EVALUATION OF EPSTEIN BARR VIRAL LOAD AND ANTIBODIES IN CHILDREN LIVING IN THE MALARIA HOLOENDEMIC LAKE VICTORIA BASIN

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July 2010.
DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other university or for any other award

Signature

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We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

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DEDICATION

To my loving, Dad Hazael Otieno, Mother Margret Otieno, fiancée Dorcas, brothers John, Walter and Gilbert, Sister Diana, sisters in-law Mary, Maureen Ochieng, Maureen Otieno and Baby Dave and Gloria.
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ABBREVIATIONS AND ACRONYMS

BL: Burkitt’s lymphoma
CFS: Chronic Fatigue Syndrome
DNA: Deoxyribonucleic acid
dNTPs: Deoxynucleotide triphosphates
EBV: Epstein Barr Virus.
eBL: Endemic Burkitt’s lymphoma
EBNA: Epstein Barr Virus nuclear antigen.
EDTA: Ethylenediaminetetraacetic Acid.
IgG,M and H: immunoglobulin G, M and H
IM: infectious mononucleosis
KEMRI: Kenya Medical Research Institute
KCl: Potassium chloride.
MgCl₂: Magnesium chloride.
mRNA: messenger ribonucleic acid
PCR: Polymerase chain reaction.
Poly A site: Polyadenylation site
q-PCR: Real time PCR
SMA: severe malaria anemia
TRAFs: Tumor Receptor Associated Factors
Tris-HCl: Tris (Hydroxymethyl) amino methane hydrochloric acid
UTR: Untranslated region
UM: uncomplicated malaria
VCA: viral caspid antigen
NK – T cells Natural killer cells
PTLD- Post transplant Lymphoproliferative Disorder
NPC- nasopharyngeal carcinoma
HLA- human leukocyte antigen
LCLs- lymphoblastoid cell lines
LMP- latent membrane protein
BCRs- B- cell receptors
RBC- red blood cell
WBC- white blood cells
PfEMP- Plasmodium falciparum membrane protein
bHLH- basic helix loop helix
TPA- tumor promoting antigen
TNF- tumor necrosis factor
Epstein Barr virus (EBV) and *P. falciparum* malaria have an overlapping distribution and are incriminated as the primary causal agents for endemic Burkitt’s lymphoma. Nevertheless, the exact mechanism utilized by these two pathogens to cause endemic Burkitt’s lymphoma has remained elusive. This study therefore sought to provide insights as to whether presence of Epstein Barr Virus in children who develop severe malaria reveals unique serological/nucleic acid signatures that would allow future development of predictive markers for evolution of Burkitt’s lymphoma. In this study we determined the viral loads as well as the serological signatures to various Epstein Barr virus antigens in children with complicated and uncomplicated malaria. Children with severe malarial anemia were age and sex matched to those with uncomplicated malaria. Epstein Barr virus -DNA viral load was quantified by real time quantitative PCR and viral load extrapolated from a standard graph using an Epstein Barr virus sample of known copy numbers. IgG and IgM antibody levels to viral capsid antigen and Epstein Barr virus nuclear antigen were determined by enzyme linked immuno-assay. B-cell numbers that are the target for Epstein Barr virus was enumerated by flow cytometry. Of the 94 subjects analyzed, 53% (N=25 in each arm) had detectable EBV load. The geometric mean viral copy number/mL was higher in children with severe malaria (27,556± 25,377 SE) compared to those with mild malaria (21,703±17,434 SE). The mean B-cell numbers were higher in cases (2307± 551 SE) than controls (1273± 426 SE). The mean viral capsid antigen IgG titers were significantly higher in the cases (0.97± 0.05 SE) compared to controls (0.79± 0.06 SE ± P<0.04). There was no difference in the titers of the other antibodies analyzed. Findings of this study are consistent with early childhood exposure to Epstein Barr virus. The higher viral load and viral capsid antigen IgG antibody in children with severe malaria is attributed to the expansion of infected B-lymphocytes, probably fueled by malaria antigenic stimuli.
CHAPTER ONE
1. INTRODUCTION AND LITRETURE REVIEW

1.1 Background

Epstein-Barr virus (EBV) was discovered about three decades ago by Epstein, Achong, and Barr in cells cultured from Burkitt’s lymphoma tissues (Epstein et al., 1964). Four years later, EBV was shown to be the etiological agent for infectious mononucleosis (IM) (Henle et al., 1970). EBV DNA was subsequently detected in tissues from patients with nasopharyngeal carcinoma (Zur et al., 1970). In the 1980s, EBV was also found to be associated with non-Hodgkin’s lymphoma and oral hairy leukoplakia in patients with acquired immunodeficiency syndrome, AIDS (Zeigler et al., 1982). Since then, EBV DNA has been found in tissues from other cancers, including T cell lymphomas and Hodgkin’s disease.

EBV infections and those of malaria are recognized as major cofactors in the genesis of African endemic Burkitt’s lymphoma (eBL), the most common pediatric cancer that accounts for 74% of childhood malignances in equatorial Africa (Burkitt, 1977). Burkitt’s lymphoma (BL) is a high grade Non-Hodgkin lymphoma comprising small, noncleaved B-cells that occurs sporadically worldwide, but has also been shown to be endemic in Papua New Guinea and in the lymphoma belt of Africa (Bishop et al., 2000; Ranney, 2007). This belt stretches from about 10° north and 10° south of the equator (Fig 1 Panel A). Within these latitudes, the lymphoma is found in regions where the mean minimum temperature exceeds 15.5 °C and the rainfall is higher than 50 mm per year (Haddow, 1963). It is more common in damp humid regions and is absent in arid areas.
The incidence rates of BL vary from being as low as 1 or none per 100,000 children in industrialized countries such as United States of America and in Western Europe (Blum et al., 2004), through intermediate rates in countries such as Algeria, to being as high as (4-10 per 100,000) in children aged younger than 15 years and who live in the so called lymphoma belt (Figure 1 Panel A) It is within some parts this belt that malaria has been seen to be endemic with more than 100 people per km² at risk of malaria infection (Figure 1 Panel B)

![Figure 1. Map of Africa showing “The lymphoma belt” (panel A) and malaria distribution together with the population at risk (panel B) (Van Den Bosch et al., 1993).](image)

With regard to EBV, African children are infected early in life and nearly all will have sero-converted by three years of age (Raneyy et al., 2007). In Africa, there is an uneven geographical distribution of eBL, with a strong correlation between residence in areas of intense, perennial malaria transmission and the incidence of eBL (Raneyy et al., 2007), suggesting that malaria may be a risk factor in the genesis of the lymphoma.
It has therefore been hypothesized that early EBV infections in equatorial Africa, coupled with holoendemic malaria causes temporary immune-suppression and polyclonal expansion of B cells, that leads to an increase in the number of EBV infected cell clones that have potential to develop into EBV associated eBL (Magrath et al., 1992).

Following primary infections, EBV establishes a life-long persistent infection usually without adverse health consequences. This relies on a balance of viral latency, viral replication and host immune system (Goldstein and Bernstein, 1990). Latent infection occurs in resting memory B lymphocytes (Laichalk et al., 2002) and is accompanied by low level chronic lytic reactivation in the oral cavity of healthier carriers (Niederman et al., 1976 ; Yao et al., 1985), where the virus is constantly shed at low levels in saliva. Persistent infection is probably due to continual re-infection of B-lymphocytes in the oral lymphoid tissues, which contribute to viral shedding (Sitki-Green et al., 2003).

1.2 Etiology of Malaria

Malaria is caused by infection with any of the four species of malaria parasites that infect humans: *Plasmodium falciparum, P. malariae, P. ovale* and *P. vivax*. Most illness and death in sub-Saharan Africa are however caused almost solely by *P. falciparum* (Good et al., 2002) that causes life threatening complications that are the main cause of over 1-3 million deaths annually mainly in children under the age of 5 years (Snow et al., 1999). The disease results from complex interactions between the human host, the mosquito vector and the parasite.
Transmission of the malaria parasites is by the bite of female mosquitoes of the genus *Anopheles*, of which the anthropophagic *Anopheles gambiae* complex is the most efficient vector in Africa (Nchinda, 2000). Sporozoites in the salivary glands are inoculated into the host and quickly enter the liver cells where they reside and multiply for 10-14 days without causing disease. Thereafter, merozoites are released and invade the red blood cells (RBC’s). The invasion of RBCs marks the beginning of the pathogenic erythrocytic stage of the life cycle. After about 30-40 hours growth in the RBC, the trophozoites divide and grow to form schizonts that contain 8-16 merozoites which are released when the RBC ruptures. It is the synchronous rupture of RBCs which gives the periodic fever, and in particular it is thought that the haemozoin or malarial pigment which is released when the RBC's rupture is responsible for the fever. For the first few cycles the fever can be irregular, then the parasites synchronise their cycles.

Apart from release of hemozoin, another phenomenon that is associated with disease involves rosetting where infected RBC adheres to non-infected RBC, (Rowe *et al.*, 1997), thus clogging small capillaries and causing cytoadherence. The latter is said to be mediated by *P. falciparum* erythrocytic membrane protein (PfEMP), located on the membranes of parasitized erythrocytes (Howard *et al.*, 1990).

Malaria may manifest itself as either mild or severe disease. In cases of mild malaria, the patients have few symptoms such as fever, chills, and headache but in acute malaria, the common clinical symptoms are progressive anemia, hypoglycemia, multi-organ dysfunction failure and unconsciousness (Marsh *et al.*, 1996). In malaria holoendemic
areas, partial protective immunity is acquired during the first 5-10 years of life such that most of the severe disease is found in younger children (Marsh et al., 1999). Severe malaria anemia is a major health problem in endemic areas, morbidity and mortality is highest in children less than 5 years old as well as in pregnant women. The pathogenesis of malaria anemia is not well understood, but studies have shown that it is caused by excess removal of non-parasitized RBC, immune destruction of parasitized RBC’s and impaired erythropoiesis (Hakan, 2003).

1.3 Epstein Barr Virus (EBV)

1.3.1 EBV epidemiology

EBV has co-evolved with human beings over millions of years and during this long association, the virus has become well adapted to its human host, thereby becoming one of the most astute virus in the herpes family. More than 90% of adults worldwide are infected with EBV (Henle et al., 1969) and carry the virus as a life long persistent infection (Babcock et al., 1998). In most cases primary infections occurs sub-clinically during childhood when EBV is horizontally transmitted via salivary contact among family members (Gratama et al., 1990). Epidemiological studies in the 1970s showed that primary infections occurred earlier in life in non-industrialized countries and in low socio-economic groups (Henle et al., 1969 ; Biggar et al., 1978) compared to affluent societies where sero-conversion appeared to be delayed until adolescence, presenting as infectious mononucleosis (IM). Many studies have shown between 50% and 74% adolescence infections (Henle et al., 1971). It is commonly assumed that those who remain uninfected throughout childhood generally become infected at adolescence.
through kissing. Consequently, IM is termed the kissing disease. However there are single reports of EBV detection in male and female genital secretions, suggesting the possibility of sexual transmission.

1.3.2 EBV entry into B—cell, latency and transformation

EBV preferentially infects B lymphocytes through binding of major viral envelope glycoprotein gp350 to the CD21 receptor found on the surface of the B-cells (Nemerow et al., 1987). A second binding site has also been described that involves glycoprotein gp42 to human HLA antigen (HLA class II as a co-receptor (Borza and Hutt, 2002). EBV is transmitted in saliva and infects B cells that are located in the oral-pharyngeal epithelium (Miller, 1990). It then travels to the mucosal lymphoid tissues, and the latent infection of naïve B cells by EBV, and subsequent entry to germinal centers is required to seed the memory B cell compartment (Babcock et al., 2000; Joseph et al., 2000). It however remains unclear as to how EBV manipulates the human B-cell compartment to achieve persistence in memory B cells (Babcock et al., 1998).

The EBV genome consists of a linear DNA molecule that encodes nearly 100 viral proteins (Kieff, 2006). During viral replication, these proteins play an important role for regulation of the expression of viral genes, replicating viral DNA, formation of structural components of the virion, and modulation of the host immune response.

Infection of epithelial cells by EBV in vitro results in active replication, with production of virus and lysis of the cell.
EBV can express four different phases of gene usage depending on the location and the differentiation state of the infected B cell (Babcock et al., 1998; Laichalk and Thorley, 2005). The lytic phase results in infectious virus particle production. The remaining three phases are all associated with latent infection, in which no infectious viral particles are produced, and occur as follows: 1) The growth phase, in which all eight known latent proteins are expressed and stimulate proliferation of the infected host cell, 2) The default phase, in which a restricted set of three latent proteins are expressed and help infected B cells survive the germinal center reaction by mimicking B-cell receptor signaling and T-cell help and, 3) The latency phase, in which the virus persists in memory B cells without EBV protein expression (Babcock et al., 1998; Laichalk and Thorley, 2005). All these phases confer resistance of infected B-cell to apoptosis induction thereby increasing the number of expressed latent EBV antigens (Kelly et al., 2006).

EBV has a unique ability to transform resting B-cells into permanent, latently infected lymphoblastoid cell lines (LCLs) in in vitro system. In EBV transformed LCLs, every cell carries copies of the viral episome and constitutively expresses a limited set of viral gene products, the so called latent proteins (LP), which comprise six EBV antigens: EBNA 1, 2, 3A, 3B, 3C and LP and 3 latent membrane proteins (LMP) 1, 2A and 2B. In addition to the latent proteins, LCLs also show expression of small non-polyadenylated (non-coding) RNAs, EBER 1 and 2; whose functions are not fully understood (Kleiff and Rickinson, 2001). This pattern of latent EBV gene expression, which appears to be activated only in B-cells infections, is referred to as latency III.
B cell activation markers CD23, CD30, CD39, and CD70 are highly expressed in LCLS. These markers are usually absent, or expressed at low levels in resting B-cells. Nevertheless, they are transiently induced at high levels when cells are activated by antigenic or mitogenic stimulation, indicating that EBV-induced immortalization can be elicited through the constitutive activation of the same cellular pathways that drive physiological B-cell proliferation. The ability of EBNA 2, EBNA 3C, and LMP1 to induce LCL-like phenotypic changes when expressed individually in human B-cell lines indicate that these viral proteins are key effectors of the immortalization process. (Wang et al., 1995)

1.3.3 EBV–encoded nuclear antigens.

EBV infected cells have a tendency to express a group of nuclear proteins that influence both viral and cellular transcriptions. EBNA 1 is expressed in all virus infected cells. Its role is primarily to aid in maintenances and replication of the episomal EBV genome which is achieved through sequence specific binding to the plasmid origin of replication OriP (kleiff and Rickinson, 2001). EBNA 1 also interacts with certain viral promoters thereby contributing to transcriptional regulation of EBNAs (including EBNA 1 itself) and of LMP 1. EBNA 1 is separated into amino- and carboxy terminal domains by a Gly-Ala repeat sequence, the main function of which seems to stabilize the mature protein preventing its proteosomal break down (Levitskaya et al., 1995). Rather than functioning in its original form, it’s suggested to be an immune evasion domain (Voo et al., 2004).
Gene knock down studies indicates that EBNA 1 does not have a crucial function in vitro B-cell transformation beyond the maintenance of viral genome (Humme et al., 2003). A more direct involvement in onco-genesis is indicated by the ability of B-cell directed EBNA 1 expression to produce B-cell lymphoma in transgenic mice (Wilson et al., 1996) and by its possible contribution to the burkitt’s lymphoma cells in vitro. Among more than 80 lytic and 8 latent EBV gene products, the latency associated EBNA 1 is the only protein consistently expressed in infected proliferating memory B cells in healthy virus carriers (Hochberg et al., 2005). During homeostatic B-cell division, EBNA1 initiates viral replication by binding to the EBV circular DNA or episome with its C-terminal domain, and cross-links the episome to mitotic chromosomes as a protein anchor, thereby accomplishing the transmission of the episome into progeny cells (Humme et al., 2003). Persistent infections are characterized by stable numbers of latently infected B cells in the blood (0.5 to 50 per million) and the steady shedding of infectious virus into saliva (Khan et al., 1996). EBNA1, the crucial EBV antigen for viral persistence, constitutes a dominant antigen for both humoral and cell mediated immune responses to the virus.

Consequently, T cells specific for EBNA1 are considered important components of EBV-specific immune control. EBNA1 represents a key target antigen for CD4 T cell-mediated immune control mechanisms of EBV infection in healthy individuals. EBV also expresses EBNA-LP and EBNA 2, EBNA 2 interacts with sequence specific DNA binding proteins to transcriptionally activate cellular genes such as CD23 and the key viral genes LMP1 and LMP2A (Kleiff and Rickinson, 2001).
EBNA-LP interacts with EBNA 2 and is required for the efficient out growth of virus transformed B cell \textit{in vitro} (Mannic \textit{et al}., 1991). The roles of EBNA 3A and EBNA 3C in B cell transformation are not clearly understood all though they are expressed in EBV transformed cells.

\textbf{1.3.4 EBV encoded latent membrane proteins (LMP1)}

The main transforming protein of EBV is LMP1; it has pleiotropic effects when it is expressed in cells, resulting in the induction of cell surface adhesion molecules and activation antigens (Wang \textit{et al}., 1990) and up regulation of anti-apoptotic proteins (Eliopoulous, 1997). LMP1 functions as a constitutively activated member of tumor necrosis factor receptor (TNFR) super family and activates several signaling pathways in a ligand-independent manner (Kilger \textit{et al}., 1998). Functionally LMP1 resembles CD40, another member of TNFR super family, and can be partially substituted for CD40 \textit{in vivo} providing both growth and differentiation signals to B-cells (Uchida \textit{et al}., 1999). LMP1 proteins activates several down stream signaling pathways that contribute to many phenotypic consequences of LMP 1 expression including the induction of various genes that encode anti-apoptotic proteins and cytokines (Eliopoulos, 2000).

The LMP2 proteins, LMP2A and LMP2B are essential for EBV induced B cell transformation \textit{in vivo} (Babcock \textit{et al}., 1998).
However, expression of LMP 2A in B-cell in transgenic mice abrogates normal B-cell development allowing immunoglobulin negative cells to colonize peripheral lymphoid organs, indicating that LMP 2A can drive the proliferation and survival of B-cell in the absence of signaling, through the B-cell receptor (BCR). LMP 2A are also known to transform epithelial cells and enhance their adhesion and motility. Repressive effects of LMP 2A expression have recently been reported in human and murine B cells and many of these target B cell specific factors, resulting in a phenotype that is similar to those of malignant Hodgkin cells in hodgkins lymphoma and germinal center B cells (Portis and Longnecker, 2003). In addition to these effects LMP 2A has also been found to induce expression of a range of genes that are involved in the cell-cycle induction, inhibition of apoptosis and suppression of cell mediated immunity.

1.3.5 EBV-specific immunity during malaria

The imbalance in the immune responses produced by malaria infection directly impacts on EBV specific immune responses. Adults living in malaria holoendemic regions show impaired EBV specific T cell responses (Moss et al., 1983). Peripheral blood lymphocytes from adults and children with acute malaria are unable to control the \textit{in vitro} outgrowth of EBV transformed cells in a colony regression assay (Whittle et al., 1984). This is thought to reflect the underlying loss of T cell dependent IFN-\(\gamma\) responses against EBV. As a consequence, the number of B lymphocytes latently infected with EBV increases, while the ability of T cells to suppress the out-growth of EBV infected lymphoblastoid cells becomes impaired (Moss et al., 1983).
The mechanism by which recurrent malaria infection impairs EBV immunity is not clear. A new hypothesis has thus emerged proposing that the frequency of infection can have a greater effect on immune function, i.e. the greater the number of antigenically heterologous infection, the greater the decrease in overall memory T cell activity (Liu et al., 2003). The model was extended when it was shown, in a mouse model, that an acute and persistent infection can induce loss of memory T-cell function to previously encountered non cross-reactive viruses (Kim and Welsh, 2004). Even though this model focuses on heterologous viral infections, it is intriguing to speculate on the role of repeated malaria infection on the reduction of T cell responses against EBV in children living in malaria holoendemic areas.

As previously mentioned, IFN-γ induced CTL responses versus EBV are thought to be crucial for immune surveillance. EBV specific CD8+ T cells recognize most of viral antigens, although probably not the EBNA 1 antigen as a result of viral immune escape mechanism. EBNA 1 is the only antigen expressed by eBL and dividing memory B cells (Leviskaya et al., 1995) that may have an immunological impact during malaria infection. Moreover BL cells have abroad range of intrinsic defects in class 1 antigen processing pathway (Rowe et al., 1995), which appears to indicate that the EBV specific CTL surveillance is likely to be inefficient. *P. falciparum* malaria severely damages CD4+ T cells responses, which possibly contributes to the loss of CTL control of EBV infections on B cells (Whittle et al., 1984).
Furthermore, high levels of IL-10 during malaria infection have an inhibitory effect on T-cell mediated immune control of B cell transformation by EBV. IL 10 also induces the proliferation of EBV infected B-lymphocytes (Bejarano and Masucci, 1998). Activation of T cell, as well as IFN-γ production during early phase of EBV infection is crucial in determining the outcome of out-growth inhibition (Bejarano and Masucci, 1998). IL-10 can function to: (i) inhibit the production of IFN γ and (ii) induce antigen specific unresponsiveness (Groux et al., 1996). It can also be assumed that the reported hindrances of dendritic cell (DC) maturation by infected erythrocytes would have an impact on the generation and maintenance of EBV-specific responses (Urban et al., 1999).

1.3.6 Malaria and EBV interaction on B-cell

*P. falciparum* is recognized as one of the major cofactors that lead to the genesis of eBL, although data is limited on the mechanisms that underlie this phenomenon. The transmission dynamics of *P. falciparum* affect both the frequency of the infection and the severity of the disease. For example, in regions of equatorial Africa where malaria is holoendemic, there is a stable transmission of *P. falciparum* that is characterized by recurrent exposure and repeated malaria infection throughout the year (Mbogo et al., 1997; Snow et al., 1997).

Parasite prevalence can be as high as 90% in children under 5 years of age. Moreover, the highest parasite loads are found among children 6-11 months of age, which is also the age that primary EBV infection is most likely to occur.
Unlike many infectious diseases, malaria only elicits protective immunity in children after several years of cumulative exposure and therefore exposure to holoendemic malaria is characterized by recurrent (perhaps chronic) infections, and is likely that during this period the child’s immune system is under constant stress from repeated infection and high parasite burden. It is during this season that the immune system becomes stressed and this is when EBV infection sets in leading to lytic proliferative stage where most B cells may become infected prior to viral progression to latency stage.

Studies have also shown that *P. falciparum* induces hypergammaglobulinaemia which is indicative that the parasite acts as a B cell mitogen (Greenwood, 1994). Cultured *P. falciparum* have been found to potentiate B cell proliferation and antibody production *ex vivo* (Kataaha *et al.*, 1984; Greenwood, 1994). However, the exact mechanism for this polyclonal B cell activation still remains elusive. *P. falciparum*-infected erythrocytes have been shown to induce both proliferation, and immunoglobulin M (IgM) secretion by IgM-positive B cells via *falciparum* erythrocyte membrane protein 1 (PfEMP1) (Donati *et al.*, 2004). *P.falciparum* also expresses a Toll-like receptor 9 (TLR9) ligand during erythrocytic schizogony (Pichyangkul *et al.*, 2004). The ability of B cell to respond to pattern recognition stimuli such as bacterial derived motifs through TLRs have also been shown in the past.

When B cells are separated into the naive (CD19+IgD+) and memory (CD19+CD27+) compartments, several differences emerge in their response to stimuli through the TLRs. Naive B cells require B-cell receptor engagement before TLR9 expression is induced,
whereas memory B cells constitutively express TLR9 (Bernsconi et al., 2004). Moreover, it has been found that memory B cells rapidly proliferate in response to stimulation with CpG, a TLR9 ligand. Following expansion, memory B cells can differentiate into antibody-secreting plasma cells. Consequently, *P. falciparum* schizonts and infected erythrocytes have the potential to directly interact with B cells and thereby induce proliferation and differentiation into antibody-secreting cells. Engaging TLR9 might stimulate the proliferation of the latently infected memory B cells, thereby expanding this pool and increasing the risk of emergence of a malignant B-cell clone (Rochford et al., 2005). The capacity of latently infected memory B cells to undergo terminal differentiation in response to TLR9 engagement could result in a switch from latent to lytic viral replication and the release of infectious virus, which could infect new populations of B cells, thus further increasing the number of latently infected B cells. Although it seems likely that *P. falciparum* may stimulate EBV latently infected memory B cells via TLR9 engagement, the impact on naive B cells is less apparent. In addition, interleukin-10 (IL-10), the expression of which is increased after infection with *P. falciparum* induces the expression of TLR9 in naïve B cells (Whittle et al., 1990).

The potential therefore exists for *P. falciparum* to interact with both naive and memory B cells, both of which can be latently infected with EBV. Kataaha et al., 1984 also showed that addition of *P. falciparum* extracts to peripheral blood lymphocytes *ex vivo* increased the rate of EBV cell transformation. The number of EBV-infected B cells was also shown to increase in children during an episode of acute *P. falciparum* malaria (Lam et al., 1991). EBV elevated viral load has also been found in symptomatic children 1-4 years of
age living in malaria holoendemic regions which indicate that even in a symptomatic children, recurrent malaria infection increases the number of EBV infected cells in peripheral blood.

### 1.4 Malignancies associated with EBV infections

EBV has been shown to be notorious in its association with cancers and auto immune diseases (Casseta and Grannieri, 2000) and the first such association was with eBL. But EBV is now linked with several other malignant tumors of lymphoid (BL, Hodgkin’s disease, Nasal NK-T cell lymphoma), or epithelial origin (nasopharyngeal carcinoma (NPC), leiomyosarcoma in immuno-suppressed patients (Rickinson and Kieff, 2001). The precise role of EBV in the pathogenesis of the tumor is unknown, but clinical studies confirm that the virus is intrinsically linked to the development of B- cell malignancies in immuno-suppressed patients (Rooney, 2000). Tumors most consistently associated with EBV are NPC and eBL.

#### 1.4.1 Burkitt’s lymphoma

Burkitt’s lymphoma (BL) is a rare, aggressive B-cell lymphoma that accounts for 30 to 50% of lymphomas in children; however, only 1 to 2% of such lymphomas occur in adults (Harris et al., 2006).

BL was first described in the middle of the last century by Denis Burkitt, a surgeon, working in Kampala, Uganda. He noted children with grossly distorted faces, with lesions involving one or both sides of the face and upper and lower jaws, with some children having huge abdominal masses, sometimes accompanied by disease in the facial
bones, with no lymph node involvement (Burkitt, 1983). This malignancy initially thought to be a sarcoma (Burkitt, 1958) was later established to be a lymphoma and named Burkitt’s lymphoma (Burkitt, 1963).

BL was later found to occur throughout tropical Africa except in areas of high altitudes or in areas where the climate was relatively cool and high incidences were reported in areas with rainfall greater than 50 mm per year. These geographic and climatic associations suggested an association with *P. falciparum* malaria. In 1961, Burkitt made acquaintance with Epstein, an experimental pathologist, and shared samples of the lymphoma with him. Within these lymphomas, Epstein and colleagues identified the virus that has come to be known as Epstein-Barr virus (EBV) (Burkitt, 1963). This was the first description of a virus involvement in the pathogenesis of a tumor in humans. In the setting of the florid reactive lymphoid hyperplasia that occurs in response to malaria, it was proposed that EBV could be oncogenic (Burkitt, 1983).

Although only limited chemotherapeutic agents were available at that time, an excellent response could be obtained with immune suppressive drugs (Burkitt, 1983).

Burkitt originally used cyclophosphamide and because of the usual short hospitalization times of pediatric patient at Mulago Hospital in Uganda, he was forced to consolidate the recommended dose of 40 mg per Kg body weight into a single injection. This exigency proved fortunate. He observed a dramatic tumor regression in the ensuing days, with majority of the cases achieving complete remission after one or two doses (Burkitt,
1867). To the present-day Burkitt’s lymphoma continues to account for most childhood malignancies in Africa (Van Den Bolsch, 2004). A second form of Burkitt’s lymphoma in adults infected with HIV is now common in Africa (Van Den Bosch, 2004).

1.4.2 Pathogenesis of Burkitt’s lymphoma

BL is a distinct category of peripheral cell lymphoma whose pathogenesis is not satisfactorily elucidated although is invariably associated with chromosomal translocation that de-regulates the expression of c-myc gene (Taub et al., 1982; Dalla et al., 1982). Klein (1979) proposed a multi-step hypothesis for the pathogenesis of eBL with early, heavy infection with EBV in children as the first stage resulting into the immortalization of B lymphocytes and some immune tolerance, permitting proliferation of infected cells. Heavy malarial infection would stimulate the expansion of the B cell pool and suppress T cells involved in EBV control. The final stage of tumor genesis would be the development of chromosomal translocation leading to deregulation of c-myc and the development of malignant clone (Jonathan and Hecht, 2000). Chromosomal translocation associated with BL involves the c-myc locus and the IgH locus on chromosome 8 and 14 respectively.

The c-myc proto oncogene is a family of retrovirus-associated DNA sequences (myc) originally isolated from an avian myelocytomatosis virus. The gene is located at position 8q24 on the long arm of chromosome 8 and c-myc gene encodes a basic helix loop helix (bHLH) transcription factor, a 64 kd protein that bind single strand DNA in a sequence specific fashion. The c-myc normally plays a central role in the peripheral transcriptional regulation of an emerging set of down stream genes that control the diverse cellular
processes including cell cycle progression and programmed cell death (apoptosis) (Jonathan and Hecht, 2000). In BL there is inappropriately high activity of c-myc gene expression consequently leading to an up regulation of c-myc proteins through several mechanisms. In 80% of BL cases, the translocation partner of c-myc is the IgH locus leading to a (8:14) q24q32 translocation. In 15% of the cases, the translocation partner is the locus at chromosome 2q11 whereas the remaining 5%, the translocation partner is the locus at chromosome 22q11.

1.4.3 The role of Epstein Barr Virus (EBV) in eBL

EBV infects and immortalizes B lymphocytes in vivo and in vitro resulting into polyclonal activation and proliferation, which are usually controlled by inhibitory immunological mechanisms, with EBV specific cytotoxic T cell playing a crucial part (Thorley and Gross, 2004).
Primary EBV infection is usually followed by latency, but in immunodeficiency, the proliferation of cells can proceed unchecked, and cells sometimes evolve from a polyclonal reactive process to a monoclonal malignant lymphoma (Knowles et al., 1995). In fatal IM, moreover, multiple distinct clones, some of which involve MYC rearrangement, have been detected within a few days of primary EBV infection (Brichacek et al., 1987).

EBV could act as a co-factor by increasing the size of the B cell pool and by transforming lymphocytes, thereby increasing the chances of a translocation occurring, or it could have a more direct role in tumorigenesis, acting together with the changes induced by the MYC translocation (Joab, 1999). eBL seems to be associated with raised concentrations of some EBV antibodies, suggesting that EBV is not merely a passenger in this disease. Compared with matched controls, patients with EBV were shown to have significantly higher titres of IgG antibody to EBV VCA up to 6 years before the onset of the lymphoma (De-The, 1997). However the VCA antibody titres did not rise after the onset of eBL, suggesting that chronic rather than acute infection with EBV was relevant to tumorigenesis. No other EBV antibody showed significant differences between pre-endemic BL cases and controls. EBV early antigen concentrations rise as the disease develops, and declines after treatment (Henle et al., 1971). The amount of cellular cytotoxicity mediated by EBV specific antibodies seems to be important in the outlook of the patient with eBL, further confirming the relation between this disease and EBV (Pearsons et al., 1979)
EBNA 1 an EBV antigen expressed during EBV latency; that is indispensable for the B-cell transformation seems to maintain the EBV episome in latent infected cells (Yates et al., 1984). EBNA1 can bind both RNA and DNA, which could affect expression of viral or cellular genes (Snudden et al., 1994), upregulate the recombinase activating genes that indicate variable diversity joining (VDJ) that can enhance B cell immortalization by several thousand times (Hume et al., 2003). In transgenic mice, EBNA 1 and Myc the mouse analogue of the human oncogene MYC, cooperates in the development of lymphomas, suggesting that the same process could occur in humans (Drotar et al., 2003). However, EBNA 1 does not seem to be oncogenic on its own, since it is expressed in cells infected with EBV, including latently infected cells, and EBER transcripts seem to be needed for a malignant phenotype and resistance to apoptosis (Komano et al., 1999). EBER1 and EBER 2 are small, non-polyadenylated nuclear RNAs. They upregulate BCL 2 to inhibit apoptosis by binding to the protein kinase, blocking the signaling of interferon α and inducing colony growth of cells in agar (Niller et al., 2003). Furthermore, a binding site for MYC has been found in the promoter for EBER1, suggesting that it cooperates with MYC, and that EBV has a role in the lymphoma development. (Niller et al., 2003).
1.4.4 Other factors implicated in the genesis of BL

Use of tumor promoting herbal remedies in Africa has been incriminated in the development of eBL. *Euphorbia tirucalli*, a herbal remedy commonly used in Africa contains a substance closely related to the tumor promoter TPA (12-o-tetradecanoylphorbol 1-3 Acetate (Van den Bolsch, 2004). *E. tirucalli* which is also used widely in Africa as a fencing hedge (Van den Bolch, 2004), has rubber like latex commonly used by children to make stickers (Macneil *et al.*, 2003). This herb is thought to produce EBV promoting substances that are present in plant parts and secreted into the soil around the plant in active form (Mizuno *et al.*, 1986). These secreted substances can also enhance EBV mediated cell transformation (Mizuno *et al.*, 1986; Imai *et al.*, 1994), modulate EBV-specific T-cell activity and cellular immunity (Mizuno *et al.*, 1983) and can also induce chromosomal translocations in B cells (Imai *et al.*, 1994), thus suggesting that substances secreted by the plant have a role in development of lymphoma associated with EBV and with eBL.

Arboviruses have also been suggested as having a role in the development of BL. Arboviruses are RNA viruses transmitted by insect vectors such as mosquitoes and are found in both temperate and tropical regions. They belong to several different families such as alphaviruses, flaviviruses, and Bwamba viruses. Arbovirus epidemics occur regularly in Africa, causing febrile illnesses with varied symptoms. An increased number of cases of endemic Burkitt’s lymphoma, most of which were positive for the arbovirus chikungunya, were seen in 1987–88 in Malawi (Van den Bolch *et al.*, 1993) when an epidemic of chikungunya fever spread from north to south.
Clusters of cases of eBL which were closer together in time and space than would occur from chance were seen in children older than 7 years in Uganda (Morrow et al., 1971) in 1961–65 and 1972–73 and in Malawi in 1987–89 (Van den Bolch et al., 1993). About 69% of the Malawian patients seen during the chikungunya fever epidemic, including some in clusters, who were tested for antibodies were sero-positive for chikungunya virus IgM or IgG at high titers on admission, or sero-converted to IgG during their first admission for eBL, suggesting that they had recently acquired the infection (Van den Bolch et al., 1994).

The case clusters described in Uganda in 1961–65 (Pike et al., 1961) occurred at times of O’nyong-nyong virus activity (Simpson et al., 1964) and in 1972–73 during times of Bwamba virus activity. In these outbreaks, it was found that antibodies to several arboviruses were much more common in patients with endemic Burkitt’s lymphoma and their families than in controls, but no one arbovirus predominated (Simpson et al., 1964; Iversen et al., 1972). Although these findings suggest a correlation between these arboviruses and eBL, the temporal outbreaks of arboviruses amidst permanent occurrence of eBL suggests otherwise. Given that more than 90% of the areas where eBL occur are endemic for malaria, it is thought that that malaria plays the primary role in the progression of EBV viraemia to lymphoma stage.
1.5 Other clinical syndrome associated with EBV

1.5.1 Infectious Mononucleosis (IM)

Whereas most EBV infections of infants and children are asymptomatic or have non-specific symptoms, infections of adolescents and adults frequently results in IM (Henke et al., 1973). Over 50% of patients with IM manifest the triad of fever, lymphadenopathy and pharyngitis. Splenomegaly, portal petechiae, and hepatomegaly are each present in more than 10% of patients. Less common complication includes hemolytic anemia thrombocytopenia and a plastic anemia among others.

Most patients with IM have leukocytosis with an absolute increase in the number of peripheral blood mononuclear cells, heterophile antibodies, elevated serum aminotransferase levels, and atypical lymphocyte. The atypical lymphocytes are primarily T cells, many of which are responding to the infected B cells. Most common symptoms of IM are attributed to the proliferation and activation of T cell in response to infections. A small percentage of the peripheral B cells may be infected by EBV in IM. Activation of B cells by EBV, with resultant production of polyclonal antibodies causes elevated titers of heterophile antibodies and occasionally causes increases in cold agglutinins, antinuclear antibodies or rheumatoid factors.
1.5.2 Chronic active EBV infection (CAEBV)

Chronic active EBV infection (CAEBV) is a very rare disorder that has the following three features: severe illness of more than six months duration that begins as a primary EBV infection or that is associated with abnormal EBV antibody titres; histologic evidence of organ disease, such as pneumonitis, hepatitis, and demonstrations of EBV antigens or EBV DNA on tissues (Straus, 1998). There are often extreme elevations of virus specific antibody titres. In contrast, chronic fatigue syndrome is a different disorder in which patients can have slightly elevated antibody titres to EBV and other viruses.

1.5.3 X-linked lymphoproliferative diseases.

Patients with X-linked lymphoproliferative disease, an inherited disease of males, are unable to control infections with EBV. Patients with this disease have a mutated gene on the X chromosome identified as SAP (signaling lymphocyte activation molecule) that encodes a protein located on the surface of T cells (Sayos et al., 1998). The absence of a functional SAP in these patients is thought to impair the normal interactions of T and B cells, resulting in unregulated growth of EBV infected B cells.

1.6 Rationale of the study

Epstein Barr virus (EBV) and *P. falciparum* malaria have an overlapping distribution and are incriminated as the primary causal agents for endemic Burkitt’s lymphoma. The results from this study will provide insights as to whether presence of EBV in children who develop severe malaria reveals unique serological/nucleic acid signatures that would allow future development of predictive markers for evolution of Burkitt’s lymphoma.
1.7 Research questions

I. Is there a difference in EBV viral load between children with mild malaria and those with severe malaria?

II. Are there specific serological signature to VCA IgG and IgM s and EBNA IgG antibodies unique to children with severe malaria or mild malaria?

III. Is there a difference in B cell numbers between children with mild malaria and those with severe malaria?

1.8 Hypothesis

EBV viral load and viral antibodies are significantly different between children with severe malaria and those with mild malaria.

1.9 Objectives of the study

1.9.1 General Objective

To determine whether severity of malaria influences EBV viral load, viral antibodies and B cell numbers

1.9.2 Specific Objectives

i) To determine whether there are differences in EBV viral load between children with mild malaria and those with severe malaria.

ii) To determine whether there are relationships in VCA IgG IgMs and EBNA IgG antibodies in children with mild malaria and those with severe malaria.

iii) To determine B numbers in children with mild malaria and those with severe malaria.
CHAPTER TWO
MATERIALS AND METHODS

2.1 Study Design

2.1.1 Study site and Sample size calculation

The assays will be performed on archived DNA and plasma specimens that were collected from a prospective case control study that enrolled patients with severe malaria (cases) and their age and sex matched controls with uncomplicated malaria. The patients were recruited from the pediatric ward of Kisumu District Hospital, Nyanza Province.

This sample size was calculated using StatMate software (Graph Pad, San Diego CA). The sample size was based on results of CR1 molecules on RBC from a previous study that enrolled children with severe and uncomplicated malaria (Nyakoe et al., 2009). In that study, a standard deviation of 114 CR1 molecules was obtained between cases of severe malarial anemia and controls. Assuming the real difference between the groups is 84 molecules, giving a sample size of 60 in each group of the two groups will give 80% power to detect that difference, $\alpha = 0.05$ (two-sided).

2.1.2 Ethical Consideration

The study was voluntary and each parent or guardian of the participants signed an informed consent before the child was enrolled. Scientific and ethical approval for the study was obtained from the Ethical Review Committee of the Kenya Medical Research Institute, Nairobi and the Walter Reed Army Research Institute of Human Use Research Committee, Silver Spring, Maryland, USA.
2.2 Study Population

Two groups of children were enrolled. Group one comprised children (age ≤ 5 yrs) admitted to the participating hospital with asexual *P. falciparum* parasitemia confirmed by a positive Giemsa-stained blood smear and anemia defined as hemoglobin ≤ 6 g/dL. Group two were age and sex matched children with symptomatic mild malaria.

2.3 Inclusion and exclusion criteria

Patients with severe anemia were defined as children with asexual *P. falciparum* parasitemia as determined by either thin or thick Giemsa-stain and hemoglobin level ≤ 6 g/dL. Each patient was matched by age (±2 months) and sex to a child with uncomplicated malaria, fevers of axillaries temperature ≥ 37.5°C or, in absence of fever, presence of any 2 of the following: nausea and/or vomiting, diarrhea, irritability, or poor feeding). Subjects were excluded from participation if there was evidence of concomitant infections (i.e. pneumonia meningitis, sepsis etc), evidence of immune-comprised status (e.g thrush and tuberculosis), and history of blood transfusion within the preceding six months. Children were also excluded if they had a history of malignancy (e.g. Burkitt’s lymphoma), evidence of severe calorie imbalance (wasting < 70% of 50th percentile weight/height ratio for age) and evidence of protein deficiency (edema, hair discoloration, abdominal distention).

2.4 Collection of blood samples

Trained clinical personnel collected all the blood samples using sterile technique. Approximately 2 mL of blood was collected in 3 mL EDTA tubes. Whole blood was then
separated from plasma by centrifuging at 2000g for 5 min at 4°C. The plasma was aspirated, aliquoted and kept at -70°C until required. For use, samples were thawed at room temperature and kept on ice during the entire assay.

2.5 Enumeration of patient’s lymphocytes.
Complete blood count was performed on patient’s samples to identify normal and abnormal blood parameter results. This was to act as a guideline for the physician/clinician on the appropriate course of therapeutic management of patients.

In this study, Patients samples were analyzed were analyzed by ACT diff.coulter for the absolute lymphocyte count. This utilizes the coulter principle. This principle is based on facts that cells which are poor conductors of electricity interrupt current flow. The impedance variation generated by the passage of non conductive cells through a small calibrated aperture is used to determine the count. The instrument calculates a three-population leukocyte count (Lymphocytes (LY %), Monocytes (MO %) and granulocyte (GR %) from the WBC histogram based on cell size. Cells between 35 (fento liters) fL and 90fL were classified as lymphocytes.

2.6 Isolation of DNA
DNA was isolated from EDTA blood using QIAamp DNA Blood mini Kits (QIAGEN Inc., CA) according to the manufacturers guidelines. Briefly, 200 μL of EDTA blood was
placed in a sterile 1.5 mL micro centrifuge tubes. This was followed by addition of 200 μL of lysis buffer and 20 μL QIAGEN protease enzymes in order to digest the proteins. The mixture was vortexed and incubated on a hotplate for 10 minutes at 57 °C. The de-proteinized DNA preparation was then purified on QIAamp spin columns after addition of 200 μL of ethanol. This was achieved by centrifugation for one minute at 8000 rpm in a micro centrifuge and the tube containing filtrate discarded. The spin column was then transferred into another 1.5 mL collection tube and 500 μL of buffer AW1 added and centrifuged at 8000 rpm for one minute and the filtrate discarded. Approximately 500 μL of buffer AW2 was added into the spin column and the preparation centrifuged for three minutes at 14,000 rpm. Finally, the purified DNA was eluted from the spin column by addition of 200 μL elution buffer (AE) and stored at –20 °C until required.

2.7 Real-time quantitative assay (qPCR)

2.7.1 EBV viral load determination.

Real time quantitative polymerase chain reaction (q-PCR) was used as described by Kimura et al (1998) with slight modifications. The PCR primers for this assay were selected from the BALF5 gene encoding the EBV DNA.

The following primers and probes were obtained and synthesized from Applied Biosystems (Foster City, CA, USA): 5’-CGGAAGCCCTCTGGACTTC-3’ for upstream primer and 5’-CCCTGTATTATCCGATGGAATG-3’ for the downstream primer, 5’-TGTACACGCACGAGAAATGCGCC-3’ with FAM as the reporter and TAMRA as the
Amplification was done on a 7300 Real Time PCR machine (Applied Biosystems Foster City, CA). PCR reactions were performed in a total volume of 50 µL containing 1x Taqman universal PCR master mix, 0.2 µM of forward and reverse primers each, 0.1 µM of probe and 2 µL of DNA template. The cycling conditions were 10 min at 95 °C, 50 cycles of 15 sec at 95 °C and 1 min at 62 °C. All samples were tested in duplicate. Each run included a set of positive and negative controls for Q/A. A standard curve of Ct values and the viral copy numbers from a two fold serial dilution of an EBV DNA sample with known copy numbers (10,000 copies/µL). Viral load of samples was extrapolated from the standard graph.

2.8 Enzyme linked immunosorbent assay (ELISA) for measurements of VCA and EBNA IgG and IgM

Serum levels of IgG and IgM antibodies against VCA and EBNA were measured using ELISA commercial kits (Vircell Microbiolgist, Ireland). For the assay, the plates were supplied with pre-coated VCA antigen for IgG and IgM and EBNA antigens for IgG determination. Test samples were then diluted at 1:20 with sample diluents provided by the manufacturer. A volume of 5 µL of each test sample was added to 100 µL of sample diluent per antibody tested. Controls were similarly prepared. The plate was then sealed and incubated at 37°C for 45 minutes.

Following incubation, the seal was removed and liquid from all the wells aspirated, the plate was then washed 5 times with 0.3 mL washing buffer. Immediately after washing, 100 µL of IgG or IgM conjugate (6A for IgG and 6B for IgM) solution was added to each well and the plate covered and incubated for a further 30 minutes. After this incubation
period, the seal was removed, liquid from all wells aspirated and the ELISA plate washed 5 times with 0.3 mL washing buffer. 100 µL of substrate solution was added to each well and finally incubated away from light at room temperature for 20 minutes. Immediately following the incubation, 50 µL of stopping solution was added into each well and the OD values read at 450/620nm (Spectra Max340PC Molecular Devices, USA) within one hour of stopping. The antibody index value was calculated as follows:

\[
\text{Antibody index value} = (\text{Sample OD / Cut off mean OD})*10
\]

Samples with indices below 9 were considered as not having either IgG or IgM specific antigens to VCA or EBNA. Samples with indices above 11 were considered as being positive for the target antibodies. Sample indices were used as surrogate measure of antibody titers.

2.9 Measurement of B-Cell CR2 by flow cytometer

A 50 µL aliquot of EDTA blood was placed into 3 wells of a round-bottom 96-well plate. Each sample was washed twice with wash buffer (PBS containing 1% BSA and 0.1% sodium Azide) followed by incubation in 100 µL of 10% mouse IgG in PBS for 30 min at room temperature.

Following centrifugation, the cell pellet was re-suspended in 100 µL of antibody cocktail CD20-PerCP/CD21-PE. Isotype controls were also used as a way of evaluating non-specific staining. After a 30 min incubation period, the plate was centrifuged to wash the
cells as described above. Thereafter, the cells were re-suspended in 200 µL of red blood cell lysing buffer (150 mM NH₄Cl, 10 mM NaHCO₃ and 1 mM EDTA) and incubated for 3 minutes at room temperature followed by centrifugation and discarding of the supernatant. The cell pellet containing blood leukocytes was washed twice as above and finally re-suspended in 100 µL of 1% paraformaldehyde and stored at 4 °C until required. The lymphocyte population was identified on the basis of forward and side scatter light characteristics. CD21 expression was determined on CD20+ cells. Acquisition was carried out using the BD FaCscan, equipped with winFCM software and analysis done with Expo software.

3.0 Statistical analysis

Data was routinely entered into Microsoft excel sheet a database for querying and reporting summaries. Computational analysis was performed with Graphpad prism 4 (GraphPad Software Inc. San Diego CA). EBV viral load was log transformed and a value of 1 was added to all samples to make all values positive, these data were then summarized as geometric mean. Differences between groups for both ELISA and EBV viral load data were calculated and analyzed using parametric student t-test. All tests were two- tailed with a 95% confidence interval.
CHAPTER THREE
RESULTS

3.1 Subjects enrolled.

The study enrolled 57 cases and 44 controls whose characteristics are shown in Table 1. As is usual with case control studies, cases were enrolled first and matching controls later. Therefore, more cases got enrolled than controls. The mean age for the cases was 16.67±9.21 and 17.02±9.60 months in the controls. The mean Hgb level in cases was 4.5±1.0 g/dL and 8.80±1.40 g/dL in the controls.

Table 1: Demographics and clinical characteristics of patients enrolled in a malarial anemia case control study.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Severe Malaria</th>
<th>Mild Malaria</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (N)</td>
<td>57</td>
<td>44</td>
<td>ND</td>
</tr>
<tr>
<td>Age (months)</td>
<td>16.67±9.21</td>
<td>17.02±9.5</td>
<td>ND</td>
</tr>
<tr>
<td>Parasitemia (mean ± SD)</td>
<td>97,100±114,000</td>
<td>80700±106,000</td>
<td>0.661</td>
</tr>
<tr>
<td>Hb level (g/dl, mean ± SD)</td>
<td>4.5±1.0</td>
<td>8.9±1.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

3.2 Viral load determination in cases and controls.

3.2.1 Generation of standard graph for calibration of viral load in patient samples

A standard graph for calibration of viral load in patient samples was generated using 7300 real time PCR machine. A serially diluted standard QA sample of known copy numbers (1.0 x 10^4 copies per µL) was used. A standard curve was then generated by plotting C_t values against the log concentration of the QA sample. Viral loads of patient samples were extrapolated from the standard graph that was run concurrently.
Figure 2 shows relative fluorescent intensities plotted against PCR cycle numbers of a serially diluted QA sample (6.0 x 10^{-1} - 2.0 x 10^{4} copies per µL). The fluorescent chemistries show a strong correlation between fluorescence intensity and viral copy numbers.

![Figure 2: A typical amplification plot generated in a 7300 real time PCR machine for amplification of EBV DNA.](image)

Each of the qPCR amplification curve can be broken into three phases i.e. the base line, exponential and plateau phases. At base line phase, fluorescent emissions from PCR products are below detection (Figure 2). Once PCR product accumulation reaches a critical threshold, fluorescent emissions rise above the background and further increase linearly in subsequent PCR cycles to produce the log-linear phase. At the beginning of the log phase, there occurs a threshold fluorescent intensity level where PCR reactions crosses the threshold. The PCR cycle at which this occurs is known as the threshold cycle (C_{t}). During the final plateau phase, fluorescent intensity slowly reaches the upper
limit and is no longer useful for data calculation. This is because PCR reaction components became increasingly limiting and therefore the product amplification rate are no longer proportional to the starting concentration.

Figure 3 shows a standard curve generated by plotting $C_t$ values against the log concentration of the QA sample. Viral load of samples was extrapolated from the standard graph that was run whenever patient samples were amplified.

![Figure 3: A typical standard curve for measurement of EBV copy numbers generated in a 7300 real time PCR machine.](image)

Two fold serial dilutions of EBV DNA control ranging from $6.0 \times 10^1$ - $2.0 \times 10^4$ copies per µL was used to generate threshold values ($C_t$). The $C_t$ values were plotted against the log copy numbers of each viral standard. ($R^2 = 0.9925$, slope= -3.2150.)
3.2.2 Viral load in children with severe malaria and with mild malaria

Of the 101 patients enrolled (Table 1), only 94 were evaluable by PCR: 47 in the severe malaria group and 47 in the mild malaria group. Of these, 25 (53%) children in each arm had detectable EBV load. Children in the severe malaria anemia group had a higher mean viral copy number/µL (27,556±25,377 SE) (geometric mean ± SE) compared to children who had mild malaria that had 21,703±17,434 SE although the difference between these two groups was not significant (p=0.46) (Figure 4).

Figure 4: Scatter plot showing the DNA copy numbers in children with severe and mild malaria.
3.2.3  **VCA and EBNA IgG in Children with severe malaria and those with mild malaria.**

As shown in Figure 3.4, children with severe malaria anemia had significantly higher VCA IgG antibodies levels (0.97± 0.05 SE) compared to children with mild malaria (0.79± 0.06 SE, \(P = 0.04\) paired t-test).

![Box plot showing the log mean VCA IgG antibody titers in children with severe malaria.](image)

**Figure 5.** Box plot showing the log mean VCA IgG antibody titers in children with severe malaria.

Likewise, EBNA IgG antibodies titers in the severe malaria anemia group were higher (1.02±0.06 SE) compared to children with mild malaria (0.90± 0.07) although this difference was not significant (\(P=0.28\), paired t-test) (Figure 6).
Figure 6: Box plot showing the log mean EBNA IgG antibody titers in children with severe and mild malaria.

3.2.4 VCA IgM level in Children with severe malaria anemia and those with mild malaria.

Figure 7 below shows levels of VCA IgM antibody titers in children with mild compared with those with severe malaria. There were no differences in VCA IgM antibody titers between the groups. Mean± SE index value in cases was 1.18±0.05 compared to 1.12±0.06 in controls (P=0.72, paired t-test)

Figure 7: Box plot showing the Log mean VCA IgM antibody titers for in children with severe and mild malaria.
3.2.5 EBV Antibodies levels in children of different categories

Since malaria morbidity is age dependent and malaria immunity is gradually acquired, the study determined whether there was an age related difference in EBV antibody levels in children with severe malaria anemia and mild malaria. Children were categorized to the following age brackets in months: 0-6, 6-12 and 12-65. Children in the 0-6 month category had the lower levels of both VCA and EBNA IgG (Figure 9) these antibodies compared to the older children who >6 months, with the cases having higher antibody titers compared to their respective controls.

Relatively higher levels of VCA IgG antibodies was observed with children in the severe malaria anemia group having higher titers than those with mild malaria were observed in the 6-12 and 12-65 month age category compared to those seen in the 0-6 month’s category. (Figure 8) with low antibody levels in age group 0-6 month and highest levels in children with >12 months. There were no observable differences in IgM antibody. The antibody titers for Viral Capsid Antigen (VCA) IgG were much higher in cases compared to their respective controls (Figure 8).
3.2.6 Enumeration of total lymphocyte in cases and control

As shown in Figure 10, children with severe malaria anemia had more lymphocyte population (2584 ± 494.2 SEM) than children with mild malaria (1668 ± 277.9 SEM) total lymphocyte numbers, and the numbers were significantly different (P<0.05), student t– test)
Figure 10: Box plot showing the Log total lymphocyte count in children with severe and mild malaria.

As EBV is a B cell mitogen and uses B-cell surface receptor CD21 for entry, this study determined B-cell levels in a subset of study subjects (N=26). The study observed an expanded B-cell pool (2,307±551 SE) in the children with severe malaria anemia compared to those with mild malaria (1,273±426 SE), although this difference did not reach statistical significance (P = 0.173).

Figure 11: Box plot showing the mean B cell count for in children with severe and mild malaria.
Table 2. Shows a summary of results obtained from all assays carried out in this study

<table>
<thead>
<tr>
<th>Signature</th>
<th>Severe Malaria</th>
<th>Mild Malaria</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral load (Geometric mean)</td>
<td>27,556± 25,377</td>
<td>21,703±17,434</td>
<td>NS</td>
</tr>
<tr>
<td>VCA IgG (Log mean)</td>
<td>0.97± 0.05</td>
<td>0.79± 0.06</td>
<td>P=0.04</td>
</tr>
<tr>
<td>EBNA IgG (Log mean)</td>
<td>1.18±0.05</td>
<td>1.12± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>VCA IgM (Log mean)</td>
<td>1.18±0.05</td>
<td>1.12± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Total lymphocyte (mean)</td>
<td>2584 ± 494.2</td>
<td>1668 ± 277.9</td>
<td>P=0.0013</td>
</tr>
<tr>
<td>B cell count (Log mean)</td>
<td>2,307±551</td>
<td>1,273±426</td>
<td>NS</td>
</tr>
</tbody>
</table>
CHAPTER FOUR
DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 DISCUSSION

Epstein-Barr virus (EBV) and Plasmodium falciparum malaria have overlapping distributions and are thought to have causal interactions, particularly with regard to the etiology of eBL. It has been previously suggested that eBL may arise as a consequence of a combination of multiple mechanisms trigged by malaria antigenic stimuli, which may involve chronic stimulation of the B cell compartment, increased viral production, and suppressed EBV-specific immune responses (Lam et al., 1997). Nevertheless, the exact mechanism utilized by these two pathogens to cause the B cell malignancy has remained elusive. Most children in the malaria holoendemic regions are exposed to EBV and malaria early in life, although it remains unclear why some children develop eBL while others do not. It is hypothesized that there could be unique permutations and combination that arise as a result of an intricate association between these two pathogens leading to the carcinogenesis. This study therefore sought to determine whether presence of EBV in children who develop severe malaria reveals unique serological/nucleic acid signatures that would allow future development of predictive markers for evolution of Burkitt’s lymphoma.

In this study, differences in EBV viral load were determined in children with mild and those with severe malaria. A higher viral load (geometric mean copy numbers/μl) was observed among children with severe malaria compared to those with mild malaria. It is speculated
that the elevated viral load seen amongst children with severe malaria may be due to a polyclonal B cell expansion that increases the number of circulating EBV infected B cells. Whittle et al. (1990) demonstrated that, during episodes of acute malaria, spontaneous outgrowth of EBV-transformed B-cells occur at a greater frequency in children suffering from acute malaria and this could lead to augmented numbers of circulating EBV carrying B cells. Reduced T cell responses could also lead to higher EBV loads, as is seen in patients after transplant and or those with AIDS (Rose et al., 2001). In summary a number of mechanisms may be in place that control the over proliferations of B cell that subsequently lead to a higher viral loads.

Children with severe malaria anemia had a higher lymphocyte count compared to children with mild malaria. These results clearly indicated that severe malaria expands total lymphocyte pool. This expansion may lead to an increase in circulating EBV infected cells leading to a higher viral load as was seen in this study.

Since EBV infects B-cells using CD21 as the ligand, B cell numbers of a subset of the study population was enumerated by flow cytometry, an expanded B-cell pool was observed in children with severe malaria anemia compared to those with mild malaria.
This observation consistent with those of Lam *et al.* (1991) who demonstrated that episodes of acute malaria leads to augmented EBV carrying B cells apparently due to polyclonal B cell expansion rather than viral replication.

This study also determined the influence of malaria severity on Epstein Barr viral capsid (VCA), Epstein Barr Nuclear Antigen IgG and VCA IgM antibodies. Such determinations, as well as identification of the immunoglobulin class of the antibodies, have been found useful in the sero-diagnosis of various EBV induced diseases (Henle and Henle, 1982). ELISA measurements for VCA IgG antibodies were significantly higher in cases compared to controls. The higher presence of VCA IgG is probably due to the fact that it persists over a longer period following primary infection. The Presence of higher VCA IgG antibody titers is also indicative of a higher viral replication rate, showing that the virus may be in its lytic form compared to being in the latent phase (Henle and Henle, 1982). Elevated antibodies against VCA were reported by De The (1978) to precede the development of eBL. It is therefore speculated here that children with severe malaria anemia are more likely than the controls to develop eBL. Unfortunately, the design of the current study did not allow us to test this hypothesis as children were only evaluated once.

This study also reported higher EBNA IgG antibodies in children with severe malaria compared to those with mild malarial.
EBNA antibodies are thought to be indicative of virus entering latency and therefore indicate that the viral form of this infection was in the active lytic form (Henle and Henle, 1982). Results from these two serological signatures are suggestive of recurrence of malaria infections affecting either the establishment and/or the maintenance of EBV latency.

There was no significant difference in VCA IgM between the groups studied. VCA IgM antibodies are early antibodies made in response to primary infection but are known to diminish soon after, although in some cases they may re-appear after further exposure. The presence of three VCA IgG, EBNA IgG and VCA IgG indicate viral reactivation (Callan, 1998). This suggests that episodes of malaria reactivate latently infected cell to enter active replication.

Since malaria morbidity and mortality is age dependent and malaria immunity is gradually acquired, (Marsh and Snow, 1999), this study determined whether there was an age related difference in EBV antibody levels. EBNA IgG titers increased with increase in corresponding increase in age. Children in the younger age group (0-6 months) showed relatively lower levels of both VCA IgG and EBNA IgG antibodies titers compared to the older children. In most cases primary infections occur sub-clinically during childhood when EBV is horizontally transmitted via salivary contact among children and family members (Gratama et al., 1990). These observations seem to suggest that younger children in the ages between 0-6 months have a negligible or a lower level of EBV exposure.
Older children are much more exposed to EBV and most would have seroconverted by the age of 3 years. Therefore these gradually increasing antibody profiles seen across the groups are clear indicators of exposure. It is speculated here that primary EBV exposure possibly occurs in children older than 6 months and the exposure rate may be commensurate with the level of activity of the child.
4.2 CONCLUSIONS

1. The findings of this study are consistent with early childhood exposure to EBV in the malaria holoendemic lake Victoria Basin. Fifty three percent of the study population (mean age of 18 months) had detectable EBV viral load as determined by nucleic acid assay. This finding support other studies that indicate that African children are infected by EBV early in life and nearly all will have sero-converted by three years of age as opposed to European population who sero convert to EBV at adulthood. This study concludes that because children in the Lake Victoria basin have an early exposure to EBV coupled with high repeated and protracted malaria probably increases the likelihood of these children to develop eBL if the right conditions exist. Because prevalence of eBL is very low in the population, there are other factors that predispose to progression of eBV to eBL stage.

2. This study has showed that children with severe malaria anemia have higher viral load compared to their age and sex matched controls with mild malaria. We attribute the higher viral load in the SMA to malaria antigenic stimuli fueling polyclonal B cell expansion and thereby increasing the number of circulating EBV infected cells. Severity of malaria may also on the other hand trigger latently infected B-cell to enter lytic/ replication leading to a higher viral load. Nevertheless the difference in viral load was not statistically significant.

3. Elevated antibodies against VCA IgG have been shown in a study by De the (1978) to precede the development of eBL. Children with severe malaria had high levels VCA IgG and this could be a serological indicator that they could develop eBL if the right conditions exist.
Based on the findings of a 6 year epidemiological study carried out between 1999-2004 in Nyanza province, an incidence rate of 2 out 100,000 cases of eBL occurred annually (Jeanette et al., 2006). Since the current study only enrolled 120 study participants, the probability that any of the children will come down with eBL is low (Jeanette et al., 2006). Some of the children in our study could come down with other EBV related lymphoproliferative disorders as VCA IgG are associated with a number of such disorders.

4 This study also showed an age related increase in VCA and EBNA IgG antibodies and the increase was higher in the cases compared to controls. We argue that this indicates the time point at which primary infections occur. This finding also clearly indicate that younger children between the age of 0-6 months are not as frequently exposed to the pathogen as the older children. Higher antibody levels as seen in the cases compared to their respective controls could be due to viral reactivation fueled by malaria antigenic stimuli. It is also at this age that there is increase in malaria transmission that is thought to perturb EBV specific immunity leading EBV lytic activity and consequent B cell infections.
4.3 RECOMMENDATIONS

I. Based on an epidemiological study that was carried out between 2004-2006 in the malaria holoendemic lake region (Jeanette et al., 2006), it appears that the incidence of eBL is approximately 2/100,000 children per annum. It is therefore recommended that studies with bigger sample sizes be designed to evaluate whether any of the viral and serological signatures described in the current study have relevance to the evolution of eBL.

II. This study reported presence of elevated levels of anti-VCA IgG antibodies, which together with other EBV associated antibodies are useful in serological diagnosis of other EBV associated diseases such as including Hodgkin’s disease. Studies should be designed to investigate whether children with particular viral and serological signatures have other underlying EBV related lymphoproliferative diseases.

III. Finally, although EBV and malaria are considered important in development of eBL, there are clearly other factors that act in concert with malaria and EBV to cause eBL tumorigenesis. It is recommended that future studies be designed to evaluate what these “other factors”. Implicated environmental factors include exposure to plant such as Euphobias, deficiency in trace elements such as selenium among many others.
REFERENCES


## APPENDICES

### Appendix I: Worksheet for analysis EBV viral by Real Time Quantitative PCR

PCR: DATE: __/__/2007

<table>
<thead>
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<th>Reagent</th>
<th>Final Concentration</th>
<th>50 μL reaction mix</th>
<th>___Samples</th>
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<tr>
<td>2× Taq Man MasterMix</td>
<td>1×</td>
<td>25 μL</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.2 μM</td>
<td>1.0 μL</td>
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<tr>
<td>Reverse Primer</td>
<td>0.2 μM</td>
<td>1.0 μL</td>
<td></td>
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<tr>
<td>Probe (10nM)</td>
<td>0.1 μM</td>
<td>0.5 μL</td>
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</tr>
<tr>
<td>DNA</td>
<td>--------</td>
<td>2.0 μL</td>
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<tr>
<td>PCR H₂O</td>
<td>q.s to 50 μL</td>
<td>q.s to 50 μL</td>
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<tr>
<td>TOTAL</td>
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<td>50 μL</td>
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### SAMPLES

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### CONTROLS

- Positive control
- Denaturation
- Negative Control (NTC)
- Negative Human Genomic DNA

### CYCLING CONDITIONS

- 10 Mins. at 95°C initial Denaturation
- 15 Seconds at 95°C Denaturation
- 1 Min. at 60°C annealing and Ext.

### COMMENT
CONSENT DOCUMENT FOR YOUR CHILDREN'S PARTICIPATION IN THE STUDY

Title of the study: activation of the immune system and pathogenic micro-organism in children with malaria.

You are requested to allow your child participate in a study that is being done at Kisumu District hospital by Dr John N. Waitumbi of the Kenya Medical Research Institute in collaboration with the Walter Reed project Kisumu, Kenya, P.O. Box 54, Tel 057 2022942. Kindly read this document and ask any question(s) on any issue(s) that may be unclear to you, before consenting to having your child is recruited into this study.

Purpose of the study

This study is to determine factors that predispose children with malarial anemia to other micro-organisms. The experimental part of this study will be the research related tests done on your child's blood after it is drawn. Otherwise your child will receive standard medical care.

Study methodology

In the event that you consent that your child be recruited into the study, a doctor will examine the child to determine whether or not your child meets the criteria set for recruitment into the study. A little blood will be taken from the child's finger for malarial parasite diagnosis. Incase your child is diagnosed with malarial anemia; he/she will be categorized into group 1 and will be admitted into hospital. Those children diagnosed as having mild malaria and found to have a body temperature of 37.5°C or lower but with any two of these symptoms; headache, nausea, vomiting, diarrhea, lack of appetite and itching of the body, will be categorized into group 2 of the study and will be treated and allowed to go home with their parents/guardians. Your child will not be recruited into the study even if you consent if the child is found to have certain diseases that may compromise the data and/or study objective. Such diseases include pneumonia, throat infections, cancer and malnutrition. On recruitment, 2.5 ml of blood will be drawn from the arm or heel of the child. A quarter of the blood will be used by doctors for diagnostic tests and the remaining blood taken to Walter Reed Research laboratory-kondele within Kisumu town for determining:

Incase your child is examined by a doctor and found to be very sick and requires admission into the hospital, he/she will be admitted for treatment and all chargeable costs of medication will be paid by the study. Incase the child is required to continue with treatment after being discharged from hospital, such costs shall be paid by the parent or guardian. If your child is not admitted into hospital, he/she will be given malaria drugs and allowed to go home with you. Occasionally, your child may be required to either give more blood for more laboratory analysis or give stool for checking worm infections so as to facilitate better medical treatment to your child.
Things that may harm the child in the study

The child will feel some little pain at time of pricking the finger to take blood. Sometimes, an infection may occur at the point of pricking, however, all care and precautions will be taken to ensure the child feel the least pain during the time of drawing blood. Blood will be drawn using new sterile needles and syringes which will be only used once and then disposed.

Benefits of participating in the study

Your child will get close and quick medical attention free of charge, free medical treatment for malaria and other infections that he/she may come down with during the period he/she is in the study.

Alternative to participation

The fact that your child is not recruited into the study will not deny your child the right of getting medical services at Kisumu District Hospital.

Payment for participation

Your child will receive free inpatient and outpatient treatment for malaria or other infectious diseases while they are participating in this study. This may include laboratory tests, and supplies such as needles, syringes, gauze, and tape used for blood drawing for which the hospital normally charges. No other compensation will be provided for your child’s participation in this research.

Confidentiality in the study

The identities and or names of the children participating in the study will not be in any way revealed during the reporting and discussions of data from this study. No pictures, videos or audiotapes will be used in the study. On the day of recruitment, the child will be assigned a study number that will be used in all communications and reporting. The study numbers will bear the letters "csa" meaning complement and severe anemia. This letters will also bear numbers starting with 001. The first child to be recruited into the study will have study number csa001. These numbers will increase as more children get recruited into the study i.e. csa001, csa002, csa003 etc. The study numbers and the names of the child will be written on a piece of paper used for recruiting the child into the study and will be kept in a safe cabinet at Walter Reed project-kondele under lock and key and accessible to only authorized personnel of the study.
Joining and withdrawing from the study:
Your child will not be forced to join the study but will do so voluntarily. In case you do not allow your child to join this study, he/she will not be denied any medical services or any other services that the child is entitled to at the kisumu district hospital. If your child is recruited in the study, you are allowed to withdraw him/her from participating in the study at any time without suffering any consequences or prejudice.

Names and contacts of investigators:
if you have any question(s) about the study, please contact Dr. Lennah Nyabiage of kisumu district hospital. if you are unable to reach Dr. Nyabiage, feel free to contact Dr. John N. waitumbi of Walter Reed Project/KEMRI P.O. Box 54, kisumu, Kenya using telephone number 057- 2022942 during the day or 0733-616 548 during the night.

Things that volunteers are not prohibited from doing:
You can withdraw your child from the study at any time without any repercussions whatsoever. Doing that will not deny you the opportunity of seeking any legal redress/action over whatever issue you want addressed. in case you have a question, kindly contact Dr. John N. Waitumbi, Walter Reed project, P.O Box 54, Kisumu, Kenya. Telephone numbers: 057-2022942 during the day or 0733-616548 during the night.

Signature of research subject
i have read the information provided above. i have been given an opportunity to ask questions and all of my questions have been answered to my satisfaction. i have been given a copy of this form.

Parent or legal guardian’s name

Parent or legal guardian’s signature date

Signature of witness
my signature as a witness certifies that the legal guardian signed this consent form in my presence as his/her voluntary act and deed.

Name of the witness

Signature of the witness----------------------------------Date -----------------------------