yudo arita kod thieth ekar thieth kaka pile kaichiwo kod migawo maochung' ne thieth epiny Kenya. Kaigolo nyathini enonro esa asaya bang' pim maokuongo edala, nyathini pod biro yudo thieth kaoa enonro nyaka ekinde nonro duto.

Onge chudo moro main kata nyathini biro yudo kuom bedo enonroni.

Penjo
Kain kod penjo moro amora kaluwore kod nonroni to inyalo tudori Laktar Peter Odada Sumba, maen mineja mar nonroni, kaen ekar timo nonro mar Center for Global Health Research, Migawo mar timo nonro etuoche dhano epiny Kenya (KEMRI), esanduku mar barua PO Box 1578, Kisumu enamba mar simu 254-733-746854 / 254-720-766550 kata jatelo maduong' ekambi mar timo nonrono CGHR, KEMRI mani Kisumu enamba mar simu 254-57-2022924. Penjo kaluwore kod ratiro mar janonro nyalo yudore e migawo mar timo nonro etuoche dhano epiny Kenya KEMRI/ duol maochung' ne ng'iyo ratiro mar dhano enonro ERC, esanduku mar baruwa PO Box 54840, Nairobi kata enamba mar simu 254- 2-02722541 kata jatelo maduong' e migawo mar timo nonro epiny Kenya, esanduku mar baruwa PO Box 54840, Nairobi kata namba mar simu 254-2-02722541.

Hinyruok moro amora:

Kadipo ni tuo kata hinyruok moro amora owuok kaluwore kod bedoni enonro, to yie itudri kod Jatelo mar kambi mar timo nonro e Center for Global Health and Research (CGHR) KEMRI maniKisumu esanduku mar barua PO Box 1578, Kisumu 40100. Enamba mar simu: 254-57-2022924 kata Laktar Peter Odada Sumba enamba mar simu 254-57-2022989/ 254-733746854/254-720-766550

Mbalariany mar SUNY Upstate Medical onge chenro mar miyi omuom moro amora kahinyori. Oki ibi ketho ratiro mar chike mari kaigoyo sei ekalatas yieni.

Maling'ling' mar weche kod yie mar tiyogo/riwo weche mag ngima enonro:

Kaiyie donjo enonroni kendo kayiene nyathini donjo enonroni, to wechen ni kata weche nyathini maluwore kod nyima ibi Kan maling'ling'. Wabiro keto namba koum gigo duto maokaw mar pimo kuomi kod kuom nyathini. Nyingi ok bi ndiki egigo maokaw mag pimgo. Mawatimo mondo warit weche mag ngimani kata mar nyathini. Katakamano nitie ji manok mabiro bedo kod nying kod namba mari kata mar nyathini. Magi biro bedo ji kaka Laktar Rochford, Laktar Odada Sumba kod jogo makonyo enonroni. Kadipo ni pimo mamoko nyalo dwaro timore epinje ulaya to gigo maokaw mar pimgo ibi ketie namba kende kendo ok bi ketie nying. Kapo ninitie andiko moro amora madindiki kaluwore kod nonroni, to nyingi ok bi yangi. Weche duto maochoki kuomi ibikan e sanduku maolor motegno ekar timo nonro mar CGHR, KEMRI – Kisian maji manok enonro kende emanyalo yawo. Mawatimo kama mondo wabed gi adiera mar rito maling'ling' mari kata mar nyathini kuom weche maluwore kod thieth kata ngimau.

Yie mar bedo achiel kuom janonro:

Weche duto kaluwore kod nonroni ese lera makare.

Goyo seyi ekalatas yieni nyiso ni oselera malong'o ewi nonroni kendo ayie ni an kata nyathina bedo achiel kuom janonroni maonge achune moro amora. Ase penjo penjo duto kaluwore kod nonroni kendo weche maomiya omiya thuolo mar yiero makare ni an kata nyathina bedo achiel kuom janonroni. Goyo seyi ekalatas yieni ok witi ratiro mar chike na kendo jotend nonroni ok okonyore ne wach moro amora. Anyalo weyo bedo enonroni sa asaya. Oboke achiel ibi miya.

Seyi mar Janyuol _____ Tarik_____

Seyi mar ng'ama kwayo yie
Yie/Chiwo yie_____ Tarik_____

Seyi mar janeno _____ Tarik_____

Appendix 8: Abstracts of Published Manuscripts

Matern Child Health J
DOI 10.1007/s10995-014-1546-4

Plasmodium falciparum Infection is Associated with Epstein–Barr Virus Reactivation in Pregnant Women Living in Malaria Holoendemic Area of Western Kenya

Ibrahim I. Daud · Sidney Ogolla · Asito S. Amolo · Eunice Namuyenga · Kenneth Simbiri · Elizabeth A. Bukusi · Zipporah W. Ng'ang'a · Robert Ploutz-Snyder · Peter O. Sumba · Arlene Dent · Rosemary Rochford

Abstract The role of *Plasmodium falciparum* malaria in Epstein–Barr virus (EBV) transmission among infants early in life remain elusive. We hypothesized that infection with malaria during pregnancy could cause EBV reactivation leading to high EBV load in circulation, which could subsequently enhance early age of EBV infection. Pregnant women in Kisumu, where *P. falciparum* malaria is holoendemic, were actively followed monthly through antenatal visits (up to 4 per mother) and delivery. Using real-time quantitative (Q)-PCR, we quantified and compared EBV and *P. falciparum* DNA levels in the blood of pregnant women with and without *P. falciparum* malaria. Pregnant women that had malaria detected during pregnancy were more likely to have detectable EBV DNA than pregnant women who had no evidence of malaria infection during pregnancy (64 vs. 36 %, $p = 0.01$). EBV load as analyzed by quantifying area under the longitudinal observation curve (AUC) was significantly higher in pregnant women with *P. falciparum* malaria than in women without evidence of malaria infection ($p = 0.01$) regardless of gestational age of pregnancy. Increase in malaria load correlated with increase in EBV load ($p < 0.0001$). EBV load was higher in third trimester ($p = 0.04$) than first and second trimester of pregnancy independent of known infections. Significantly higher frequency and elevated EBV loads were found in pregnant women with malaria than in women without evidence of *P. falciparum* infection during pregnancy. The loss of control of EBV latency following *P. falciparum* infection during pregnancy and subsequent increase in EBV load in circulation could contribute to enhanced shedding of EBV in maternal saliva and breast milk postpartum, but further studies are needed.

Keywords EBV load · Mother · Pregnancy · *Plasmodium falciparum* malaria

I. I. Daud · S. Ogolla · E. Namuyenga · P. O. Sumba
Center for Global Health Research, Kenya Medical Research Institute, Kisumu, Kenya

I. I. Daud · Z. W. Ng'ang'a
College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

S. Ogolla · E. Namuyenga
Maseno University, Kisumu, Kenya

A. S. Amolo
School of Physical and Biological Sciences, Jaramogi Oginga Odinga University of Science and Technology, Bondo, Kenya

K. Simbiri · R. Rochford (✉)
Department of Microbiology and Immunology, SUNY Upstate Medical University, 750 East Adams St., Syracuse, NY 13210, USA
e-mail: rochforr@upstate.edu

E. A. Bukusi
Center for Microbiology Research, Kenya Medical Research Institute, Nairobi, Kenya

R. Ploutz-Snyder
Universities Space Research Association, Houston, TX, USA

A. Dent
Center for Global Health and Diseases, Case Western Reserve University, Cleveland, OH, USA

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Improved Pregnancy Outcomes in a Prospective Study of Pregnant Women Enrolling in an Antenatal Clinic in Western Kenya

Ibrahim I. Daud[1,2], Fredrick O. Opinya[1], David Midem[1], Moses Kigani[3], Elizabeth A. Bukusi[4], Zipporah Ng'ang'a[2], Peter O. Sumba[1], Arlene Dent[5], Rosemary Rochford[6*]

[1]Center for Global Health Research, Kenya Medical Research Institute, Kisumu, Kenya
[2]College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
[3]Chulaimbo Sub-District Hospital, Kisumu, Kenya
[4]Center for Microbiology Research, Kenya Medical Research Institute, Nairobi, Kenya
[5]Center for Global Health and Diseases, Case Western Reserve University, Cleveland, OH, USA
[6]Department of Microbiology and Immunology, SUNY Upstate Medical University, Syracuse, NY, USA
Email: *rochforr@upstate.edu

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Abstract

In areas of sub-Saharan Africa where malaria is endemic, pregnant women are at a greater risk of malaria than non-pregnant women leading to significant adverse consequences including anemia, intrauterine growth retardation, low birth weight (LBW), and pre-term delivery. The Kenya Ministry of Health adopted Intermittent Preventive Treatment (IPT) and use of insecticide-treated nets (ITN) as a National strategy for malaria prevention in pregnancy. In this report, we evaluated the prevalence of malaria, the anthropometric measures of birth outcomes and the reasons for loss to follow up among pregnant women participating in an ongoing cohort study in Western Kenya. A total of 175 HIV-negative pregnant women enrolled at antenatal clinic of Chulaimbo sub-District hospital were longitudinally evaluated in a monthly follow-up visits through antenatal visits (up to 4 per mother) and delivery. Thirty three percent and 15% of the pregnant women were malaria positive by real-time quantitative (Q)-PCR and microscopy respectively at enrolment, while 54% and 23% of the pregnant women had malaria by Q-PCR and microscopy respectively at any time during follow-up. Of the enrolled study participants, 65% delivered at Chulaimbo hospital. Overall, 39% (69) of the pregnant women were lost to follow-up. The major reasons for loss to follow up were relocation from the study area (26%) and delivery at alternative health

*Corresponding author.

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