EPSTEIN BAR VIRUS STRAINS IN PERIPHERAL BLOOD AND SALIVA OF MOTHER-CHILD PAIRS IN MALARIA HOLOENDEMIC REGIONS WITH VARYING BURKITT'S LYMPHOMA INCIDENCE RATES

BY

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ABSTRACT

Endemic Burkitt's lymphoma is a polymicrobial childhood cancer disease mainly associated with EBV and *plasmodium falciparum* infections whose combined effects profoundly affect the B cell compartment. The exact role of these microbes in Burkitt's lymphoma pathogenesis as well as other EBV associated malignancies is not well understood. Genetic polymorphisms have led to the identification of EBV type 1 and 2 which may be specifically associated with some virus positive tumors. EBV strain prevalence and the effect of malaria infections on EBV specific immune T cell responses in children at risk for BL in malaria holoendemic areas have not been studied. This study determined the prevalence of EBV type 1 and type 2, malaria infection prevalence and interferon gamma T cell responses in children and their mothers from BL 'hot' and 'cold' spots. Since EBV can be detected in both saliva and blood, this study compared the differences in virus types between these compartments as well. In a cross sectional study design, samples were collected from 58 Burkitt's lymphoma cold spot area participants and 37 Burkitt's lymphoma hot spot area participants. DNA was extracted from peripheral blood and saliva and PCR amplification was done on all samples. PCR primers were used to amplify a region of the EBNA3C gene that can distinguish between the two strains based on the base pairs of the PCR product. EBV type 1 and 2 were identified based on length differences within the EBNA3C gene. Malaria infections were also determined by blood smears and compared with EBV infections and specific T cell responses from ELISPOT assays. EBV DNA was detected in 94.6% of the hot spot area samples and 86.2% of the cold spot area samples. 75.15% of the participants had EBV in blood compared to 66.1% in saliva. EBV type 1 and 2 multiple infections were detected in 94.6% of the hot spot area participants compared to 51.7% of the cold spot area participants. Single type 1 and type 2 EBV infections were detected at low frequencies in the cold spot area. Comparison of the type of EBV found in mothers and children showed only 61.5% match in blood and saliva all being type1 and 2 multiple infections. In the cold spot area, 38.1% had matching strains in saliva and 57.1 in blood. 37.5% had type 1 single infections, 25% type 2 single infections, and 37.5% type 1 and 2 multiple infections in saliva. On the other hand, in the hot spot samples, 8.3% had type 1 single infections, 8.3% had type 2 single infections and 83.3% type 1 and 2 multiple infections. Chi-square analysis at $P<0.05$ showed significantly high detection of EBV DNA in the hot spot area samples compared to the cold spot area samples. Type 1 and 2 co-infections were predominant in the hot spot blood and saliva samples. A high percentage of children from both study sites were co-infected with both EBV type 1 and 2. A high proportion of mother-child pairs had matching EBV types in both blood and saliva compared to the hot spot ($P< 0.05$). Malaria infection prevalence was relatively high in the hot spot area compared to the cold spot area. EBV specific T cell responses were reduced in the malaria infected individuals compared to the uninfected individuals. This study concludes that EBV type 1 and 2 co-infections are highly prevalent in the malaria holoendemic region with high BL incidence rates and there is uniform EBV type transmission from the mothers to their children. EBV type distribution was not however dependent on the prevalence of malaria. EBV specific T cell immune responses tent to be suppressed by malaria infections.

Key words: *EBV type 1and 2 DNA, Saliva, blood, Burkitt's lymphoma, endemic malaria,*

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CHAPTER ONE: GENERAL INTRODUCTION

1.1 Background Information

Burkitt's lymphoma (BL) is a highly malignant B cell tumor first described in Uganda by a medical physician called Dennis Burkitt in 1958 (Burkitt, 1983). This cancer is classified according to clinical symptoms as endemic, sporadic and acquired immunodeficiency syndrome related. Endemic BL (eBL) occurs exclusively in Africa, sporadic BL comprises 20-30% of non Hodgkin's lymphoma in children of developed countries, while the AIDS related BL accounts for the 30% of all HIV-associated lymphomas (Mwanda *et al.*, 2005).

There is growing evidence that endemic Burkitt's lymphoma is a polymicrobial disease (Chêne *et al.*, 2007). This tumour is commonly associated with Epstein Barr Virus (EBV) and *Plasmodium falciparum* infections whose combined effects profoundly affect the B cell compartment (Rasti *et al.*, 2005). A recent study by Moorman and colleagues has shown that eBL is the most common EBV associated cancer in areas with holoendemic malaria transmission (Moormann *et al.*, 2007).

EBV is a gamma herpes virus that infects and latently persists in over 90% of the human population worldwide. Primary infection occurs sub-clinically during childhood upon EBV transmission horizontally through salivary contact during kissing or sharing of feeding utensils (Slots *et al.*, 2006). Other modes of EBV transmission include sexual intercourse, where the viruses have been demonstrated in the male and female genital secretions (Leigh and Nyirjesy, 2009), blood transfusion, (Kühn, 2000; Alfieri *et al.*, 1996), organ-transplants (Hoshida *et al.*, 2001) and through the urogenital during delivery (Leigh and Nyirjesy, 2009)

In Equatorial Africa, primary EBV infections occur in young infants < 3 years old with a developing immune system following a decrease of maternal antibody levels

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(Henle, *et al.*, 1978). Most infections are asymptomatic or mild and go unnoticed, but in young adults typical symptoms of sore throat, fever, swollen glands, and fatigue appear in some cases and last for weeks or months. In symptomatic infections, eBL manifest as disfiguring enlargements of the jaw, cheeks and the abdomen at the age of 5-10 years old (Mwanda *et al.*, 2004). EBV is associated with various other malignancies including Hodgkin's lymphoma, nasopharyngeal carcinoma, and several other lymphomas caused by general immune suppression like those associated with AIDS (MacArthur and Farrell, 2007).

EBV entry into target cells results in lytic or latent infection (Cohen, 2000). In the lytic infection, the viral structural genes are all transcribed, which leads to virions production and results in lysis of host cells. Latent infection is predominantly non-productive and characterized by expression of six EBV determined nuclear antigen genes i.e. (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP); three latent membrane protein genes LMP1, 2A, and 2B, *Bam*HIA rightward transcripts, and two abundant EBV-encoded non-polyadenylated RNAs (EBER*1* and EBER2) (Cohen, 2000). This full pattern of gene expression is expressed in type III latency. In EBV-associated malignancies, latent gene expression is often more restricted (Cohen, 2000). Endemic Burkitt's lymphoma expresses EBERs, EBNA1, and *Bam*HIA rightward transcripts, which is defined as type I EBV latency.

Plasmodium falciparum infections have been associated with high EBV viral loads and lytic reactivation in EBV infected asymptomatic individuals (Chêne *et al.*, 2007). Elevated EBV viral loads have been demonstrated regardless of malaria intensity. EBV viral loads are stably high with repeated exposure to malaria infection and children in both sporadic and holoendemic transmission regions have uniformly high viral loads (Brubaker and Olwit, 1980; Moormann *et al.*, 2005).

The immune system controls the EBV viral loads by killing the infected B cells and preventing the spread of the infectious virus thus generally reducing the level of

infection. It is unable to eliminate the virus completely hence viral shedding in saliva and virus infected cells persist at low levels approximately 1 in 10^4 to 10^5 memory B cells (Babcock *et al.*, 1999).

Allelic polymorphisms in the EBNA2, 3A, 3B, and 3C genes define two broad types of EBV isolates, type 1 and 2, which are present at different frequencies in different geographic locations (Gratama *et al.*, 1990). Other minor changes within each virus type, detected by variations in the size of EBNA proteins, define distinct EBV isolates and these help to trace virus transmission within families (Gratama *et al.*, 1990).

Strain specific variations have been identified in EBNA1, BZLF1 and LMP1. LMP1 is quite variable and widely used to identify strain variants by the presence or absence of a 30bp deletion in the carboxy-terminus (Edwards *et al.*, 1999). Other significant changes in the LMP1 coding sequence include the number of 33bp repeats and the 15bp insertions within the repeats (Edwards *et al.*, 1999). Sub-strains with additional base-pair changes have been found. For example, the B95.8 strain is characterized by additional changes at position 266 of the LMP1 coding sequence (Sitki-Green *et al.*, 2003).

This thesis aimed at determining the prevalence of EBV type 1 and 2, and their transmission in mother-child pairs from holoendemic malaria transmission areas with varying BL incidence rates.

1.2 Study Rationale

Primary asymptomatic EBV infections, mostly associated with endemic Burkitt's lymphoma in African children (Biggar, *et al.*, 1978), are mainly transmitted through saliva especially when mothers pre-chew food for their children and also through kissing and sharing eating utensils (Cohen, 2000). This virus persists in the lymphoid or epithelial cells within the oral cavity and intermittently reactivates leading

to viral shedding in the saliva (Cohen, 2000). Recent studies have shown that Kenyan children 1-4 years old from a holoendemic malaria area have high EBV viral loads in their peripheral blood, as compared to children of the same age from an area with sporadic malaria transmission (Moormann *et al.*, 2005).

There are two main EBV strains namely EBV type 1 and type 2 with major genotypic differences in the expression of their EBNA 3C genes. EBV type 1 is highly virulent compared to type 2 and it is common in developed countries (Zimber *et al.*, 1986). EBV type 2 was first described in Kenya (Yao *et al.*, 1996). Mixed infections are common in immuno-compromised individuals (Sculley *et al.*, 1990; Yao *et al.*, 1996). Different EBV type 1 and 2 levels of expression were associated with Hodgkin's lymphoma in Kenya (Weinreb *et al.*, 1996).

To gain a better understanding of Burkitt's lymphoma etiology, it was necessary to characterize EBV types and determine their prevalence and transmission dynamics in areas with varying BL incidence rates and holoendemic malaria. This study aimed at determining *Pf* malaria prevalence, characterizing the different EBV types based on variations in the size of EBNA proteins in various DNA isolates obtained from whole blood and saliva from healthy children and their mothers in BL high and low risk regions.

1.3 Problem Statement

EBV transmission most probably occurs from mothers to children. Transmission of EBV through the saliva occurs at a high efficiency when they pre-chew food for their children during kissing or sharing feeding utensils (Slots *et al.*, 2006). Many of these children live as healthy carriers (asymptomatic population) with high EBV viral loads in their peripheral blood while only few present with Burkitt's lymphoma (symptomatic population) within the same geographical location (Mwanda

et al., 2005). Burkitt's lymphoma is the most common EBV associated cancer in holoendemic malaria transmission areas (Moormann *et al.*, 2007) and it's etiology has long been associated with EBV and malaria infection (Rasti *et al.*, 2005; Rainey *et al.*, 2007), but there is no uniformity in their case distribution (Mwanda *et al.*, 2004). EBV strain characterization and transmission dynamics have not been done in areas with varying BL incidence rates and holoendemic malaria transmission. This is why this study was designed to characterize EBV strains in mother child pairs from a malaria holoendemic region.

1.3 Study Significance

EBV strain verification in mother-child pairs will provide added knowledge in understanding the etiology of eBL. This knowledge will help in designing appropriate surveillance and preventive interventions aimed at altering BL disease progression to reduce EBV associated cancer burden world wide. It will further identify knowledge gaps to be addressed by future researchers.

1.4 General Objective

The general objective of this study aimed at determining the prevalence of EBV type 1 and 2, and their transmission in mother-child pairs from holoendemic malaria transmission areas with varying BL incidence rates. The effect of malaria infections on EBV specific T cell responses were also determined by Enzyme linked immunospot (ELISPOT) assay.

1.4.1 Specific Objectives

a) To identify and compare EBV types in blood and saliva from different mother-child pairs and males and females in the high and low BL risk regions.

b) To determine the prevalence of EBV infection and type distribution in relation to malaria prevalence in high and low BL risk regions

CHAPTER TWO: LITERATURE REVIEW

2.1 Transmission of EBV

Epstein Barr virus is a ubiquitous human gamma-herpes virus that infects more than 95% of the world's population (Young and Rickinson, 2004). In contrast to sporadic Burkitt's lymphoma (sBL), eBL is associated with EBV infection in almost 100% of cases, as evidenced by the presence of EBV in tumor cells (Brady *et al.*, 2007)

EBV is mainly transmitted through saliva and mainly infects B cells in the oropharyngeal epithelium (Slots *et al.*, 2006). Other modes of EBV transmission include sexual intercourse as the virus has been demonstrated in the male and female genital secretions (Leigh and Nyirjesy 2009), blood transfusion, organ-transplants (Alfieri *et al.*, 1996) and through the urogenital during delivery (Meyohas *et al.*, 1996).

2.2 Epidemiology of EBV infections: Burkitt's lymphoma.

EBV infection is said to be one of the Burkitt's lymphoma etiological agents besides malaria infection (Chene *et al.*, 2009). EBV infection prevalence differs based on differences in genetic and environmental exposure (Koriyama *et al.*, 2005), nutritional status (Williams, 1975) and presence or absence of disease (Zandman *et al.*, *2009)*

Burkitt's lymphoma, the most common EBV associated B-lymphocyte malignant tumor occurring in children (Mwanda *et al.*, 2004), is endemic in Equatorial Africa. There are different types of BL including; endemic Burkitt's lymphoma (eBL) in Africa, sporadic Burkitt's lymphoma (sBL) in developed countries, and AIDS-related BL worldwide. Endemic Burkitt's lymphoma (eBL) distribution is well understood in Africa, where Burkitt discovered the disease's association with EBV and traveled throughout the continent visiting hospitals and documenting the prevalence of eBL (Burkitt, 1983). This led to the naming of the region of high incidence in Africa as

the "lymphoma belt". This region extends from West Africa to East Africa between 10° North and South of the Equator and continues down the eastern coast. Temperature and humidity were associated with the belt and this lead to the association of BL with malaria (Burkitt, 1983). The geographical distribution of BL has further been determined in Uganda (Geser *et al.,* 1980), Democratic Republic of Congo (Renoirte *et al.,* 1971), Sudan (Veress *et al.*, 1976), Nigeria (Durodola, 1978), E. Africa (Geser *et al.,* 1980) and Kenya (Mwanda *et al.*, 2004; Rainey *et al.*, 2007) (see Figure 2.1). Malaria incidences have also been compared to the BL distribution in Kenya (Figure 2.2) (Rainey *et al.*, 2007). EBV infections greatly vary depending on environmental exposure, genetic factors, nutritional status and the presence and absence of disease in different geographical locations (Koriyama *et al.,* 2005; Naithani *et al.*, 2007; Williams, 1975).

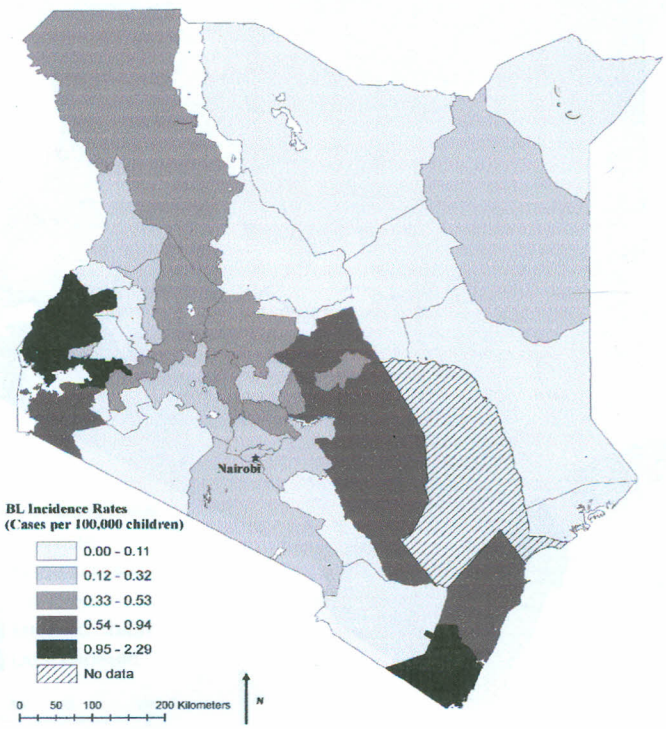

Figure 2.1: Ten-year average annual BL incidence rates, Kenya 1988 – 1997

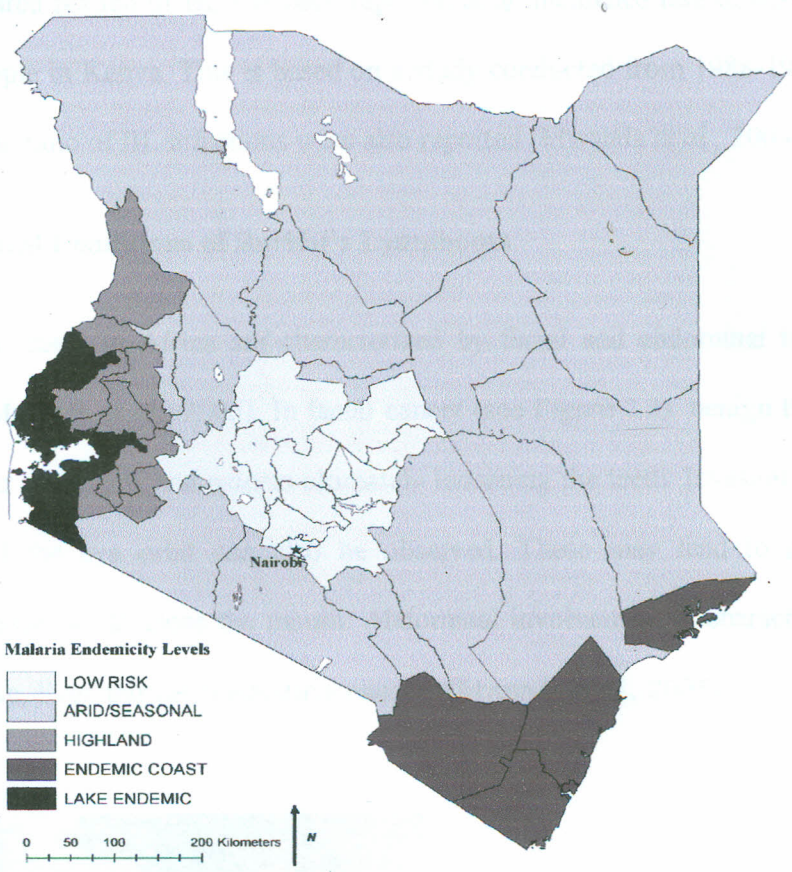

Figure 2.2: Malaria endemicity levels in Kenya, by 1989 district boundaries

An estimated burden of BL has been reported at an incidence rate of 8.3 cases per 1 million people in Kenya. This is based on a study conducted from 1988-1997. A 2:1 male to female ratio of BL infections were also reported (Mwanda *et al.*, 2004).

2.3 Pathological Conditions of Burkitt's Lymphoma

Most eBL cases in Africa are characterized by facial and abdominal tumors (Burkitt, 1958; Mwanda *et al.*, 2005). In facial cancer (see Figure 2.3), benign tumors affect the alveolar of the jaw and extents ultimately loosening the teeth. Invasion of the nasopharynx and the eye orbit can also be observed. These may lead to airway obstruction and inability to close the mouth. Abdominal involvement is characterized by kidney, ovarian, liver and gastrointestinal cancers (Mwanda *et al.*, 2005).

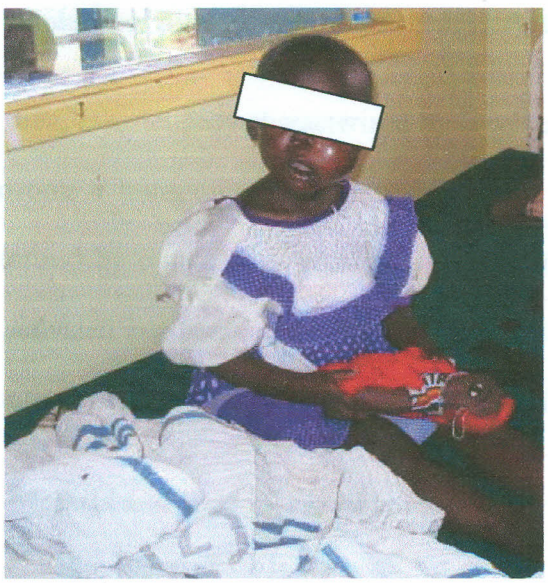

Figure 2.3: A Child with Burkitt's lymphoma (Jaw Tumor)

The EBV viral genome can be detected in eBL tumor cells using molecular techniques such as the polymerase chain reaction (PCR) (Brady *et al.*, 2007). Biopsy preparations show a monomorphic outgrowth of undifferentiated lymphoid cells with little variation in size and shape, an amphophilic cytoplasm with clear vacuoles, and a non-cleaved nucleus containing two to five nucleoli at low magnification and a starry

sky pattern is observed as a result of macrophage invasion (Figure 2.4) (Brady *et al.*, 2007).

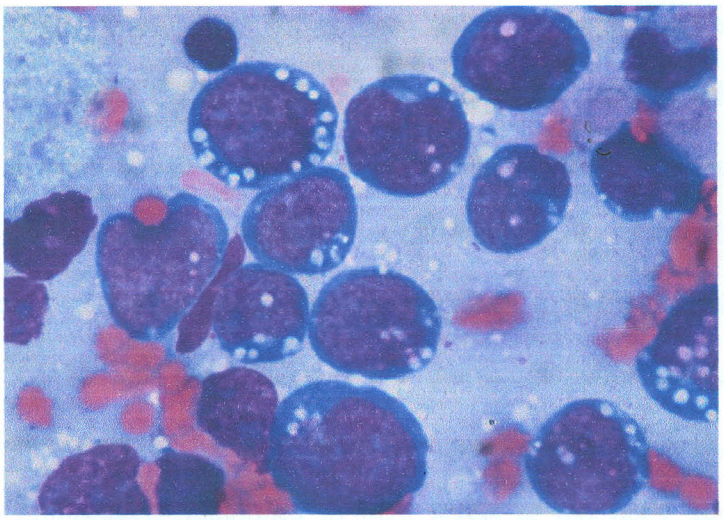

Figure 2.4: Burkitt's lymphoma; Touch Preparation, Wright stain

2.4 Molecular Biology of Endemic Burkitt's lymphoma

EBL is genetically characterized by B cell chromosomal translocation involving chromosome 8 transposing with chromosome 14. Burkitt's lymphoma is particularly characterized by c-myc proto-oncogene translocation (Brady *et al.*, 2007) to a more easily activated position during the chromosomal shift inducing uncontrolled B cell proliferation (de Thé, 1993) forming tumors in the lymph nodes.

2.5 Malaria and Burkitt's lymphoma

The etiology of eBL is highly attributed to *P. falciparum* and EBV infections (Rasti *et al.*, 2005); (Rainey *et al.*, 2007). *P. falciparum* infection cause massive B cell proliferation, mainly through mitogenic effects, thereby increasing the chances that a chromosomally altered B cell will be increased in number and then no longer subjected to growth control mechanisms (Morrow, 1985).

Plasmodium falciparum antigens such as cistein rich interdomain region I alpha (CIDR1α) can directly induce EBV reactivation during malaria infection that may increase the risk of BL development for children living in malaria-endemic areas (Chêne *et al.*, 2007). During the blood stage of *P. falciparum* malaria, infected red blood cells (iRBCs) express high levels of PfEMP1 reaching their maximum at the trophozoite stage (28-32hours post invasion). The CIDR1α domain of PfEMP1 (clone FCR3S1.2-varl) has a mult-adhesive phenotype and binds to different cell surface receptors, such as CD36, PECAM-1 (CD31) and immunoglobulins (Donati *et al.*, 2004). When immune B cells infected with latent EBV interact with CIDR1α, the virus is reactivated and immediately multiplies to high levels in the body significantly increasing the risk of BL development.

Primary infection with EBV leads to the immortalization of large numbers of B lymphocytes. Severe falciparum malaria then leads to an intense host response with particular proliferation of the EBV-infected B lymphocytes and finally, the great increase in the B lymphocytes provides a much higher statistical opportunity for the emergence of the cytogenetically abnormal BL cell non responsive to the normal growth mechanisms (Morrow, 1985).

The trend of malaria mainly relate to the overall high incidence rate of EBV infections in malaria endemic regions (Rainey *et al.*, 2007). High EBV loads are associated with high malaria cases (Moormann *et al.*, 2005). At 1-4 years, children experience high malaria attacks and thus have high EBV loads, and at 5-7 years old, they develop malaria immunity and therefore the incidence of malaria attacks reduce, consequently, the EBV viral load also reduce and remain at a constant level as in children 10-14 years (Moormann *et al.*, 2005)

2.6 The EBV Antigens and Life Cycle

On oral transmission, EBV replicates in the oropharynx within specialized epithelial cells that directly bind to the virus or indirectly get it through transfer from the surface of adjacent infected B cells (Walling *et al.*, 2007). Lytic infection leads to increased viral shedding into the throat (de Souza *et al.*, 2007). These viruses infect mucosal B cells and initiate a latent growth transforming infection in lymphoid tissues and appearance of large numbers of infected cells (de Souza *et al.*, 2007).

Following infection, the body mounts cellular immune response to both lytic and latent antigens (Young and Rickinson, 2004). Most infected B cells are eliminated, while some down regulate latent antigens and remain in circulation as members of the long-lived memory B cell pool. Reactivation into the lytic cycle occurs through antigen stimulation (Chêne *et al.*, 2007) or reception of plasma cell differentiation signals for antibody production (Thorley-Lawson, 2001). This leads to virus shedding in the throat of healthy carriers, and enables transmission of virus to new hosts through saliva and other modes of transmission. At this stage, the lytic antigen specific CD8 T cells come into play to regulate the production of the virions via the plasma cells from the memory B cells (Young and Rickinson, 2004).

2.7 EBV-encoded Latent Antigen Expression and Survival Strategies

EBV latent infection has been characterized into three different pathways i.e. type 1 latency, type 2 latency and type 3 latency (Kelly *et al.*, 2006; Werner *et al.*, 2007).

2.7.1 Type 1 latency

Type 1 EBV expression does not influence the differentiation of B cells and therefore, there is no virus proliferation. Cells displaying type 1 latency are found among the memory B cell pool, in which expression of all EBV-encoded proteins is

down regulated, with the exception of EBNA 1, which is essential for the replication of EBV episomes (Walling *et al.*, 2001a). The full set of the virally encoded proteins can be induced upon activation of the cell through antigen stimulation (Chêne *et al.*, 2007) or plasma cell differentiation signals for antibody production (Laichalk *et al.*, 2005).

EBV specific T cell immunity can not recognize type 1 cells in the absence of co-stimulatory molecules in the memory compartment. EBNA 1 has long glycine-alanine repeats that prevent the antigen processing in the proteosome to generate peptides that associate with MHC class 1 molecule (Levitskaya *et al.*, 1995), hence not recognized by the $CD8^+$ cells. This enhances EBV persistence through maintenance of infected B cells in healthy individuals.

2.7.2 Type II latency

This is divided into IIa and IIb with respect to antigen expression. Type IIa latency program seen in nasopharyngeal carcinoma (NPC) and Hodgkin's lymphoma (HL) expresses EBNA 1, LMP 1 and LMP 2 EBV antigens (Walling *et al.*, 2001a); (Sitki-Green *et al.*, 2003). Cells in type II latency are not induced to proliferation unless additional cellular changes occur in the cell and cell growth promoting signals are provided by the microenvironment. EBNA 2 not present is required to induce proliferation of EBV infected B cells (Werner *et al.*, 2007).

Cells with type IIb latency express all EBNAs except LMP 1 (Sitki-Green *et al.*, 2003). Like IIa cells, they do not induce proliferation due to the absence of LMP 1 required for B cell transformation. From studies, EBNA 2 is responsible for activating the LMP 1 promoter region in B cells but its role in the absence of LMP1 is not well defined (Sitki-Green *et al.*, 2003).

2.7.3 Type III latency

Type III EBV latency is defined by the expression of all EBV encoded latent proteins including all the EBNAs, i.e. EBNA 1, 2, 3A, 3B, 3C, LP (Thorley-Lawson,

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2001) and the membrane associated proteins LMP1, LMP2a and LMP2b (Kelly *et al.*, 2006). This state of EBV expression is only found in the B lymphocytes where LMP 1 is able to induce activation markers and co-stimulatory molecules making it more immunogenic (Young and Rickinson, 2004). This type of latency is typical of *in vitro* EBV-immortalized B-lymphoblastoid cell-lines. *In vivo*, type III latency cells only exist during the acute phase of primary infection, before EBV-specific T cell responses develop and in patients with impaired immune functions e.g. transplant recipients treated with immunosuppressive agents (Young and Rickinson, 2004).

2.8 EBV Strains and Genetic Polymorphism

Genetic analysis has shown that the EBV genome consists of highly conserved regions, regions of unique variants, and highly polymorphic regions. Highly polymorphic regions transform over time to give different EBV genotypes associated or non-associated with disease (Levitskaya *et al.*, 1995). Allelic polymorphisms in the EBNA2, 3A, 3B, and 3C genes define two broad types of EBV isolates, type 1 and 2 which are present at different frequencies within different geographic locations (Gratama *et al.*, 1990). Other minor changes within each virus type, detected as variations in the size of the EBNA proteins, give rise to other distinct EBV strains, which allow tracing virus transmission within families (Gratama *et al.*, 1990). Multiple and single strain EBV transmission occur in different individuals within varied families (Gratama *et al.*, 1990).

Strain specific variations have been identified in EBNA1, BZLF1 and LMP1 (Edwards *et al.*, 1999). LMP1 is the most discriminating locus for EBV genotyping. It is widely used to identify strain variants by the presence or absence of a 30 bp deletion in the carboxy-terminus. Other significant changes in the LMP1 coding sequence include the number of 33bp repeats and the 15bp insertions within the repeats (Edwards *et al.*, 1999). Sub-strains have been found to exist with additional base-pair changes in

addition to the sequence changes in the known strains. This has been demonstrated by the identification of a B95.8 strain with additional changes at position 266 of the LMP1 coding sequence (Sitki-Green *et al.*, 2003).

This study mainly focused on the latent-cycle gene encoding the virus genome maintenance protein EBNA3C which is involved in EBV transformation of primary human B lymphocytes. EBV DNA present in peripheral blood and saliva was extracted using QIAamp DNA mini kit, to determine EBV types by PCR (Brady *et al.*, 2007). *P. falciparum* infections were determined by microscopy and EBV specific CTL responses determined by IFN-γ ELISPOT assay (Moormann *et al.*, 2007).

2.9 Immune Response to EBV

Generally, EBV infection elicits innate, humoral and cellular immune responses to fight against the virus and its pathological effects. This helps the body to control the virus and mainly prevent lymph proliferation.

2.9.1 Innate Immune Responses

Natural Killer cells form the major component of the early innate immune response to many microbial agents including EBV. NK cell populations inversely correlate with EBV viral load (Williams *et al.*, 2005). NK cells are known to inhibit EBV- induced resting B cell transformation through release of IFNγ in the presence of dendritic cells. IL-12 produced by dendritic cells promotes differentiation of T cells into T_H 1 cells. Like NK cells, Th 1 cells also produce the IFNγ which acts to activate the functional activity of macrophages to kill microbes (Lotz *et al.*, 1985).

Reduction in surface HLA class I expression in lytically infected cells *in vitro* is accompanied by increased sensitivity to NK cell recognition. Unlike Cytomegalovirus, EBV has not evolved any NK immune evasion strategies (Lotz *et al.*, 1985).

2.9.2 Adaptive Immune Responses to EBV Infections.

2.9.2.1 Humoral Immune Responses to EBV Infections

Humoral immune responses to EBV infections involve the individuals' antibody production against EBV. People infected with the virus have high antibody titers against viral capsid antigens (VCA) and early antigens (EA). Following primary infection IgM to the VCA are first detected followed by VCA-specific IgG antibodies (Hislop *et al.*, 2007a). Later, there is IgG to EBNA 1 and in some cases, antibodies to early antigen EA also appear, but not in all infected individuals. In healthy EBV carriers, IgG to VCA and EBNA 1 are always present (Hislop *et al.*, 2007b).

2.9.2.2 Cellular Immune Responses to EBV Infections

EBV infection induces HLA class 1 restricted primary and memory $CD8^+$ T cell responses. In acute infection, there is high viral replication coupled with high levels of EBV epitope specific reactivities detectable functionally in ex-vivo cytotoxicity assays and finally, they are visible through HLA-class 1 tetramer staining as numerically dominant populations (Hislop *et al.*, 2007b).

During acute infection, 1%-40% of the total $CD8^+$ T cell populations in circulation have reactivity to Immediate Early (IE) and early (E) antigens of the lytic cycle whereas 0.1%-5% to latent cycle antigens specifically EBNA 3A, 3B and 3C antigens. These $CD8^+$ T cells are perforin positive with direct *ex-vivo* function in cytotoxicity (Hislop *et al.*, 2007a) and cytokine secretion assays (Catalina *et al.*, 2002; Woodberry *et al.*, 2005). They express activation markers (CD38), cell cycling markers (Ki-67) and the CD45RO isoform indicating recent antigenic stimulation and die rapidly by apoptosis *in vitro* when antigen stimulation is reduced (Hislop *et al.*, 2007c). After acute infection, $CD8^+$ T cells express low levels of anti-apoptotic Bcl-2 and Bcl-x proteins and high levels of pro-apoptotic Bax proteins (Hislop *et al.*, 2007b). Lytic

epitope-specific responses are heavily culled leading to a dramatic reduction in the $CD8^+$ T cell numbers. The low latent epitope responses are less heavily reduced and remain highly represented in the circulating pool of EBV-specific $CD8^+$ T cells. This remaining $CD8^+$ T cell pool loose expression of the activation and cell cycling markers of the activated state (Hislop *et al.*, 2007a).

EBV antigen-specific $CD4^+$ T cell responses are HLA II restricted and have not been analyzed in so much detail as the $CD8^+$ T-cell responses (Walling *et al.*, 2001b). This is due to several factors, including lower frequencies of EBV-specific $CD4^+$ T cells, as the pool of EBV-specific $CD4^+$ T cells does not expand as markedly as the $CD8^+$ T cell pool during acute EBV infection (Hislop *et al.*, 2007c). Therefore, HLA class II-restricted EBV epitopes have only recently started to be determined. In addition, production of HLA-II tetrameric reagents has proven much more difficult than for HLA class-I (Hislop *et al.*, 2007a). EBV elicits primary and memory $CD4^+$ T cell responses and during acute EBV infection, $CD4^+$ T cell responses to the immediate early (IE) protein BZLF1 are more common compared to BMLF1 or latent protein EBNA3A-specific responses, while EBNA1-specific responses are least detected. EBV-specific $CD4^+$ T cell populations rapidly increase during acute infection and markedly decline thereafter. Therefore, low frequencies of EBV specific $CD4^+$ T cells are detected in healthy virus carriers (Hislop *et al.*, 2007a).

Most EBV specific $CD4^+$ T cells produce IFNγ and TNFα, while few produce IL-2, which is important for T cell proliferation and potentiation of apoptotic cell death of antigen activated T cells and express $CD45RO^+$, $CD27^+$ and $CD28^+$ surface molecules. EBNA1 and EBNA 3C specific $CD4^+$T cells are mostly Th1 and secrete IFN-γ (Hislop *et al.*, 2007c).

CHAPTER THREE: MATERIALS AND METHODS.

3.1 Study Area and Population

This study was conducted in Kanyawegi location (hot spot area) with high BL risk and Seme location (cold spot area) with low risk for BL within Kisumu District, Western Kenya (Figure 3.1) (Mwanda *et al.*, 2004). Hot and cold spot eBL areas have been defined depending on the eBL incidences per 10,000 children. Hot spots have high risk of eBL cases compared to the cold spots with no eBL incidence reported. (Rainey *et al.*, 2007). Earlier reports have shown that both sites in Western Kenya have holoendemic malaria transmission patterns with residents getting 100-300 *Plasmodium falciparum* mosquito bites annually causing 97% of the malaria infections (Bloland *et al.*, 1999) and more intense malaria transmission seasons in these areas coincide with the rainfall seasons occurring within April-August and November-January as earlier reported by (Bloland *et al.*, 1999).

The population of Kanyawegi (Burlitt's lymphoma 'Hot spot area') was 8,891 and that of Seme location (Burkitt's lymphoma 'cold spot area') was 9,464 according to the population census activity conducted in the year 2006. BL incidence rates determined in a study carried out by Mwanda and colleagues from 1988 to 1997 revealed that; in Kanyawegi location, the BL incidence rates were between 0.95 and 2.29 cases per 100,000 children and in Seme location, the BL incidence rates were between 0.00 and 0.11 per 100,000 children. Both study areas are located in a stable malaria holoendemic transmission region of Western Kenya.

This cross-sectional study began by community mobilization and demographic survey. The GPS mapping was done for ease of house hold location (Figure 3.2 and 3.3). Excel computer based random sampling was employed. The random numbers were attained by selecting the empty column from study identity and then state the start

and end of selection [=RAND (A1:A100). Hold and drug down the curser to generate the random numbers.

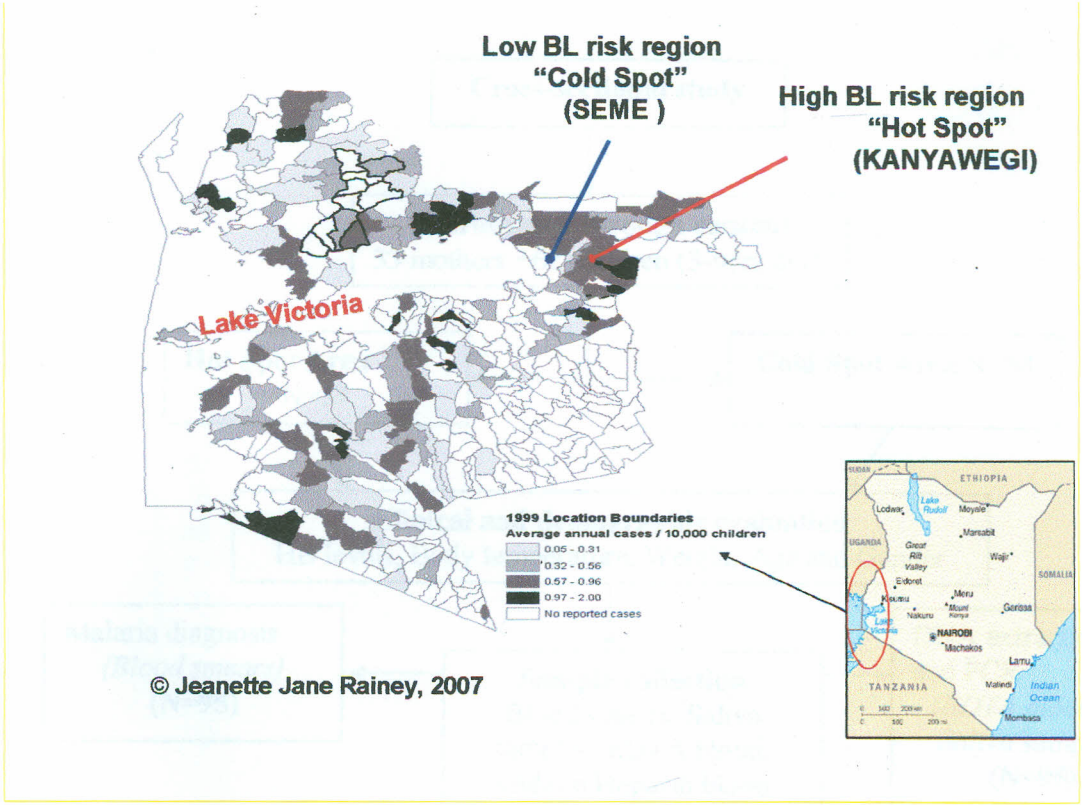

Figure 3.1: A Map of Nyanza Province in Kenya Showing 'Hot' and 'Cold' Spot Areas

3.2 Study Design

This cross sectional study recruited 58 participants from the cold spot region and 37 participants from the hot spot region. Figure 3.2 gives a flow diagram which summarizes the study design.

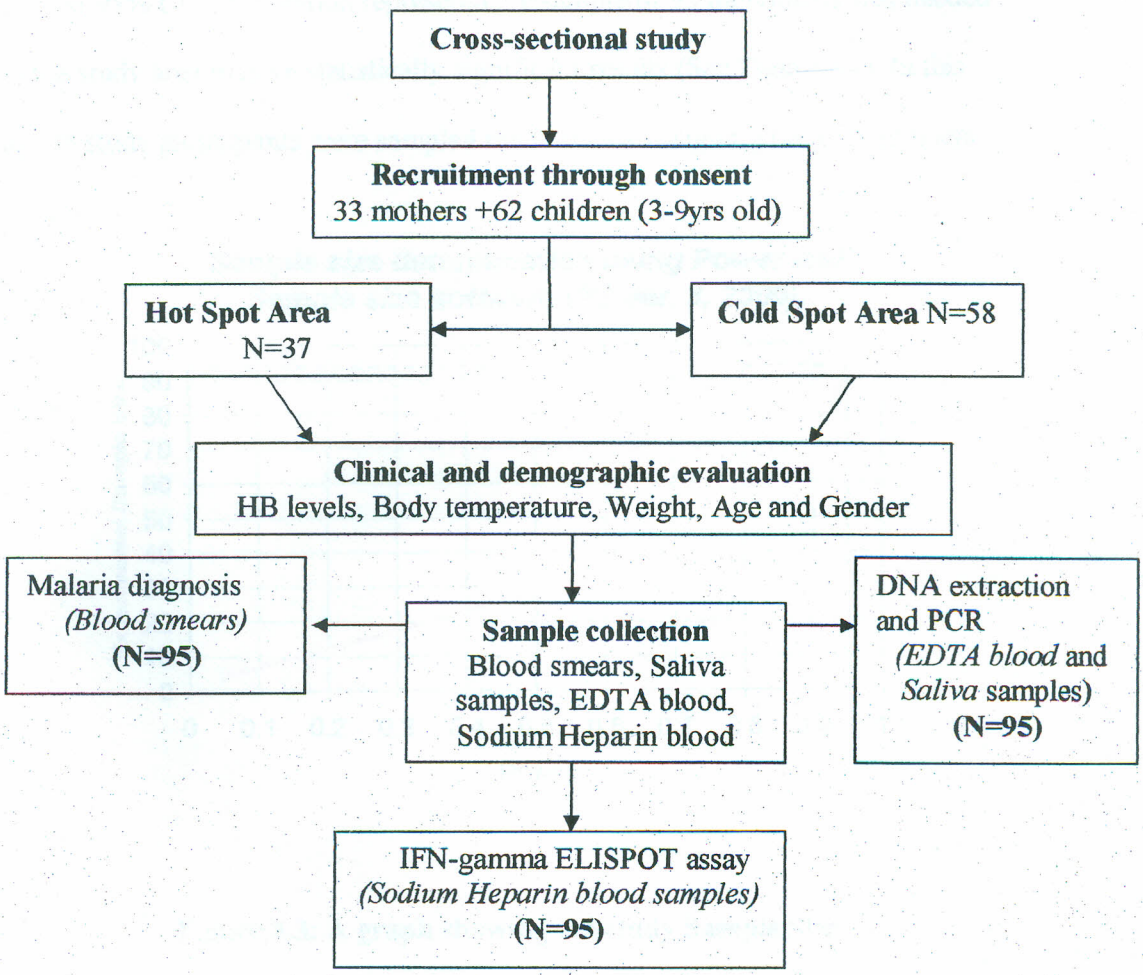

Figure 3.2: Study Design Flow Chart

3.3 Sample Size Determination

The sample size determination was done using power and sample size program software that performs Power and Sample size calculations for many different study designs (Dupont and Plummer, 1990 and 1998)

At 95% CI, a population representative sample of 93 individuals was needed from the study area to have statistically significant results (See Figure 3.3). In this study, 95 study participants were sampled randomly by assigning random numbers.

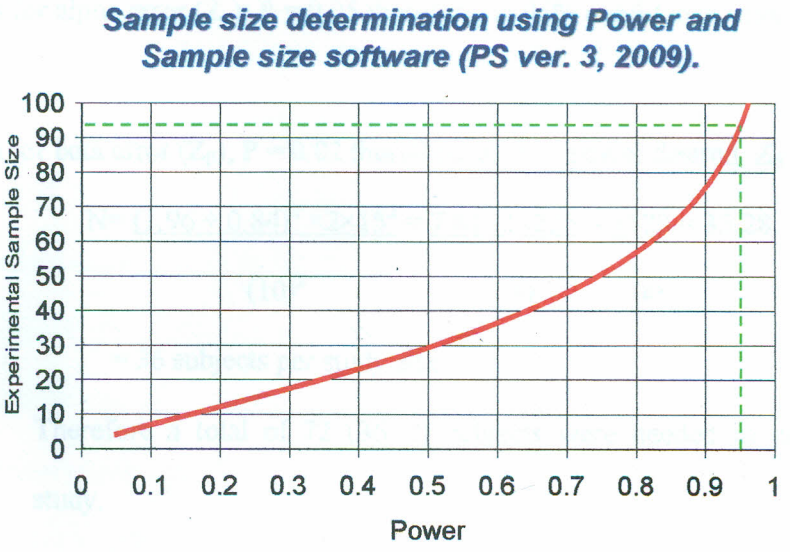

Figure 3.3: A graph showing the study Sample Size

Freeman and Jekel's formular for sample size calculation for to test differences in proportions was used to determine the sample size for the hot and cold spot study regions (Freiman et al., 1978; Jekel et al., 1983).

Formula: $\underline{N= (Z_\alpha + Z_\beta)^2 *2*S^2}$

$(d)^2$

Where; N is the sample size, Z_α is the alpha error, Z_β is the beta error, S^2 is the variance and $(d)^2$ is the difference to be detected.

Recent data has shown that BL incidence rates in the hot spot are between 0.95 and 2.29 cases per 100,000 children and in the cold spot area 0.00- 0.11 cases per 100,000 children (Mwanda *et al.*, 2004; Rainey *et al.*, 2007). Assuming that the predictor of the BL clusters is based on Nyanza province cancer data where more than 30 % of the children present with cancer. The assumption for this study is that a minimum of 40% of the children have BL and 50% of the children are asymptomatic. The expected standard deviation (S) among the BL cases in the two study sites was 15 and the expected variance (S^2) was 225. The difference (d^2) to be detected is therefore 10%.

Data for alpha error (Z_α); P = 0.05 therefore, at 95% confidence Interval, Z_α = 1.96.

Data for beta error (Z_β); P = 0.02 therefore, at 80% power desired, Z_β = 0.84

$$N = \frac{(1.96 + 0.84)^2 \times 2 \times 15^2}{(10)^2} = \frac{7.84 \times 2 \times 225}{100} = \frac{3528}{100} = 35.28.$$

= 36 subjects per study site.

Therefore a total of 72 (36×2) subjects were needed to conduct the study.

3.4 Inclusion Criteria

Children aged between 3-9 years old with their mothers who had stayed together for the last one year were recruited. Only those with normal body temperatures of 36-37.4°C and permanent residents of this study area were recruited. Before sample collection, study participants with fever were given anti-malarial drugs for malaria prophylaxis. All children were required to have consent from their parents/guardians to participate in the study.

3.5 Exclusion Criteria

Participants presenting with severe illness including anemia (less than 6g/dl) were excluded from the study. Children above the age of 9 years were excluded from the study.

3. 6 Collection and Processing of Blood and Saliva Samples

Hemoglobin levels, weight and body temperatures of the participants were recorded prior to the collection of samples to detect any health aberration. Five milliliters of venous blood was collected and 500µL aliquots stored in EDTA tubes at -80°C for DNA extraction. 4.5 mLs were liquated into sodium heparin tubes for peripheral blood mononuclear cells (PBMC) isolation and culture techniques. Thick and thin blood smears were prepared from peripheral blood immediately after collection before dispensing in to the anti-coagulant tubes. This were air dried, the thin films were then fixed in absolute methanol and then stained in 3% giemsa stain for 1 hour. Microscopy was then done at ×100 magnification in oil immersion to detect the malaria parasites (Barcia, 2007).

For saliva collection, communication was very important and therefore an age of at least 3 years was appropriate for ease of collection. The saliva specimen was collected by placing a cellulose pad affixed to a polypropylene stem (sterile saliva collector) under the tongue of an individual until the saturation of the cellulose pad, the defined volume of the saliva taken up by the cellulose pad was indicated by blue coloration indicator in a window on the stem as described by the manufacturer (Malvern Medical Developments Company). Saliva was squeezed out of the cellulose filter paper pad into a 1.5ml tube and stored at -20 ^{0} C until DNA extraction.

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3.6.1 Sample Preservation and Storage

Blood samples for DNA extraction were preserved with ethylene di-amine triacetic acid (EDTA). EDTA has a high affinity towards divalent ions like Ca2+, Mn2+, and Mg2+ which are co-factors for many active enzymes inside the cells including nucleases which digest the DNA molecules. Once the cell is disrupted nuclear envelope goes off and the nuclear contents come to contact with the cellular content which is rich in nucleases. The broken cell is treated with EDTA that chalets the ions so that the nucleases loose their function hence enhances the DNA yields.

Blood for peripheral blood mononuclear cell (PBMC) isolation were preserved in sodium heparin which ensures high recovery, viability and functionality of PBMC's required for reliable assessment of cell mediated immune responses.

3. 7 DNA Extraction

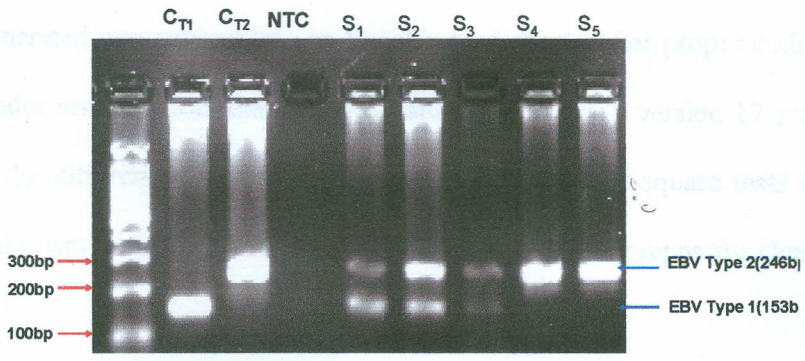
DNA extraction from the saliva and peripheral blood samples was done using the Q1Aamp DNA blood mini kit (QIAGEN Sample and Assay Technologies, USA). Processed samples from saliva and blood were stored at -20°C and -80°C respectively, and thawed at room temperature before DNA extraction. 200µL of sample was mixed in a micro centrifuge tube with 20µL of Q1AGEN protease mainly to digest the DNA proteins within the cell. 200µL of the lysing buffer was added to the contents in the micro-centrifuge tube and votexed for 15 seconds and then incubated at 56^0C for 10 minutes to enhance DNA yield. 200µL of ethanol (96-100%) was added to facilitate dehydration and precipitation of all the impurities such as the lipids. At this point, the mixture was applied to a Q1Aamp spin column and centrifuged at 6000xg for 1 minute (Eppendorf Centrifuge 5417R). Washing was done using 500µL of wash buffer twice to purify the extracted DNA. Finally, elution was done by liquating 100µL of the eluting buffer twice to make a final volume of 200µL of the DNA collected. The

eluting buffer mainly repercolates the DNA from the Q1Aamp spin column into the collecting tube (Clausen *et al.*, 2007)

3. 8 Conventional Polymerase Chain Reaction for EBV Genotyping

The EBV viral genome can be detected in eBL tumor cells using molecular techniques such as the PCR (Brady *et al.*, 2007). Two micro-liters (2μL) of the DNA were used for DNA amplification on a PTC-100™ machine. Samples were tested in singlet using primers specific for a portion of the EBNA 3C gene (Hassan et al. 2006). EBNA3C: product size: (type I - 153bp / type II - 246bp): Forward primer; 5`-AGAAGGGGAGCGTGTGTTG-3` and reverse primer: 5`GGCTCGTTTTTGACGTCGG-3. These primers were designed using Primer Express® Software (PE Applied Biosystems; Invitrogen laboratories, Foster City, CA) to detect the 153bp and 246 bp regions of the EBV EBNA3C genes, respectively, according to the methods of Hassan and colleagues (Hassan *et al.*, 2006).

The PCR reaction cycle was as follows: 2 min at 95°C for initialization; 1 min at 95°C for denaturation, 1min at 60°C for annealing and exponential amplification for 39 cycles; and final extension at 72°C for 7 min. JumpStart™ ReadyMix™ (Sigma) was used for processing and digesting the DNA. DNA bands were analyzed using a UV trans-illuminator and photographs taken for all samples (see Figure 3.4 for a representative gel) (Hassan *et al.*, 2006)



(Intensity of the bands suggest the EBV type quantity in the sample.)

Figure 3.4: Representative Gel Showing EBV Type 1 and Type 2 Bands.

3.9 Ethical Considerations

Study approval was obtained from the Kenya Medical Research Institute National Ethical Review Committee and Ethical Review board of SUNY Upstate Medical University hospital, U.S.A. Written informed consent was obtained from parents or guardians of study participants. Blood collection was carried out by trained and qualified phlebotomists from the Ministry of Health, Kenya, to reduce risks of bleeding incidents. All sharps were stored in biohazard sharps' containers before disposal at the KEMRI incinerator. Study participation was voluntary; participants were allowed to withdraw at any time during the study. Health care access was not dependent on participation. Any child presenting with Burkitt's lymphoma was taken to New Nyanza Provincial General Hospital (Kisumu, Kenya) for further investigation and treatment. To ensure confidentiality, all the samples collected were coded for identification using the study ID number and investigators controlled access to the data. No sample was labeled with the participants' name.

3.10 Data Management and Analysis

Data generated was entered into an Excel spread sheet. After proper coding for the variables under investigation, the data was transferred to SPSS version 17 software for analysis of the differences in proportions using Pearson's Chi-square tests at $P < 0.05$. Graph-pad prism 5 analytical software was used to generate graphs for clear data presentation.

CHAPTER FOUR: RESULTS

4.1 Study Sample Characteristics and Basic results

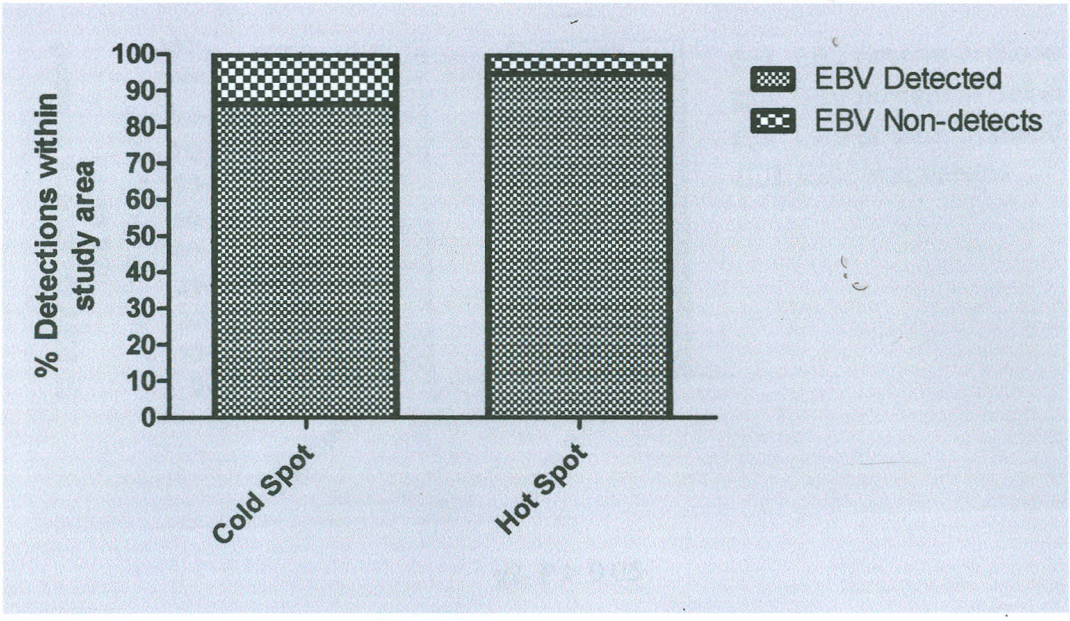
Table 4.0 shows study sample characteristics and basic results. The study sample comprised of 37 and 58 participants from the BL high risk region (hot spot) and the BL low risk region (cold spot), respectively. Thirteen (13) mothers and their 24 children were recruited from the hot spot; 20 mothers and their 38 children were recruited from the cold spot. In total, 39 mothers and their 62 children were recruited in this study. Considering the gender, 15 male and 22 female participants were recruited from the hot spot, 24 male and 34 female participants were recruited from the cold spot. In total, 39 male and 56 female participants were recruited in this study. A total of 190 blood and saliva samples were collected from the participants each giving blood and saliva. After PCR analysis, (85) i.e. 35 and 50 participants from the hot spot and cold spot, respectively, had detectable levels of EBV DNA (EBV positive). Two (2) participants from the hot spot and 8 participants from cold spot had non detectable levels of EBV DNA (EBV negative). Malaria parasites were detected in 43 participants while 52 did not have the parasites. Interferon gamma responses were observed in 10 malaria positive and 40 malaria negative individuals (Table 4.0).

Table 4.0: The study sample characteristics and basic results.

Study region	*Hot Spot*	*Cold Spot*	*N*
Study Population	***37***	***58***	***95***
Age: Adults	*13*	*20*	*33*
Children	*24*	*38*	*62*
Gender: *Male*	*15*	*24*	*39*
Female	*22*	*34*	*56*
Samples: *Saliva*	*37*	*58*	*95*
Blood	*37*	*58*	*95*
Total No. of samples	***74***	***116***	***190***
PCR; *EBV +ve*	***35***	***50***	***85***
EBV -ve	2	8	10
Microscopy;	***Malaria Positive***	***Malaria Negative***	
	43	***52***	***95***

4.2 Overall Prevalence of EBV Detections in the Study Area

EBV DNA was detected at a higher frequency in samples from the hot spot (94.6%) compared to cold spot (86.2%) (P = 0.018) (Figure 4.1). EBV DNA detection in both hot and cold spots dominated in the blood compartment with a mean prevalence of 75.15%, compared to the saliva compartment with a mean prevalence of 66.1%. EBV DNA prevalence in blood from the hot spot was 86.5%, compared to 63.8% in the cold spot (P = 0.001). In the saliva compartment, the hot spot had a prevalence of 81.1% compared to the cold spot with a prevalence of 51.1% (P = 0.001), Table 4.1. Chi-square analysis showed a high significant difference for EBV detection in both blood and saliva samples from the hot compared to the cold spot indicating high EBV detection in hot spot blood and saliva samples compared to the cold spot samples ($\chi 2$, P < 0.05) (Figure 4.2).



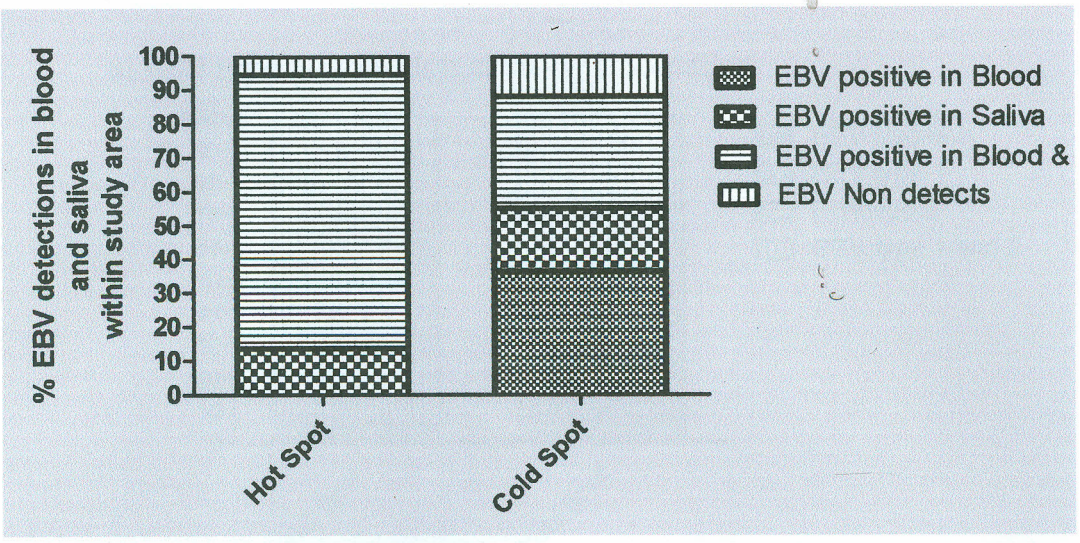
$\chi 2$, $P < 0.05$

Figure 4.1: EBV detection in the study area

Table 4.1: Prevalence of EBV DNA in blood and saliva samples from hot spot and cold spot areas

	Total No.	EBV positive in Blood and / or saliva	EBV positive in Blood	EBV positive in saliva
Hot spot	37	35 (94.6%)	32 (86.5%)	30 (81.1%)
Cold spot	58	50 (86.2%)	37 (63.8%)	30 (51.1%)
Mean prevalence		90.4%	75.15%	66.1%

$\chi 2$, $P < 0.05$



$\chi 2$, $P > 0.05$

Figure 4.2: EBV detections in blood and saliva between the two study regions

4.3 EBV Type Distributions

EBV type 1 and 2 multiple infections were highly detected in the hot spot (94.6%) compared to the cold spot (51.7%) ($P = 0.001$). Singe EBV type 1 and type 2 were detected in the cold spot at low frequencies i.e. 31% and 3.4% respectively. There were no single infections evident in the hot spot (Figure 4.3a).

High EBV type 1 and 2 multiple infections were evident in 66.7% of the adults and 69.4% of the children ($P = 0.927$). Single infections were detected at low levels in both adults and children. Type 1 was detected in 21.2% of the adults and 17.7% of the children and type 2 in 3.0% of the adults and 1.6% of the children ($P > 0.05$) (Figure 4.3b).

Like in adults and children, EBV type 1 and 2 multiple infections were highly detected in 71.8% of the males and 66.1% of the females ($P = 0.0553$). Type 1 single infections were detected in 20.5% of the males and 17.9% of the females. Type 2 single infection was detected in 3.6% of females and not in males ($P > 0.05$) (Figure 4.3c).

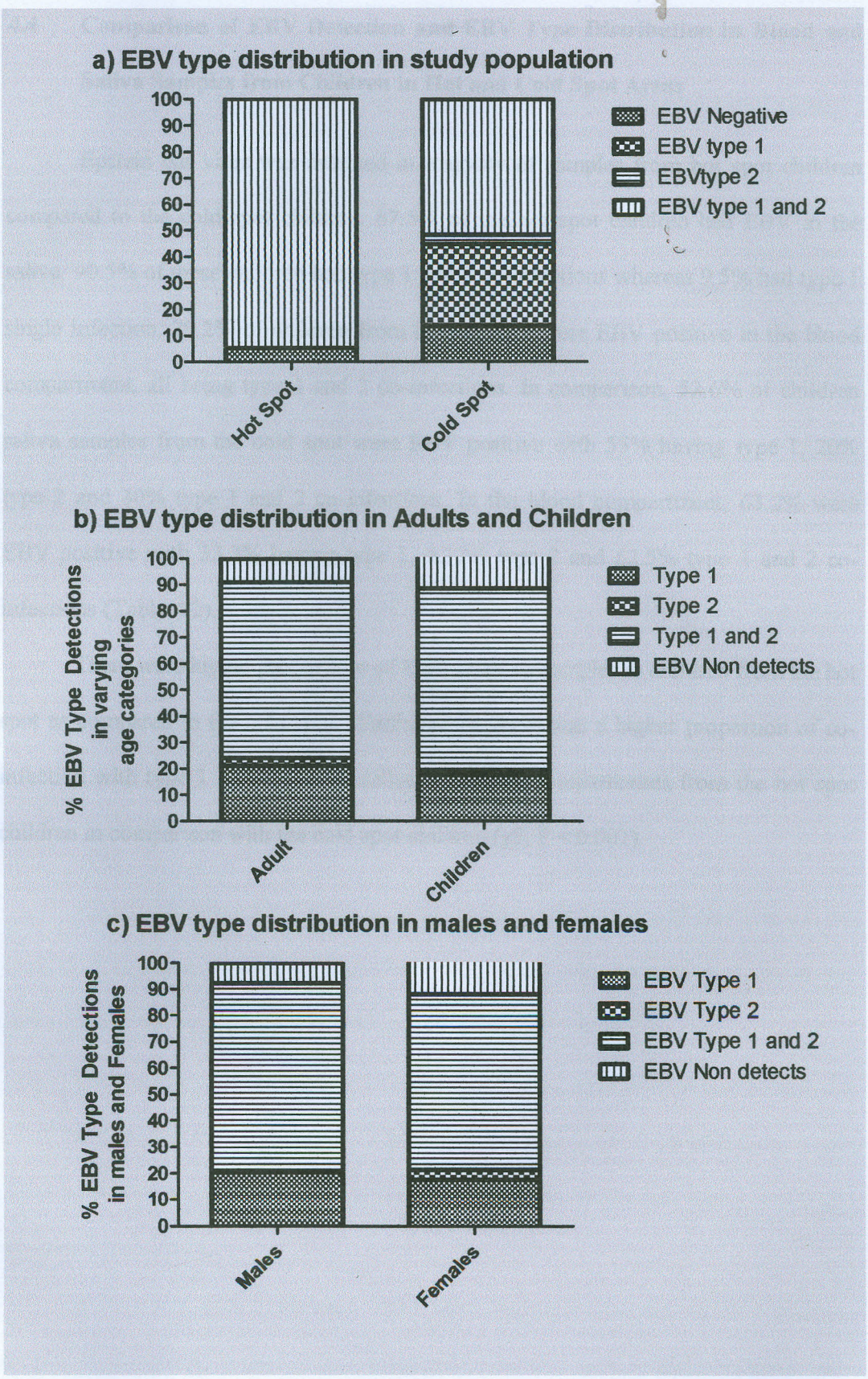


Figure 4.3: EBV type distributions

4.4 Comparison of EBV Detection and EBV Type Distribution in Blood and Saliva Samples from Children in Hot and Cold Spot Areas

Epstein bar virus was detected in majority of samples from hot spot children compared to the cold spot children. 87.5% of the hot spot children had EBV in the saliva. 90.5% of these children had type 1 and 2 co-infections whereas 9.5% had type 1 single infection. 79.2% of children from the hot spot were EBV positive in the blood compartment, all being type 1 and 2 co-infections. In comparison, 52.6% of children saliva samples from the cold spot were EBV positive with 55% having type 1, 20% type 2 and 30% type 1 and 2 co-infections. In the blood compartment, 63.2% were EBV positive with 33.3% having type 1, 4.17% type 2 and 62.5% type 1 and 2 co-infections (Table 4.2).

There were higher proportions of EBV positive samples in children from the hot spot as compared to the cold spot. Furthermore, there was a higher proportion of co-infection with type 1 and 2 in both saliva and blood compartments from the hot spot children in comparison with the cold spot children ($\chi 2$, $P < 0.001$).

Table 4.2: EBV detection and type distribution in children's blood and saliva samples from hot and cold spot areas

Study site	N	Compartment	EBV positive	Type 1	Type 2	Type 1 and 2 co-infections
Hot Spot	24	Saliva	21 (87.5%)	2 (9.5%)	0 (0%)	19 (90.5%)
		Blood	21 (87.5%)	2 (9.5%)	0 (0%)	19 (90.5%)
Cold Spot	38	Saliva	20 (52.6%)	11 (55%)	4 (20%)	6 (30%)
		Blood	24 (63.2%)	8 (33.3%)	1 (4.17%)	9 (62.5%)

$\chi 2$, $P < 0.001$

4.5 Comparison of EBV Detection and Type Distribution in Mothers' Blood and Saliva Samples from Hot and Cold Spot Areas

Blood and saliva EBV prevalence in mothers was uniform (84.6%) in the hot spot, all being type 1 and 2 multiple infections (100%). In the cold spot, blood and saliva EBV DNA prevalence was 65% and 50%, respectively.

Investigation of EBV type distribution in the cold spot showed that 30% of the saliva samples had type 1, 40% type 2 and 30% had type 1 and 2 co-infections. In the blood compartment, 23% had type 1, 67.7% type 2, and 69.2% had type 1 and 2 co-infections (Table 4.3).

In summary, like in children, there were higher proportions of EBV positive samples from mothers in the hot spot compared to the cold spot. In addition, there were higher proportions of EBV type 1 and 2 co-infections in both blood and saliva samples from the hot spot mothers compared to samples from cold spot mothers ($\chi 2$, $P<0.05$).

Table 4.3: EBV detection and type distribution in mothers' blood and saliva samples from hot and cold spots.

Study site	N	Compartment	EBV positive	Type 1	Type 2	Type 1 and 2 co-infections
Hot Spot	13	Saliva	11 (84.6%)	0	0	11 (100%)
		Blood	11 (84.6%)	0	0	11 (100%)
Cold Spot	20	Saliva	10 (50%)	3 (30%)	4 (40%)	3 (30%)
		Blood	13 (65%)	3 (23%)	1 (67.7%)	9 (69.2%)

$\chi 2$, $P < 0.001$

4.6 Comparison of EBV Detection and type Distribution in Blood and Saliva Samples from Males in Hot and Cold Spot Areas

There was high prevalence of saliva and blood EBV DNA detection from male participants in the hot spot. 93.3% had EBV in saliva and 86.7% in blood. In the cold spot, 45.8% had EBV DNA in saliva and 62.5% in blood.

EBV type distribution analysis showed that; 7.1% of the hot spot saliva samples had type 1 and 92.86% had type 1 and 2 co-infections. All the EBV DNA detected in blood had type 1 and 2 co-infections. In comparison, 36.36% of the cold spot saliva samples had type 1, 27.27% had type 2 and 36.36% had type 1 and 2 co-infections. 40% of the blood samples had type 1 and 60% had type 1 and 2 co-infections (Table 4.4).

Therefore, there were higher proportions of EBV positive samples from males from the hot spot as compared to the cold spot ($\chi 2$, $P < 0.001$). Higher proportion of co-infection with Type 1 and 2 in both blood and saliva compartments from the hot spot males in comparison with the cold spot males were observed ($\chi 2$, $P < 0.001$).

Table 4.4: EBV detection and type distribution in male saliva and blood samples from cold and hot spot areas

Study site	N	Compartment	EBV positive	Type 1	Type 2	Type 1 and 2 co-infections
Hot Spot	15	Saliva	14 (93.3%)	1 (7.1%)	0	13 (92.86%)
		Blood	13 (86.7%)	0	0	13 (100%)
Cold Spot	24	Saliva	11 (45.8%)	4 (36.36%)	3 (27.27%)	4 (36.36%)
		Blood	15 (62.5%)	6 (40%)	0	9 (60%)

$\chi 2$, $P < 0.001$

4.7 Comparison of EBV Detection and Type Distribution in Blood and Saliva Samples from Females in Hot and Cold Spot Areas.

There was a high prevalence of saliva and blood EBV DNA detection from female participants in the hot spot compared to the cold spot. 81.8% of the hot spot saliva samples and 77.3% blood samples were EBV positive. In the cold spot, 55.9% of the saliva samples and 64.7% of the blood samples were EBV positive.

Observations of EBV type distribution in the hot spot showed that 5.6% had type 1 and 94.4% had type 1 and 2 co-infections in saliva. All the EBV DNA detected in blood had type 1 and 2 co-infections (100%). In the cold spot, 52.6% had type 1, 26.3% type 2 and 21.1% had type 1 and 2 co-infections in saliva. In the blood compartment, 22.7% had type 1, 9.1% type 2 and 68.2% had type 1 and 2 co-infections (Table 4.5).

In summary, like in males, there were higher proportions of EBV positive samples from females from the hot spot compared to the cold spot. Higher proportion

of co-infection with Type 1 and 2 in both saliva and blood compartments from the hot spot females in comparison with the cold spot females were observed. ($\chi 2$, P <0.001)

Table 4.5: EBV types in female participants from cold and hot spot areas.

Study site	N	Compartment	EBV positive	Type 1	Type 2	Type 1 and 2 co-infections
Hot Spot	22	Saliva	18 (81.8%)	1 (5.6%)	0	17 (94.4%)
		Blood	17 (77.3%)	0	0	17 (100%)
Cold Spot	34	Saliva	19 (55.9%)	10 (52.6%)	5 (26.3%)	4 (21.1%)
		Blood	22 (64.7%)	5 (22.7%)	2 (9.1%)	15 (68.2%)

$\chi 2$, P < 0.001

Gender analysis showed that 71.8% of the male population had EBV in blood and 64.1% in saliva compared to the female population with 69.6% in blood and 66% in saliva.

EBV type distribution showed that, in male blood samples 21.4% had typeland 78.6% had type 1and 2 co-infections. In female blood samples, 12.8% had typel, 5% type 2 and 82% type 1and 2 co-infections. In saliva samples, 40% of the males had type 1, 12% type 2 and 68% type 1 and 2 co-infections. 29.7% of the females had typel, 13.5% type 2 and 56.8% type 1 and 2 co-infections (Table 4.6). The EBV detection and type distribution was not dependent on gender ($\chi 2$, P <0.001).

Both males and females from the hot spot had a high proportion of EBV positive blood samples compared to the saliva. Type 1 and 2 co-infections dominating in both blood and saliva.

Table 4.6: Comparison of male and female EBV detection and type variation in blood and saliva.

Gender	N	Compartment	EBV Positive	Type 1	Type 2	Type 1 and 2 co-infections
Male	39	Saliva	25/39 (64.1%)	5/25 (40%)	3/25 (12%)	17/25 (68%)
		Blood	28/39 (71.8%)	6/28 (21.4%)	0	22/28 (78.6%)
Female	56	Saliva	37/56 (66%)	11/37 (29.7%)	5/37 (13.5%)	21/37 (56.8%)
		Blood	39/56 (69.6%)	5/39 (12.8%)	2/39 (5%)	32/39 (82%)

$\chi 2$, $P > 0.05$

4.8 Blood and Saliva EBV Type Distribution in Mother-child Pairs.

In the hot spot, 78.13% of the EBV positive participants had matching EBV types in blood and saliva. All these were type 1& 2 co-infections. In the cold spot EBV positive participants, 16.20% had matching types of which 33.33% were type 2 and 66.67% were type 1 and 2 co-infections (Table 4.7). In summary, most participants from the hot spot population had similar EBV types in blood and saliva compartments all being type 1 and 2 co-infections compared to the cold spot samples.

Table 4.7: Blood and saliva compartments with matching EBV types

Study site	EBV positive	Percentage Match	Type 1	Type 2	Type 1 & 2
Hot spot N= 37	32	27/35 78.13%	0	0	100%
Cold spot N= 58	37	8/50 16.20%	0	33.33%	66.67%

$\chi 2$, P >0.05

4.8.1 Blood and Saliva Compartments with Similar EBV types in Mothers.

In the hot spot, 83.2% of the mothers had matching EBV type 1 and 2 co-infections in blood and saliva. In the cold spot only 17.7% of the mothers had matching EBV types. 33.33% were type 2 and 66.67% were type 1 and 2 co-infections (Table 4.8). In summary most samples from the hot spot had matching EBV types all being type 1 and 2 co-infections compared to the cold spot samples ($\chi 2$, P > 0.05).

Table 4.8: Blood and saliva compartments with similar EBV types in mothers

	EBV Positive	Percentage matches in mothers.	Type 1	Type 2	Type 1 & 2
Hot spot N=13	12	10/12 (83.3%)	0	0	100%
Cold spot N=20	17	3/17 (17.7%)	0	33.33%	66.67%

$\chi 2$, P >0.05

4.8.2 Blood and Saliva Compartments with similar EBV types in Children

In the hot spot 65.2% of the children had matching EBV types in their blood and saliva compartments all being type 1& 2 co-infections. In the cold spot, only 9.1%

had matching EBV types with 33.33% having type 2 and 66.67% type 1 and 2 co-infections (Table 4.9). A high proportion of the children blood and saliva samples from the hot spot had matching EBV types all being type 1 and 2 co-infections.

Table 4.9: Blood and saliva compartments with similar EBV types in children

Study site	EBV Positive	Percentage match in children	Type 1	Type 2	Type 1&2
Hot spot N=24	23	15/23 (65.2%)	0	0	100%
Cold spot N=38	33	3/33 (9.1%)	0	33.33%	66.67%

$\chi 2$, $P > 0.05$

4.8.3 Blood and Saliva Compartments with Similar EBV Types in Mother-Child Pairs.

In the hot spot, 61.5% of the mother-child pairs had matching EBV types in blood and saliva all being type 1 and 2 co-infections. In the cold spot, 57.1% had matching EBV types in blood and 38.1% in saliva (Figure 4.4). EBV type distribution analysis show that; 8.3% were type 1, 8.3% were type 2 and 83.3% were type 1 and 2 co-infections in the blood. In the saliva, 37.5% were type 1, 25% type 2 and 37.5% were type 1 and 2 co-infections (Table 4.10). A uniformly higher proportion of the mother – child pairs from the hot spot had matching EBV types in both blood and saliva compared to the cold spot ($\chi 2$, $P < 0.001$).

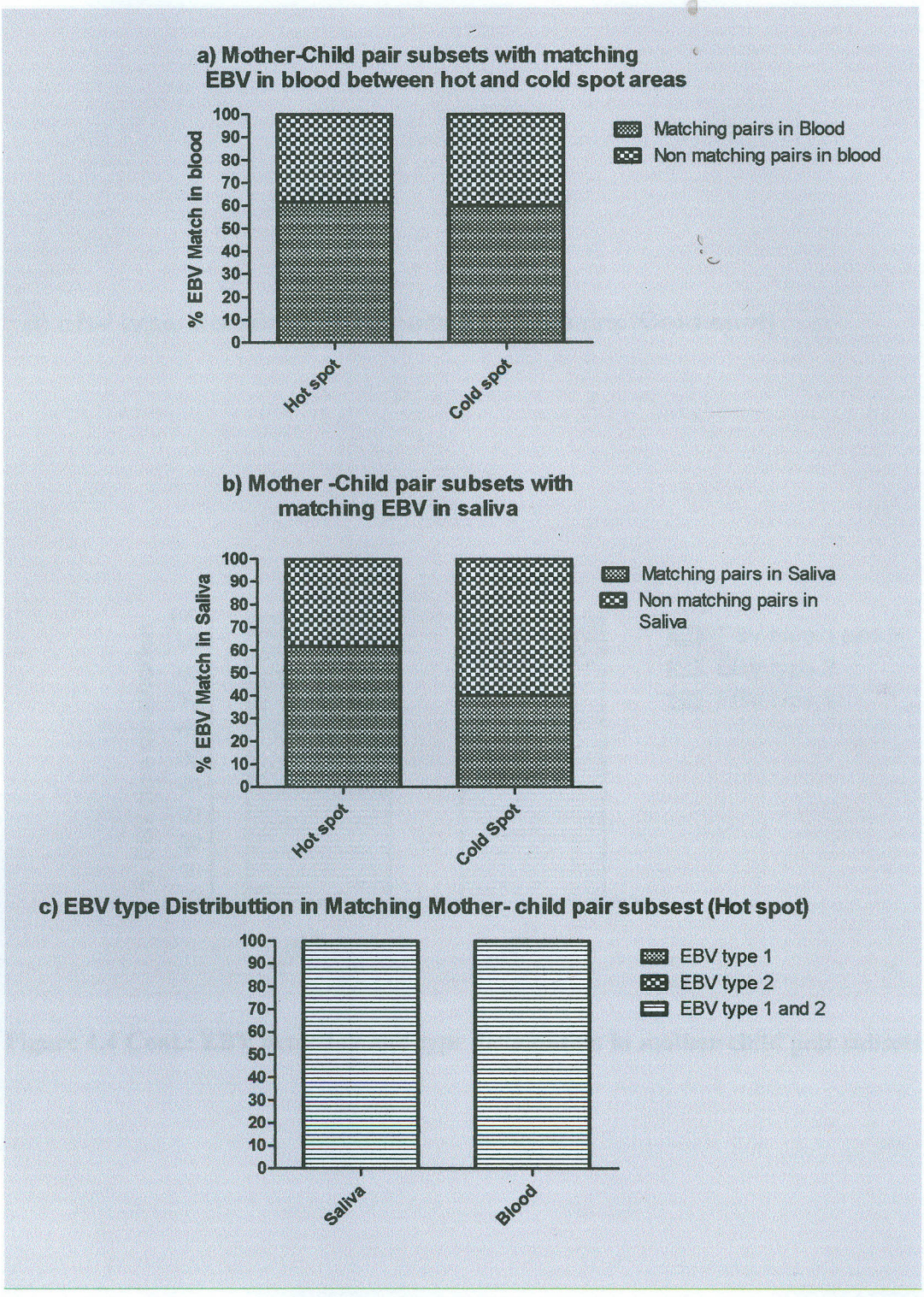


Figure 4.4: EBV detection and type distribution in mother-child pair subsets

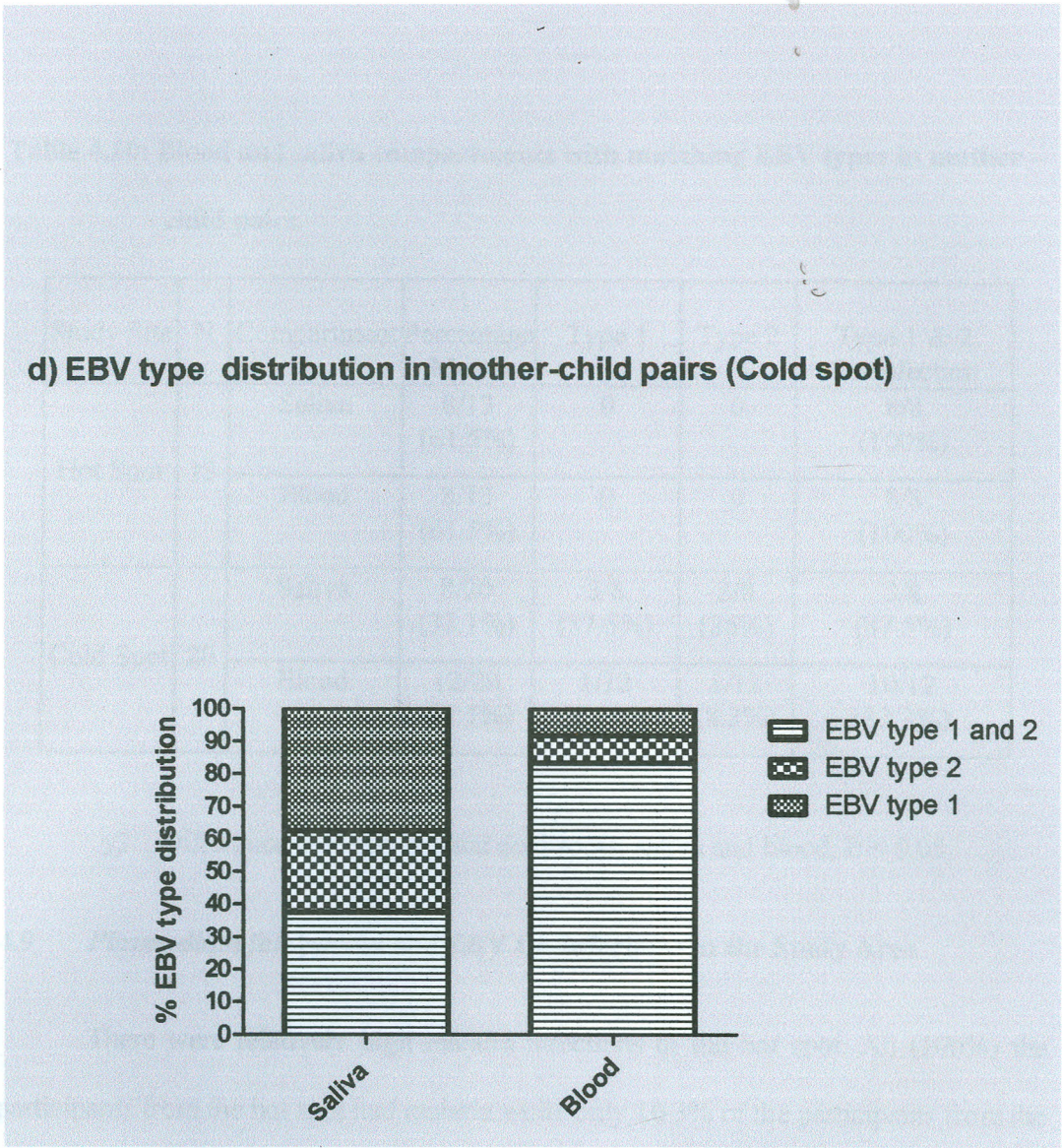


Figure 4.4 Cont.: EBV detection and type distribution in mother-child pair subsets

Table 4.10: Blood and saliva compartments with matching EBV types in mother – child pairs.

Study Site	N	Compartment	Percentage Match	Type 1	Type 2	Type 1 & 2 Co-infection
Hot Spot	13	Saliva	8/13 (61.5%)	0	0	8/8 (100%)
		Blood	8/13 (61.5%)	0	0	8/8 (100%)
Cold Spot	20	Saliva	8/20 (38.1%)	3/8 (37.5%)	2/8 (25%)	3/8 (37.5%)
		Blood	12/20 (57.1%)	1/12 (8.3%)	1/12 (8.3%)	10/12 (83.3%)

$\chi 2$; Difference between hot and cold spots: saliva and blood, $P < 0.05$

4.9 *Plasmodium falciparum* and EBV Co-infections in the Study Area

There were relatively high malaria infections in the hot spot. All (100%) the participants from the hot spot had malaria while only 10.3% of the participants from the cold spot were malaria positive ($P = 0.001$) ($\chi 2$, $P > 0.05$) (Figure 4.5a). 94.6% of the malaria positive cases were EBV positive while 5.3 % of the malaria positive individuals turned out to be EBV negative in the hot spot. In the cold spot, only 10.3% of the participants were malaria positive and all were EBV positive. 75.9% were malaria negative and EBV positive (Figure 4.5b). 95.1% of the malaria and EBV co-infected individuals had EBV type 1 and 2 multiple infection, 2.4% had EBV type 1 single infections and also EBV type 2 single infection were in 2.4% of the malaria and EBV co-infected individuals. 36.4% of the EBV positive individuals without malaria had EBV type 1 single infection, 2.3% had EBV type 2 single infections and 59.1% had EBV type 1 and 2 single infection (Figure 4.5c).

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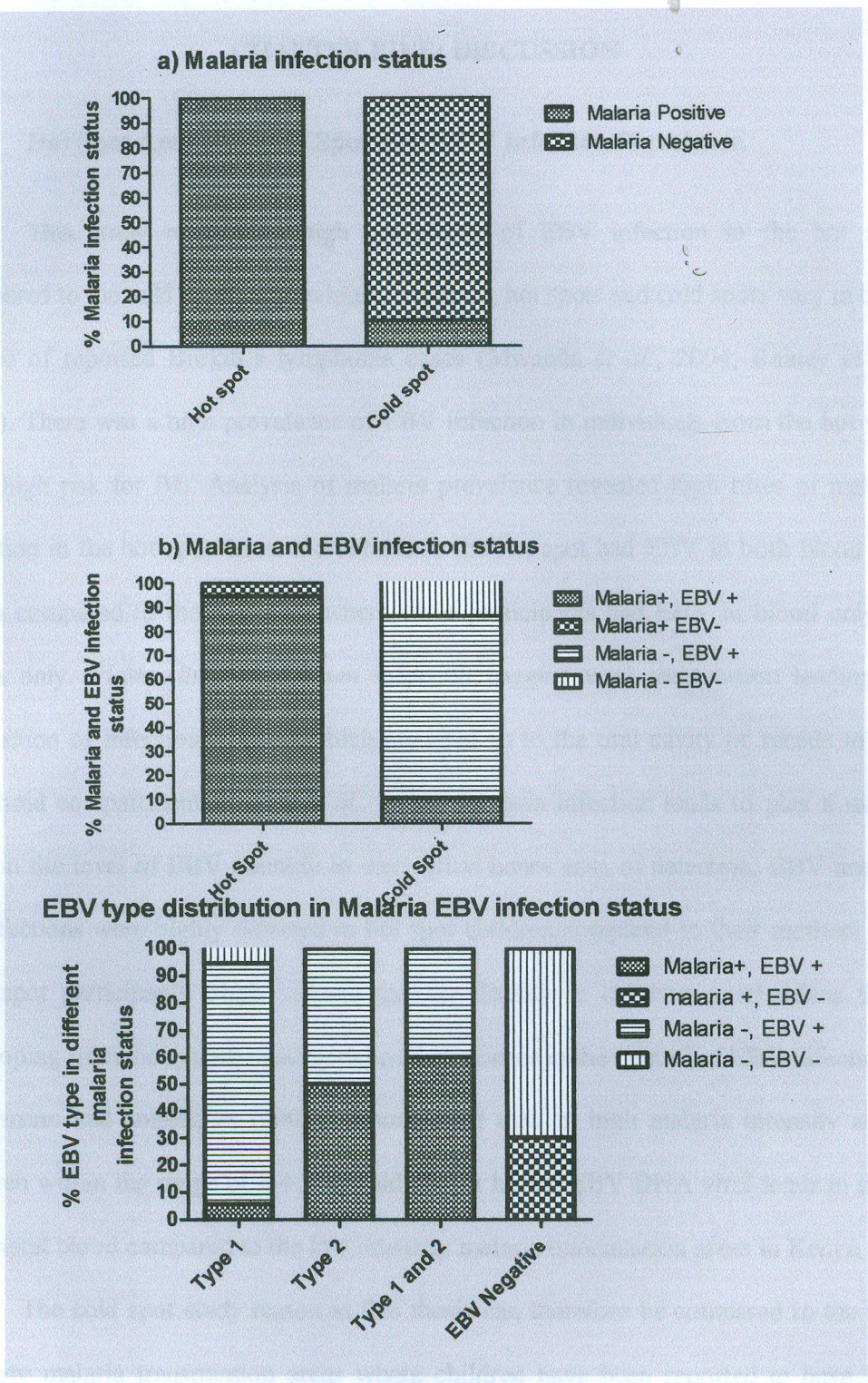

Figure 4.5: EBV detection and type distribution in different malaria and EBV infection status.

CHAPTER FIVE: DISCUSSION

5.1 Hot Spot Area and Cold Spot Area EBV Infection Prevalence.

This study revealed a high prevalence of EBV infection in the hot spot compared to the cold spot. As previously reported, hot spots and cold spots vary in their degree of reported Burkitt's lymphoma cases (Mwanda *et al.*, 2004; Rainey *et al.*, 2007). There was a high prevalence of EBV infection in individuals from the hot spot with high risk for BL. Analysis of malaria prevalence revealed high rates of malaria infection in the hot spot. Most individuals in the hot spot had EBV in both blood and saliva compared to the cold spot where some participants had EBV in blood only or saliva only. *Plasmodium falciparum* infection triggers viral reactivation leading to production of infectious virions which are shed in to the oral cavity or recede to the lymphoid compartment (Donati *et al.*, 2004). Malaria infection tends to play a major role on the level of EBV quantity in circulation hence ease of detection. EBV and Pf co-infections were highly detected in hot spot children compared to their mothers and cold spot participants. High malaria parasite density in children overburdens their developing immune system causing delayed responses in the control of EBV infections. Moormann and colleagues (2005) demonstrated that, in high malaria intensity areas children within the range of 1-4 years old harbor higher EBV DNA viral loads in their peripheral blood compared to the low intensity malaria transmission areas in Kenya.

The cold spot study region in this thesis can therefore be compared to the low intensity malaria transmission areas where children have been reported to have low EBV DNA viral loads. Vice-versa, the hot spot region with high risk for eBL can be compared to the high intensity malaria region where children are exposed to recurrent *Plasmodium falciparum* infections. Malaria induced immunosuppression and hyper activation of the B cell compartment in which the EBV latently persists, can lead to

increased EBV viral loads in circulation readily detectable by polymerase chain reaction.

5.2 EBV Type Distributions

EBV type prevalence has been reported in different geographical locations and population settings. (Zimber *et al.*, 1986) showed that EBV type 1 is common in developing countries and it is more prevalent than EBV type 2. EBV type 2 was first described in Kenyan BL cell lines by Young and colleagues in 1987. Mixed EBV type 1 and 2 multiple infections were commonly described in HIV immuno-compromised individuals by Sculley *et al.*, (1990)) and T cell immuno-compromised by (Young *et al.*, (1987). Weinreb and colleagues in 1996 depicted different EBV type 1 and 2 levels of expression in Hodkins lymphoma cases in Kenya. This study characterized different EBV types in blood and saliva compartments in asymptomatic individuals from neighboring regions with different Burkitt's lymphoma incidence rates. Multiple EBV type 1 and 2 infections were highly detected in the hot spot with no case of single infection. This may be accredited to the reduced anti-EBV immune responses in this region and high malaria parasite infections responsible for the augmented EBV viral loads in circulation that can easily be detected. Reduced immune selection pressure (the intensity with which the immune system tends to eliminate the infection) due to suppressed EBV immunity by recurrent malaria infection could be adding to the persistence of both EBV type 1 and 2 co-infections in the hot spot.

Comparatively, low levels of EBV single infections as well as multiple infections by the two EBV types were identified in the cold spot. Reduced *Plasmodium falciparum* infections present little effect to EBV immunity and hence high immune selection pressure which results to single infections of either type 1 or 2, and restricted levels of EBV type 1 and 2 multiple infections as well. Determining the influence of

immune selection pressure on viral evolution should be carried out to help identify the dominant alleles that influence viral loads in EBV infected persons and also define those that are most associated with antiviral effects.

Although endemic Burkitt's lymphoma incidence has been described to be higher in males compared to females at a ratio of 3:1 (Rainey et al. 2007); more males than females present with abdominal tumors (Biggar *et al.*, 1978), and no significant difference in the EBV type distribution in mothers/children and males/females has been reported. In the present study, gender and age disparities did not show significant association with EBV type distribution. Case control investigations should therefore be carried out to identify the most common EBV type associated with BL malignancies in the malaria holoendemic regions.

5.3 EBV Types and Malaria Infections.

From recent studies by (Chêne *et al.*, 2007)) and (Laichalk and Thorley-Lawson, 2005), it has been shown that antigenic stimulation and reception of plasma differentiation signals for antibody production induces EBV reactivation into the lytic cycle. Further, *Plasmodium falciparum* derived proteins can also lead to a direct reactivation of EBV during acute malarial infection, increasing the risk of BL development for children living in malaria endemic areas (Chêne *et al.*, 2007). Cystein-rich inter-domain region 1α (CIDR1α) of the *Plasmodium falciparum* erythrocyte membrane protein 1(PfEM-1) induces proliferation and activation of B cells. (Chêne *et al.*, 2007 ; Thorley-Lawson 2001). High detection of EBV DNA in saliva samples from the hot spot in the present investigation can be attributed to *Plasmodium falciparum* infection which stimulates virus shedding in the throat of the asymptomatic individuals promoting virus transmission through kissing or sharing of feeding utensils as also reported by Cohen. (2000). Studies by Moorman and Rasti have shown high EBV viral

loads in children from malaria endemic areas (Moormann *et al.*, 2005; Rainey *et al.*, 2007). Donati also demonstrated that acute malaria infection leads to increased levels of circulating EBV which are cleared following ant-malarial treatment. (Donati *et al.*, 2004). Therefore, ready detection of the EBV DNA is highly attributed to *Plasmodium falciparum* infection. These results suggest that malaria infection does not alter the type of EBV strain but plays a role on the level of viral detection. Co-detection of EBV type 1 and 2 is more frequently seen in malaria positive individual. EBV type 1 single infections were detected in healthy individuals with high EBV specific immunity due to its virulence as shown by Zimber and colleagues in 1986.

5.4 EBV Transmission.

EBV type 1 and 2 co-infections were significantly evident in both blood and saliva compartments of mother-child pair compartments. Single infections detected were complementary in the blood and saliva compartments suggesting horizontal transmission from mothers to their children mainly through saliva during feeding and kissing as shown by Cohen, 2000. Whether EBV transmission is intrauterine or only after bath is not well understood and needs further investigations.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

EBV types 1 and 2 multiple infections in blood and saliva were common in both sites but more prevalent in the hot spot areas. Single infections were dominant in the cold spot region. There was concordance of type 1 and 2 co-infections in blood and saliva from mother-child pairs in the hot spot. EBV detection and type distribution was independent of age and gender. EBV type distribution was not however dependent on the prevalence of malaria and EBV specific T cell responses were reduced in malaria infected individuals.

6.1.1 Recommendations from the Results

Public education should be conducted to create awareness about the medical importance of EBV. Malaria control measures should be enhanced to maintain the body's immunity against EBV infections.

6.1.2 Recommendations for Future Research Work

Further genetic typing should be done to confirm EBV strain transmission within families.

A Burkitt's lymphoma case control study should be carried out to investigate specific EBV strains viral loads, levels of EBV specific innate and adaptive immune response mediators, and essential nutrients.

Monitoring and evaluation studies should be conducted to find out the use and effect of malaria control measures implemented in malaria holoendemic areas.

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