

**DISTRIBUTION OF ANTI-EBV ANTIBODIES IN HIV  
POSITIVE INDIVIDUALS AT VARIOUS STAGES OF  
HIV/AIDS INFECTION AT MBAGATHI DISTRICT  
HOSPITAL, NAIROBI COUNTY**

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**Distribution of Anti-EBV antibodies in HIV positive individuals  
at various stages of HIV/AIDS infection at Mbagathi District  
Hospital, Nairobi County**

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**A thesis submitted in partial fulfillment for the degree of Master  
of Science in Public Health in the Jomo Kenyatta University of  
Agriculture and Technology**

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## DECLARATION

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

Signed .....Date .....

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

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### **DEDICATION**

This work, which honours all Human Immunodeficiency Virus (HIV) patients, is dedicated to my parents, Nicholas and Amy D'lima, who believed without seeing, saw but did not understand and trusted against all odds.

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## LIST OF ACRONYMS AND ABBREVIATIONS

<b>AIDS</b>	Acquired immune deficiency syndrome
<b>ART</b>	Anti-retroviral treatment
<b>BALF1</b>	Bronchoalveolar lavage fluid early protein
<b>BALT</b>	Bronchial associated lymphoid tissue
<b>BCBL-1</b>	Body cavity based lymphoma type 1 cell line
<b>Bcl2</b>	B cell line 2 antagonist killer gene product
<b>BHRF1</b>	Apoptotic inhibitor – the EBV homologue of the Bcl2 proto-oncogene
<b>BL</b>	Burkitt lymphoma
<b>CAEBV</b>	Chronic active EBV infection
<b>CCR5</b>	Chemokine Receptor 5
<b>CD4+</b>	Cluster of differentiation marker 4 positive (usually applied to lymphocytes)
<b>CD8+</b>	Cluster of differentiation marker 8 positive (as applied to lymphocytes)
<b>CLIA</b>	Chemiluminescence assay
<b>C-myc</b>	Cellular proto-oncogene activated in various animal and human tumors
<b>CMV</b>	Cytomegalovirus
<b>CNS</b>	Central Nervous System



<b>CTLs</b>	Cytotoxic T-lymphocytes
<b>CXR</b>	Chest X-ray
<b>DLBCL</b>	Diffuse large B cell lymphoma
<b>DNA</b>	Deoxyribonucleic acid
<b>EA</b>	Early antigen
<b>EA-D</b>	Early antigen diffuse
<b>EA-R</b>	Early antigen restricted
<b>EBER</b>	EBV encoded RNA
<b>EBNA</b>	Epstein Barr Nuclear antigen
<b>EBNA+</b>	Epstein Barr Nuclear antigen positive
<b>EBV</b>	Epstein Barr virus
<b>EDTA</b>	Ethylene diamine tetracetic acid
<b>EIA</b>	Enzyme immuno assay
<b>ELISA</b>	Enzyme linked immunosorbent assay
<b>ERC</b>	Ethical Review Committee
<b>ESR</b>	Erythrocyte sedimentation rate
<b>FI</b>	Fusion inhibitor
<b>HAART</b>	Highly active anti-retroviral treatment
<b>Hb</b>	Hemoglobin
<b>HC</b>	Heavy chain

<b>HHV- 4</b>	Human Herpes virus Type 4/Epstein Barr virus
<b>HHV- 8</b>	Human Herpes virus Type 8/Kaposi Sarcoma virus
<b>HIV</b>	Human immunodeficiency virus
<b>HIV+</b>	Human immunodeficiency virus positive
<b>HLA</b>	Human leucocyte antigen
<b>HSV 1</b>	Herpes simplex virus Type 1
<b>HSV 2</b>	Herpes simplex virus Type 2
<b>HVA</b>	Ateline herpesvirus
<b>HVS</b>	Herpesvirus Saimiri
<b>IFA</b>	Immunofluorescence assay
<b>IgG</b>	Immunoglobulin G
<b>IgM</b>	Immunoglobulin M
<b>IL - 6</b>	Interleukin 6
<b>IL - 7</b>	Interleukin 7
<b>IM</b>	Infectious mononucleosis
<b>Kb</b>	Kilobase
<b>KEMRI</b>	Kenya Medical Research Institute
<b>λ LC</b>	Lambda light chain
<b>LMP1</b>	Latency membrane protein 1
<b>MDH</b>	Mbagathi District Hospital

<b>MFI</b>	Multiplex flow immunoassay
<b>NHL</b>	Non Hodgkin Lymphoma
<b>NNRTI</b>	Non nucleoside reverse transcriptase inhibitors
<b>NRTI</b>	Nucleoside reverse transcriptase inhibitors
<b>ORF</b>	Open reading frame
<b>p 22</b>	An EBV viral capsid antigen
<b>PBMC</b>	Peripheral blood mononuclear cells
<b>PCR</b>	Polymerase chain reaction
<b>PCV</b>	Packed cell volume
<b>PEL</b>	Primary effusion lymphoma
<b>PI</b>	Protease inhibitor
<b>PTLD</b>	Post transplantation lymphoproliferative disorder
<b>RANTES</b>	Regulated upon Activation Normal T cell Expressed and Secreted
<b>R GNU S</b>	R computer program from open source operating system not UNIX
<b>RRV</b>	Rhesus Rhadinovirus
<b>SSC</b>	Scientific Steering Committee
<b>tat</b>	HIV encoded cytokine like transactivator of transcription protein
<b>TB</b>	Tuberculosis

<b>VCA</b>	Viral capsid antigen
<b>WBC Tot</b>	Total white blood cell count
<b>WHO</b>	World Health Organization
<b>ZEBRA</b>	Z EBV replication activator protein

## ABSTRACT

Epstein - Barr Virus (EBV) is a member of the family of Human Herpes Viruses. It may be acquired and continue to be carried without expression of any clinical disease, or following a primary disease episode, most commonly infectious mononucleosis. In susceptible individuals it causes nasopharyngeal carcinoma, Burkitt and other lymphomata and oral hairy leukoplakia. Over 90% of the Kenyan population is seropositive for EBV, but immunocompetent individuals maintain the virus in a latent phase. In the course of HIV infection, changes in EBV activity are associated with the development of a variety of lymphoproliferative disorders. The aim of this study was to identify whether or not reactivation of EBV, and its attendant risks, can be detected in HIV positive patients, and whether such reactivation might have diagnostic or prognostic value in the management of the disease. A total of 101 adult subjects were randomly selected from HIV seropositive outpatients at Mbagathi District Hospital, Nairobi. Clinical and sociodemographic data were captured using a questionnaire, followed by a clinical examination of the head, neck and mouth. Citrated venous whole blood was used for EBV antibody assays by solid phase ELISA using EBV Profile 2™ kits (Medizinische Labordiagnostika AG, Lubeck, Germany). The presence of anti EBNA-1, p22 and EA-D antibodies were regarded as diagnostic of reactivation disease, which is the stage at which AIDS lymphomas develop. The proportion of the study population who were positive for EBV antibodies was 95% while 5% were negative. Of those who were positive 18%, 60% and 11% were in early, late and reactivated infection respectively. EBV serostatus was then analyzed in relation to the subjects' stage of HIV disease. The HIV clinical stage distribution of the study patients was Stage 1: 5.9% , Stage 2 : 35.6% and Stage 3 : 57.8% . The Odds Ratio for reactivated EBV infection with HIV Stage 3 as the exposure was 0.1 (95% CI 0.03 – 1.00). Thus, no significant risk of reactivation of EBV infection in HIV stage 3 was demonstrated.

The Odds Ratio for advanced EBV disease (late and reactivated infection) with HIV stage 1 and 2 as the exposure was 0.32 (95% CI = 0.1 – 1.1). Cluster analysis of high intensity protein bands demonstrated EBNA1/EA-D as the weakest but most significantly different cluster pair in all HIV clinical stages. Whatever the stage of HIV disease or degree of overall immunosuppression, reactivation of EBV has potentially serious neoplastic consequences for the patient. Although this study did not demonstrate a significant risk of Reactivation of EBV with HIV clinical stage 3 as the exposure factor, EBNA and EA-D appear to be the strongest candidate biomarkers of EBV reactivation in this population. Further research into biomarkers of EBV reactivation would result in diagnostic tests to help in early detection of EBV associated lymphomas in HIV patients.

## **CHAPTER ONE**

### **INTRODUCTION**

#### **1.1 Background information**

The Epstein - Barr virus (EBV) is ubiquitous in humans with 80 – 90% of adults worldwide showing serological evidence of past exposure (Ryan *et al.*, 2004). Similar EBV seroprevalence has been demonstrated in patients with Hodgkin's disease in Kenya (Abwao *et al.*, 2007). The Human Immunodeficiency virus (HIV) causes Acquired Immune Deficiency Syndrome (AIDS). The prevalence of HIV in Kenya is 6%. (UNAIDS, 2014 ). EBV is associated with AIDS related lymphomas (ARL's), Twenty-five to 40 percent of HIV positive patients will develop a malignancy, with approximately 10 percent developing non-Hodgkin lymphoma (NHL) (Akanmu , 2006). As an example, a series of 1073 HIV positive patients reported a total cancer incidence of 4 percent per year; the rate of NHL was 1.2 percent per year (Johnson *et al.*, 1997). Since the start of AIDS pandemic the ARL's have been a focus of researchers worldwide. AIDS-related lymphoma (ARL) can be divided into the following three types on the basis of areas of involvement:

- a) Systemic NHL
- b) Primary central nervous system lymphoma (PCNSL)
- c) Primary effusion lymphomas ("body cavity lymphoma")

Systemic NHL is the most common variety of ARL, followed by PCNSL, which is less common but not rare, and primary effusion lymphoma, which is a rare disease

Most ARLs are high-grade, aggressive Non Hodgkin Lymphomas( NHLs). The etiology of NHL is largely unknown; however, several factors play an important role in development of the disease. These include infections with viruses, namely, Epstein-Barr virus (EBV) infection and human herpesvirus 8 (HHV-8) infection; continuous B-cell

stimulation; and immunodeficiency. Systemically arising NHL constitutes about 80% of all AIDS-related lymphomas (ARLs). (Tossing, 1996). These lymphomas are of the following varieties (Grulich & Vajdic, 2015),

- a) Burkitt lymphoma and Burkitt-like lymphoma
- b) Diffuse, large cell lymphoma, including centroblastic lymphoma, immunoblastic lymphoma, and plasmablastic lymphoma of the oral cavity.

Africa has the highest burden of AIDS, but epidemiologic data and studies on HIV and lymphopoietic neoplasm are limited if available at all in African countries. Association between NHL and HIV seems to be weaker in African countries as compared with more developed countries (Seaberg *et al.*, 2010). The Relative Risk increase of NHL in people with AIDS in Africa is 10% lower as compared with AIDS patients in developed countries. In Uganda, for example, the annual incidence of NHL was reported as 6 per 100,000 between 1995 and 1997. (Lanoy *et al.*, 2011).

Despite recent advances in the management of AIDS and its associated lymphomas, AIDS still has a poor prognosis, especially when it comes to PCNSLs and primary effusion lymphomas. The underlying severity of the HIV infection also plays a critical role in mortality and morbidity.

## **1.2 Statement of the problem**

HIV infection is associated with a number of EBV related lymphoproliferative disorders. These disorders mark a shift from EBV latency to lytic antigen profiles. Serology has been and is currently used to differentiate between acute and chronic EBV infections in immunocompetent patients. However in HIV there is an altered humoral response to antigenic challenge arising from immunosuppression, in general. Therefore standard EBV serological methods, or at least their interpretation are rendered non conclusive. EBV serology has not been used to diagnose and monitor HIV associated lymphoproliferative



disorders. In advanced centres these are monitored by Real Time Polymerase Chain Reactions and other genomic methods. These techniques are not economically viable for routine diagnostics in less equipped laboratories. There is a gap in diagnosing and monitoring HIV related lymphoproliferative disorders. However, EBV infection proteomic dynamics are being altered by concomitant HIV infection. Therefore, it is plausible that a shift from latent to lytic EBV protein profiles occurs. This manifests in reactivation of latent EBV infection in HIV disease and potentially mark the start of tumorigenesis. Currently there are no antibody markers for the risk of EBV-induced lymphoproliferative disorders in the course of HIV infection although a panel of serological and molecular tests on serum or plasma can screen for EBV associated Nasopharyngeal carcinoma in high-risk populations, assess prognosis, and monitor disease status over time (Paramita et al., 2007).

### **1.3 Justification of study**

HIV/AIDS and its Herpes virus associated lymphomas, are public health concerns in Africa and the world. Approximately 1–6% of HIV infected patients develop lymphomas each year. Herpes virus infections often pre-date HIV infection. Baseline data are required on the expression of late stage EBV antibodies during different clinical stages of HIV co-infection to establish an EBV infection – neoplastic connection. This data would help predict the risk of EBV reactivation and lymphoproliferative disease in this population. This study was designed to show the distribution of EBV reactivation stage antibodies and their possible use as markers of EBV reactivation and lymphomagenesis in an HIV positive population.

## **1.4 Objectives**

### **1.4.1 General objective**

To determine the distribution of antibodies to EBV antigens in HIV positive individuals at various stages of HIV/AIDS disease.

### **1.4.2 Specific objectives**

- i. To determine the proportions of individuals with serum EBV antibodies to various EBV antigens at different stages of EBV infection in HIV/AIDS patients.
- ii. To determine the proportions of individuals at various stages of EBV infection in different stages of HIV clinical disease in the study population.
- iii. To determine the relationship between different stages of HIV infection and EBV disease progression.

## **1.5 Research questions**

- i. What is the proportion of individuals with various serum EBV antibodies, in this HIV + study population.
- ii. What is the proportion of individuals at various stages of EBV infection in this HIV + study population?
- iii. What is the relationship between different stages of HIV infection and EBV disease progression?

## **1.6 Scope**

This was a cross sectional study which measured the qualitative distribution of Anti EBV antibodies in HIV positive individuals at various stages of HIV/AIDS presenting at Mbagathi District Hospital.

### **1.7 Study limitations**

1. Without the presence of the EuroLine Scan computer software, a digital evaluation and characterization of Antigen-Antibody bands into IgG and IgM class antibodies was not possible. The latter class is produced in early infections and their presence will confound the intensity of Antigen-Antibody bands when evaluated visually.
2. Immunosuppression leads to decreased antibody expression of B lymphocytes therefore antibody levels are not a reliable indicator of viral load of EBV, HIV and any viral infection .
3. Due to limited funds it was not possible to select equal numbers of patients in each EBV infectious stage. Although the sample size was 138 only 101 patients were recruited into the study. Thus the results have an accuracy of 73%.

## **CHAPTER TWO**

### **LITERATURE REVIEW**

#### **2.1 The Epstein Barr virus**

Epstein Barr Virus (EBV) is named after Michael Anthony Epstein and Yvonne Barr, who working with Bert Achong, in 1964, first described the virus in cells cultured from tumor specimens sent to them from Mulago Hospital in Kampala, Uganda by Denis Burkitt (Epstein *et al.*, 1964). Burkitt and Epstein had met three years earlier in London during a talk by the former on his findings regarding paediatric "African lymphomata" in tropical Africa. In the talk, Epstein hypothesized that there may be an infectious component to these lymphomata. After the presentation, Burkitt agreed to send Epstein frozen specimens for him to analyze (Coakley, 2006). Epstein, Barr and Achong were working as a team at the Middlesex Hospital at the time. The Epstein - Barr virus (EBV), also called Human herpesvirus 4 (HHV-4), is a virus of the herpes family and is one of the most common viruses in humans. Most people become infected with EBV, which is often asymptomatic but infection commonly causes infectious mononucleosis also known as glandular fever (Crawford *et al.*, 1987).

Epstein-Barr virus occurs worldwide and most people gain adaptive immunity through the development of serum immunoglobulin antibodies, preventing repeated sickness from re-infection (Ryan *et al.*, 2004). In the United States, as many as 95% of adults between 35 and 40 years of age are EBV seropositive. Infants become susceptible to EBV as soon as maternal antibody protection (present at birth) disappears. Many children become infected with EBV, and these infections usually cause no symptoms or are indistinguishable from the other mild, brief illnesses of childhood. In developed countries, many persons are not infected with EBV in their childhood years. When infection with EBV occurs during adolescence or young adulthood, it causes infectious mononucleosis in 35% to 55% of cases ( Vidrih *et al .*, 2001).

The mouth, salivary glands, and oropharynx are major reservoirs for the virus which is frequently transmitted by oral fluids – hence its common sobriquet as “the kissing disease”.

### **2.1.1 Classification of Herpesviruses and EBV**

EBV belongs to the *Herpesvirales* order of double stranded DNA viruses, *Herpesviridae* family, subfamily *Gammapherpesvirinae* and genera *Rhadinovirus* (Davison, 2010) . A characteristic feature common to all herpesviruses is that after primary infection often during childhood, a latent phase is developed that persists lifelong in the infected host (Davison, 2007). This coexistence between the herpesviruses and the host cell is the viruses ability to modulate the host immune response, interfere, and interact with different immune effectors, thereby promoting viral persistence in the host (Lisco *et al.*, 2009). Occasionally, the latent virus is reactivated resulting in recurrent symptomatic or nonsymptomatic productive infections (Pellet & Roizmann, 2007).

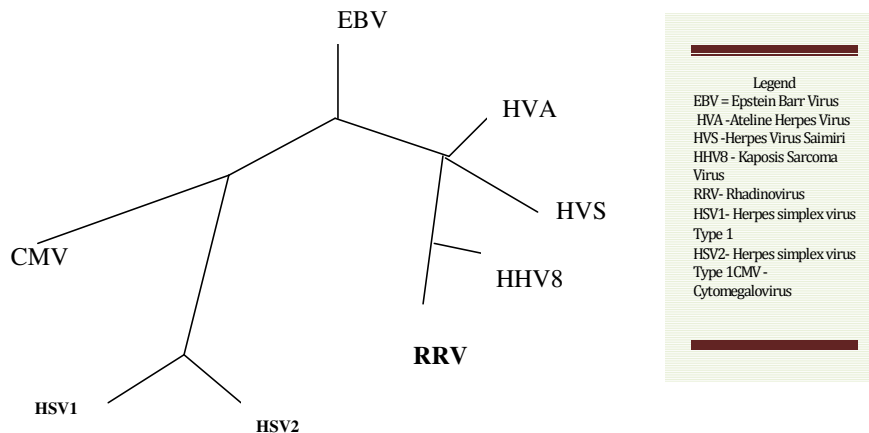
After primary infection herpesviruses have the ability to become latent and persist in their natural host (Murray, 2005). Replication of herpesviruses is by temporal control of DNA transcription and proteins synthesized include immediate (alpha) transcripts, early (beta) transcripts and late (gamma) transcripts (Ryan, 2004).

EBV is a  $\gamma 1$  Human herpes virus. EBVs closest human herpes virus relative is HHV- 8, a  $\gamma 2$  Human herpes virus (Moore *et al.*, 1996).  $\gamma$  Herpes viruses are characteristically lymphotropic, may productively infect fibroblasts, may cause latent infection of lymphocytes or progenitor stem cells and the host range of the herpesvirus species is limited to the family of the natural host. They are associated with lymphoproliferative disorders and certain cancers. HVS can induce malignant lymphomas in owl monkeys and marmosets (*Aotus and Sanguinus sps*) (Ensoli *et al.*, 2001). EBV in humans is linked to infectious mononucleosis (Purtilo, 1987) and to a potentially fatal B cell proliferation in young males with X linked susceptibility to lymphoproliferative disease. The virus is

also linked to post-transplant lymphoproliferative disease (due to immune stimulation from the transplant), to lymphomas in AIDS patients (Hanto *et al.*, 1985), Burkitt lymphoma (Epstein *et al.*, 1964), Nasopharyngeal carcinoma (Zur ausen *et al.*, 1970) and Hodgkin disease (Russo *et al.*, 1996). Whereas the proliferation of EBV-infected B cells is controlled by a potent EBV-specific immunosurveillance in immunocompetent healthy carriers, persistent EBV infection may lead to EBV driven lymphoproliferative disease in immunosuppressed patients (Cohen, 2000).

EBV is ubiquitous in humans with 80 – 90% of adults worldwide showing serological evidence of past exposure (Ryan *et al.*, 2004). Similar EBV seroprevalence has been demonstrated in patients with Hodgkin's disease in Kenya (Abwao *et al.*, 2007). Two EBV phenotypes, Type A and Type B have been identified. The latter type is commonly found in the malarial belt of Equatorial Africa, North Eastern Brazil and Papua New Guinea (Sculley, 1990). The principal target cell of EBV is the B lymphocyte and fibroblasts including those in the salivary glands.

*Herpesviridae* infect many mammals and several examples have been identified in non-human primates; *Herpesvirus saimiri*, (HVS) in *Saimiri scilireus*, Ateline Herpesvirus (HVA) in *Ateles geoffreyi*, Rhesus Rhadinovirus (RRV) and Retroperitoneal fibromatosis herpesvirus in *Macaca mulatta* share similar genetic homology with EBV (Crawford *et al.*, 1987). More distant relatives of EBV in the *Herpesviridae* family which infect humans include Cytomegalovirus (CMV) or HHV-3, Herpes Simplex Virus type 1 (HSV1) or HHV-1 and Herpes simplex Virus type 2 (HSV2) or HHV-2 (Figure 2.1)



**Figure 2.1: Herpesviridae family phylogenetic tree adapted from Crawford and Edwards, 1987**

There are eight distinct viruses in the Herpesviridae family known to cause disease in man (Murray *et al.*, 2005). These are classified into  $\alpha$  (alpha),  $\beta$  (beta) and  $\gamma$  (gamma) subfamilies designated from HHV-1 through to HHV-8 (Davison, 2010). These have distinct primary target cells, pathophysiology, sites of latency and means of spread (Table 2.1).

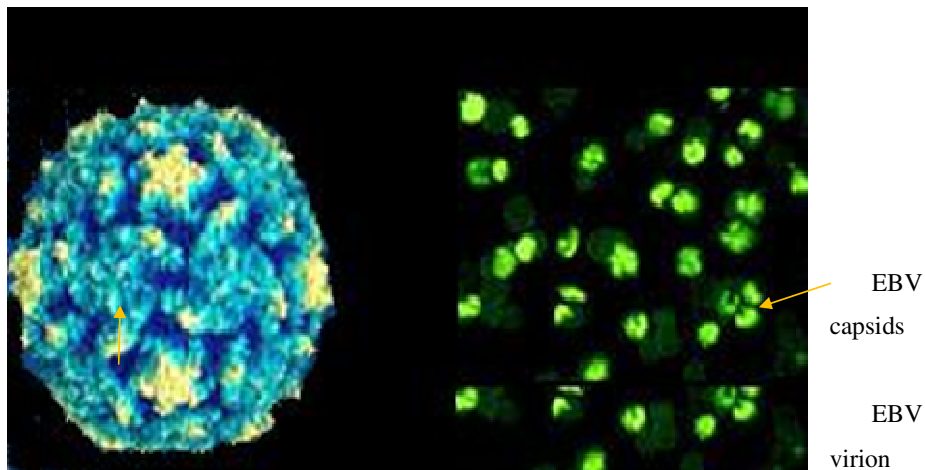
The *Herpesviridae* family is characterized by the electron microscopic architecture of the virion (De The, 1981). Herpesvirus particles are 120 - 200 nm in diameter. All virus particles comprise a capsid, tegument and envelope (Ryan *et al.*, 2004). The Herpes virus capsid consists of 162 hollow capsomeres arranged in icosahedral symmetry. The tegument is an amorphous globular structure surrounding the capsid (De The, 1981). The envelope is a pleomorphic lipid bilayer external to the tegument in which are embedded the viral glycoproteins (Figure 2.2).

**Table 2.1: Basic characteristics of human herpesviruses from Davison, 2010**

Scientific name (common name) of the virus	Genome size (Kbp) and type	Target cells	Cytopathic effects	Tissue tropism	Pathophysiology	Common modes of transmission	Global prevalence (%)
<b>Alpha herpesviruses: Short replicative cycle</b>							
HHV-1 (HSV-1)	152 dsDNA	Mucoepithelial cells (predominantly oro-facial tract)	Cytolytic	Nerve ganglia	Oral/genital herpes, latency	Close contact, sexual contact	50-90
HHV-2 (HSV-2)	155 dsDNA	Mucoepithelial cells (predominantly genital tract)	Cytolytic	Nerve ganglia	Oral and/or genital herpes, latency	Sexual/direct contact	20-60
HHV-3 (VZV)	125 dsDNA	Mucoepithelial cells and T cells	Cytolytic	Nerve ganglia	Chickenpox and Shingles, latency	Close contact, respiratory route	50-95
<b>Beta herpesviruses: Long replicative cycle</b>							
HHV-5 (CMV)	235 dsDNA	Epithelial cells, Lymphocytes, fibroblasts etc.	Cytomegalic	Leukocytes, Epithelial cells	Multiple organs are involved, mild to life threatening manifestations	Respiratory, body fluids, sexual contact, blood transfusion, organ transplants	40-100
HHV-6 (variant A and B)	168-170 dsDNA	Epithelial cells, T-lymphocytes, Fibroblasts	Not yet known	T-lymphocytes	Sixth disease (Roseola Infantum or exanthema subitum)	Salivary	60-100
HHV-7	145 dsDNA	Epithelial cells, T-lymphocytes, Fibroblasts	Not yet known	T-lymphocytes	Sixth disease (Roseola infantum or exanthema subitum)	Salivary	40-100
<b>Gamma herpesviruses: Variable replicative cycle</b>							
HHV-4 (EBV)	172 dsDNA	Mucoepithelial cells, B-lymphocytes	Lympho-proliferative	B-lymphocytes	Infectious mononucleosis, several types of lymphomas and carcinomas	Salivary	80-100
HHV-8 (KSHV)	145 dsDNA	Lymphocytes	Not yet known	Lymphocytes	Kaposi Sarcoma (KS), primary effusion lymphoma (PEL)	Salivary, sexual contact	2-50

HHV: Human herpesviruses, VZV: Varicella zoster virus, CMV: *Cytomegalovirus*, EBV: Epstein-Barr virus, KSHV: Kaposi sarcoma associated herpes virus, dsDNA: Double stranded deoxyribonucleic acid





**Figure 2.2: EBV virion and capsids (De-The, 1981)**

### **2.1.2 The EBV genome**

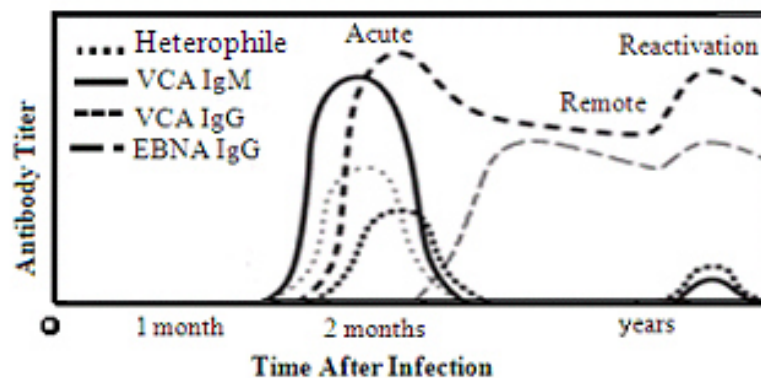
The EBV genome is a single molecule of linear, double-stranded DNA of 173 kilobase pairs in length. It may also exist as a circular episomal form during latency. Like all herpesviruses, EBV encodes a large number of proteins including enzymes involved in nucleic acid metabolism and DNA synthesis (Babcock *et al.*, 2000). Epstein Barr Virus has 80 open reading frames encoding cellular interleukin-10, *bcl-2*, and colony-stimulating factor 1-receptor homologues and a set of viral glycoproteins. In transformed lymphocytes, EBV expresses 6 nuclear proteins (EB nuclear antigens designated 1 -6), 3 integral or Latency membrane proteins (LMP1, LMP2A, LMP2B) and two highly abundant small nuclear transcripts (EBERs) (Thorley Lawson and Babcock, 1999).

### **2.1.3 The life cycle of EBV**

Epstein Barr Virus is usually acquired early in life by salivary transmission. Primary infection is usually asymptomatic. If infection is acquired in adolescence, after an

incubation period of more than 1 month it results in infectious mononucleosis (glandular fever).

Epstein Barr Virus induces virus specific antibodies in immortalized B-cells. Tests for EBV-specific antibody may be carried out. These are usually indirect immunofluorescence for antibody to early antigen (EA) or virus capsid antigen (VCA). Epstein Barr Virus transforms ("immortalizes") B-cells and an infected individual carries B-cells containing the EBV genome throughout life (Henle & Henle, 1985). Primary EBV infection in healthy hosts is accompanied (Murphy et al., 1986) by a serological response. Immunoglobulin M class antibody against viral capsid antigen (VCA) rises first. Antibodies against EBNA appear at least 1 month after primary infection and are measured, along with IgG anti-VCA, as markers of prior infection and as indicators of EBV reactivation (Svahn *et al.*, 1997). Titers against early antigen (EA) rise on primary infection and again in pathological states of EBV reactivation (Figure 2.3).



VCA =Viral capsid antigen

EBNA = Epstein Barr Nuclear antigen

**Figure 2.3: Epstein Barr Virus Serology post primary infection reflecting life cycle and infection stages from Svahn *et al.*, (1997)**

Epstein Barr Virus is associated with two well-recognized human malignancies, namely endemic Burkitt lymphoma and undifferentiated nasopharyngeal carcinoma. Severe EBV-associated lymphoproliferative disease or lymphoma may occur in immunosuppressed patients. Patients with AIDS or early symptomatic HIV infection are susceptible to EBV-associated so-called oral hairy leukoplakia (Greenspan *et al.*, 1989). This is a condition most common on the lateral borders of the tongue, though it can affect other parts of the oral mucosa. Epstein Barr Virus virions pack the cells of the upper stratum spinosum, producing the cytological appearance of choilocytosis, with distinctive hyperparakeratosis.

#### **2.1.4 The pathobiology of EBV infections**

Epstein Barr Virus has a 173-kb DNA genome for which the nucleotide sequence and predominant transcripts are well characterized. Epstein Barr Virus is capable of infecting B and T lymphocytes, squamous epithelial cells of the oropharynx and nasopharynx, glandular epithelium of the thyroid, stomach, and salivary glands, smooth muscle cells, and follicular dendritic cells in lymph nodes. Healthy virus carriers harbor 1 to 50 EBV genomes per million blood mononuclear cells, with B lymphocytes representing the major cellular reservoir (Khan *et al.*, 1996). Beyond B cells, it is nearly impossible to find infected cells of other lineages in healthy carriers, but it is presumed that the other cell types listed above are capable of being infected based on the identification of EBV DNA in lesions arising from them. Investigation of patients with EBV-infected neoplasms provides reasonable evidence that EBV was present before neoplastic transformation, raising the still unresolved question of the extent to which EBV contributes to tumorigenesis (Thompson *et al.*, 2004).

Primary infection results in transient viremia followed by rapid immune response. The virus persists for life in its human host by cleverly balancing its ability to hide from the immune system via latent infection of B lymphocytes with its ability to replicate and shed from oral mucosa. At any given time, about 20% of carriers are shedding salivary virions, leading to nearly universal propagation of the virus in human populations (Khan *et al.*, 1996).

EBV infection of B lymphocytes leads to two alternate outcomes mimicking the physiological effects of antigen stimulation. One outcome culminates in the production of memory B cells that persist long-term; the other outcome results in differentiation toward plasma cells that are destined to die. These two outcomes support latent viral persistence and lytic viral replication, respectively (Cohen, 2000). Lifelong infection of the human host relies on these dual phases of infection whereby the virus hides from the immune system in memory B cells, and a subset of these cells are diverted to produce thousands of virions that not only infect more of the host's own lymphocytes but also are shed in saliva to infect other individuals. Viral replication is naturally enriched in the oral mucosa where memory B cells are routinely stimulated to differentiate after exposure to foreign antigens (Khan *et al.*, 1996).

Lytic viral replication is accompanied by expression of about 90 viral proteins, including BZLF1 (also known as ZEBRA), and complexes of viral proteins collectively referred to as early antigen and viral capsid antigen. These lytic antigens elicit a humoral immune response, resulting in elevated antibody titers that quell rampant lytic virus production in the healthy carrier (Crawford *et al.*, 1987).

Latent infection is characterized by abundant production of EBV-encoded RNA (EBER), but it is important to mention that EBER transcripts remain untranslated. EBER transcripts are thought to function in controlling translation (Khan *et al.*, 1992). Also expressed in latently infected cells are EBV nuclear antigen (EBNA) 1 and latent

membrane protein (LMP) 2A, neither of which elicits an effective immune response. EBNA1 functions to ensure that the viral genome is propagated to daughter cells upon cell division, whereas LMP2A keeps other viral proteins from being expressed. Limited protein expression helps avert immune destruction *in vivo* (Khan *et al.*, 1992).

Naturally infected B lymphocytes can be cultured from the blood of viral carriers by co-cultivating virions with B cells from neonatal umbilical cord lymphocytes. Viral culture represents an accurate and semi-quantitative measure of EBV in clinical samples, but it is rarely used in clinical laboratories due to high costs and slow turnaround time (Cohen, 2000).

More practical laboratory tests for EBV rely on detection of viral DNA and its gene products. In EBV-infected tissues, three different patterns of latent viral gene expression are seen. Type I latency refers to a very limited spectrum of latent viral gene expression, namely EBER transcripts along with EBNA1 and LMP2A proteins. This pattern is found in circulating lymphocytes of healthy viral carriers, and it is also characteristic of Burkitt lymphoma and gastric carcinoma. Type II latency, characterized additionally by LMP1 and LMP2B coexpression, is seen in Hodgkin disease, T cell lymphoma, and nasopharyngeal carcinoma, all of which tend to occur in immunocompetent hosts. Type III latency refers to the full spectrum of latent viral gene expression, as found transiently in acute infectious mononucleosis and as seen in EBV-driven lymphoproliferations arising in immunocompromised hosts. Viral genes expressed in Type III latency include all of the EBNAs (1, 2, 3A, 3B, 3C, LP), the LMPs (1, 2A, 2B), and EBER (Babcock *et al.*, 2000).

## **2.2 The association of EBV with HIV lymphoma subtypes**

The spectrum of lymphomas occurring in HIV infected patients includes pathologic subtypes showing specific association with EBV. Burkitt Lymphoma and Diffuse Large B Cell Lymphoma (DLBCL) are often closely associated to EBV infection. Most HIV associated lymphoproliferative disorders including Primary Central Nervous System Lymphoma, Systemic DLBCL Type 1B – plasmacytoid, Primary Effusion Lymphoma and Plasmablastic Lymphoma of the oral cavity type are linked to EBV infection. Hodgkin Lymphoma in HIV infected patients appears to be an EBV driven lymphoma (Carbone *et al.*, 1999).

HIV lymphomas are classified according to those occurring in immunocompetent patients, those occurring more specifically in HIV patients and those also occurring in other immunodeficiency states (Table 2.1).

### **2.2.1 Hodgkin Lymphoma**

Hodgkin Lymphoma (HL) is the most common type of non AIDS defining lymphoma. The risk of developing HL in HIV patients is upto 11 – 18 times above the general population (Goedert *et al.*, 1998). The association of HL with EBV is considerably stronger than that seen in HL in the non-HIV infected population. HIV associated HL most often presents at an advanced clinical stage and has an aggressive course with frequent extranodal disease. In HIV typically, unusual extranodal sites such as the skin, lung and GIT may be involved. These sites are essentially never involved by HL that is not associated with HIV (Thompson *et al.*, 2004).

**Table 2.2: Classification of HIV associated lymphomas adapted from Bibas *et al* (2009)**

Clinico-histological type of lymphoma	Occurrence of lymphoma
1a. Burkitt and Burkitt like lymphoma	1.Also in immunocompetent patients
1b. DLBCL	
i. Centroblastic	
ii. Immunoblastic (including PCNSL)	
1c. Extranodal marginal zone lymphoma	2.More specifically in HIV+ patients
1d. Classical Hodgkin lymphoma	
2a. Primary Effusion Lymphoma	
2b. Plasmablastic lymphoma of oral cavity type	
3a. Polymorphic B cell lymphoma Pens	3.Also in other immunodeficiency states

### 2.2.2 Burkitt lymphoma

This high grade B cell lymphoma is important in HIV because Burkitt and Burkitt like /atypical Burkitt Lymphoma make up the largest group of HIV associated NHL comprising upto 35–50% of these neoplasms in some studies (Spina *et al.*, 1998). BL in HIV is characterized by multiple genetic lesions. In addition to translocation of CMYC, point mutations in regulatory regions associated with MYC and within the TP53 tumor suppressor gene are common (Raphael *et al.*, 2001).

Burkitt NHL comprises a heterogeneous group of highly aggressive B cell malignancies (Harris *et al.*, 1994). EBV has been detected in 90% of endemic Burkitt lymphoma (BL), 20% of sporadic BLs and 40% of HIV- associated BLs. (Magrath, 1990). Currently

Burkitt lymphoma can be divided into three main clinical variants: the endemic, the sporadic and the immunodeficiency-associated variants (Ferry, 2006).

The endemic variant occurs in equatorial Africa (Kutok and Wang, 2006). It is the most common malignancy of children in this area. Children affected with the disease often also have chronic malaria which is believed to have reduced resistance to Epstein-Barr virus allowing it to take hold. The disease characteristically involves the jaw or other facial bones, the distal ileum, caecum, ovaries, kidney or the breast (Ferry, 2006).

The sporadic type of Burkitt lymphoma (also known as "non-African") is another form of non-Hodgkin lymphoma found outside Africa. The tumor cells have a similar appearance to the cancer cells of classical African or endemic Burkitt lymphoma. Again it is believed that impaired immunity provides an opening for proliferation of the Epstein-Barr virus. Non-Hodgkin, which includes Burkitt, accounts for 30-50% of childhood lymphoma. The jaws, especially the mandible, are less commonly involved, compared with the endemic variant. The ileo-cecal region is commonly involved (Harris *et al.*, 1994). Immunodeficiency-associated Burkitt lymphoma is usually associated with HIV infection or occurs in the setting of post-transplant patients who are taking immunosuppressive drugs. Actually, Burkitt lymphoma can be the initial manifestation of AIDS (Bellan *et al.*, 2003).

Burkitt lymphoma tumour cells have a translocation between chromosome 8 and chromosomes 14, 2 or 22 (Dalla-Favera *et al.*, 1982). Each translocation involves transfer of the *c-myc* oncogene (involved in cell activation and proliferation) to chromosomes bearing the immunoglobulin producing genes. Thus EBV antibody production is in concert with production of oncogenic proteins because of constitutive expression of *c-myc* (Dalla-Favera *et al.*, 1982).



### **2.2.3 Diffuse Large B Cell lymphoma (DLBCL)**

Diffuse Large B Cell Lymphoma is a clinico-histologically heterogeneous group of lymphomas. Those with a predominance of centroblasts have been termed centroblastic DLBCL, whereas those with greater than 90% immunoblasts/plasmablasts have been termed immunoblastic DLBCL.

Epstein Barr Virus -associated DLBCLs have been considered as EBV-driven lymphoproliferations occurring in the context of a defective T-cell immunity against EBV (Rowe *et al.*, 1991). However, unlike EBV-driven lymphoproliferative disease in transplant recipients, which includes monoclonal, oligoclonal, as well as polyclonal B-cell proliferations, DLBCL is always monoclonal. This suggests that, in addition to the effects contributed by EBV LMP-1, additional factors such as genetic damage are likely to contribute to the pathogenesis of AIDS-DLBCL.

### **2.2.4 Primary Central Nervous System Lymphoma (PCNSL)**

Primary Central Nervous System Lymphoma comprises 15% of HIV associated NHLs and has a reported incidence of over 1000 times greater than in the non-HIV population (Flinn and Ambinder, 1996). These tumors have a tendency to occur late in the course of HIV infection and show EBV association in virtually 100% of the cases (Cohen, 2005). A few studies have reported that detection of EBV in the cerebrospinal fluid of HIV-positive patients with a CNS lesion infers a diagnosis of lymphoma (Ivers *et al.*, 2004). These lymphomas have been reported to express all EBV latent encoded proteins (latency III) (McMahon *et al.*, 1991). Nevertheless, the exact role of EBV in the pathogenesis of these disorders remains not completely defined.

### **2.2.5 Primary effusion lymphoma (PEL)**

Primary Effusion Lymphoma occurs almost exclusively in HIV-infected patients. This lymphoma subtype comprises less than 5% of all HIV-associated NHL. Cases of this type were first described in 1989 (Knowles *et al.*, 1989), but its distinctive features were not fully recognised until after the identification of the Kaposi sarcoma-associated herpesvirus/human herpesvirus 8 (KSHV/HHV8) in 1994 (Nador *et al.*, 1996).

Primary Effusion Lymphoma is a distinct type of B-cell non-Hodgkin lymphoma (NHL) that presents most frequently in body cavities as lymphomatous effusions without an associated tumor mass.

Primary Effusion Lymphoma is defined by its consistent association with KSHV infection. Most cases are also co-infected by EBV. It is believed that KSHV, rather than EBV, is a driving force in these tumors, as in PEL, at least 5 KESV viral genes are expressed, which provide proliferative and antiapoptotic signals. In contrast, EBV has a restricted latency pattern of gene expression in PEL, where only EBNA1 and EBERs are expressed (Carbone *et al.*, 2009).

### **2.2.6 Plasmablastic lymphoma of the oral cavity type**

Plasmablastic lymphoma is a distinct type of DLBCL that occurs most often in the oral cavity or jaw of an HIV-infected individual. This rare lymphoma subtype accounts for 2.6% of HIV-related NHL (Folk *et al.*, 2006). The first description designated this tumour as a lymphoma of the oral cavity; however, subsequent reports have described less frequent involvement of extra oral sites such as the anal cavity, gastrointestinal tract, lung, paranasal sinus, skin, spermatic cord, testicle, bone and lymph nodes (Schichman *et al.*, 2004)

### **2.2.7 Polymorphic B-cell Lymphoma (PTLD-like)**

Human Immunodeficiency Virus infection results in a reduction of T-cell immunity similar to that iatrogenically induced in transplant patients. It is not surprising that polymorphic lymphoid proliferations resembling post-transplant lymphoproliferative disorders (PTLD) have been reported in HIV-infected adults and children. According to the WHO classification, they are divided into early lesions (reactive plasmacytic hyperplasia and mononucleosis-like syndrome), polymorphic lesions, monomorphic lesions, and Hodgkin-like lesions (Harris *et al.*, 2001). Similarly to PTLD, these infiltrates are often associated with EBV infection. The wide expression of the latent EBV-encoded proteins strongly suggests an important role that EBV may play in the oncogenic process.

The similar role that EBV is thought to play in inducing the survival and neoplastic transformation of infected GC cells in both PTLD and Hodgkin lymphoma, in addition to the near 100% EBV positivity in PTLD-associated Hodgkin lymphoma, has led some investigators to speculate a connection between the 2 diseases and the possibility that EBV infection and its GC effects may be the initiating role in the pathogenesis of both entities (Timms *et al.*, 2003).

### **2.3 The role of HIV in lymphomagenesis**

Human Immunodeficiency Virus is a lentivirus of the retrovirus family and viral DNA is integrated into the host chromosome via reverse transcribed viral DNA in a random process that is not directly oncogenic (Jarret, 2006). HIV appears to have an indirect causative effect in lymphomagenesis as HIV sequences have not been found in HIV associated lymphomas. In vitro studies showed that HIV infection of B cell lines derived from EBV seropositive individuals led to B cell immortalization, dysregulation of c - myc and activation of EBV (Herndier *et al.*, 1992). Certain HIV proteins especially Transactivator of transcription protein (tat) have been implicated as potentially

oncogenic by being transactivators of cellular genes like IL-6 and IL-7 (Herndier *et al.*, 1992). Transactivator of transcription (tat) protein also directly interferes with cell cycle control by interacting with the regulatory protein Rb2/p1308. This direct role has been identified as a significant factor in the pathogenesis of HIV related Burkitt lymphoma (Bellan *et al.*, 2003).

The increased risk for lymphoma in the HIV infected is related to several molecular, immunological and infectious factors. These include duration and degree of immunosuppression, induction of cytokines leading to B cell proliferation and opportunistic infections with oncogenic herpesviruses such as EBV and HHV8 (Knowles, 2003). Several findings support this view. The relative risk of AIDS associated tumors increases progressively with the decline of CD4+ T cell counts (Mbulaiteye *et al.*, 2003). The relationship between tumor development and immune deficiency is not so straightforward. Only certain types of AIDS associated tumors arise in immunodeficient patients. In particular NHL subtypes including Immunoblastic lymphomas and PCNSL and Burkitt like lymphomas typically develop in patients with very low CD4+ counts. On the other hand the other NHL subtypes along with classic Burkitt Lymphoma, Hodgkin's Disease, cervical cancer and Kaposi sarcoma increase in patients who have higher CD4+ T cell counts (Kirk *et al.*, 2001). Several other studies also indicate that immune activation rather than immune deficiency is key in initiation of B cell lymphomas. AIDS associated B cell lymphomas are described to be preceded by chronic antigen dependent B cell stimulation, This leads to a persistent and generalized lymphadenopathy that in turn promotes the clonal expansion of pre neoplastic antigen specific B cell populations (Carbone, 2003).

#### **2.4 Epstein Barr Virus and B lymphocyte interaction in tumorigenesis**

It has been hypothesized that failure of immune surveillance leads to conversion of polyclonal virally infected proliferating B lymphocytes to monoclonal malignancy. This is due to specific cytogenic rearrangements thus causing oncogene activation and

endowing altered tumor cells with selective growth advantage over normal diploid cells (Purtilo *et al.*, 1985). Immune regulation of latent EBV

infection is one of the best-studied examples of persistent T cell-mediated immune control. More than 90% of adults are seropositive for this virus and their B cells expressing EBV encoded latency associated transforming proteins are tightly controlled by high levels of EBV specific HLA restricted cytotoxic T lymphocytes (CTLs) which persist indefinitely. The interaction of specific TLs with the target cells of latent EBV infection in immunocompetent hosts is characterized by a complex self-modulating network of cellular immune mediated interactions resulting in potent target cell lysis (Purtilo, 1987).

B lymphocytes inactivate antigen presenting cells, preventing T cell recruitment. This leads to inhibition of CD8 positive CTLs. The B cells also express ligands which kill CTLs via apoptosis. Purified CD4+ T cells eliminated outgrowth when added to EBV infected B cells. Thus, unlike the killing of EBV infected lymphoblastoid cell lines, in which CD8+ cytolytic T cells play an essential role, prevention of early phase EBV induced B cell proliferation requires CD4+ effector T cells ( Khanna & Burrows, 2000).

Viruses associated with cancers encode pro-growth/proliferation functions and anti-apoptotic functions. EBV latent nuclear protein EBNA2 manipulates the host cell Notch signaling pathway, ultimately leading to immortalization of the infected B cell in immuno-compromised individuals. Notch is a transmembrane cell signaling protein involved in the developmental signaling pathway that influences

cell fate decisions, proliferation and survival. Notch 1 affects two decision points in stem cell regulation,

favouring self-renewal over differentiation and lymphoid over myeloid lineage outcome. When Notch is activated into its intracellular form it interacts with transcriptional repressor proteins displacing them to turn on transcription, resulting in cell proliferation (Levens *et al.*, 2000).

EBNA2 mimics the anti-apoptotic and cellular proliferation functions of Notch1C. The EBV gene product LMP1 is expressed in B cell lymphomas in immunodeficient individuals: it also interacts with the mitochondrial membrane and induces apoptosis. LMP1 is expressed in nasopharyngeal carcinoma and Hodgkin's disease. LMP1 usurps the TNF cell-signaling pathway leading to activation of NF $\kappa$ B activity and transcription. Mutations in LMP1 that destroy the ability to activate NF $\kappa$ B render the cell defective in transformation. EBV infected resting memory B cells, expressing LMP2A as the only viral latency gene, accumulates in the peripheral blood of immunosuppressed patients and are associated with high EBV DNA levels. Only very small numbers of proliferating infected cells account for the portion of viral DNA levels not ascribable to resting B cells in 50% of the patients with nasopharyngeal carcinoma and Hodgkin's disease (Babcock *et al.*, 2000). The cellular Bcl-2 gene is involved in the regulation of apoptosis. EBV encodes a vBcl-2 homologue BHRF1, shown to inhibit apoptosis. This gene is expressed during the lytic cycle and thought to be important in facilitating cell survival during lytic phase replication (Wang *et al.*, 1996). A second EBV encoded vBcl-2 homolog, BALF1, which like cellular Bcl-2 and BHRF1, inhibits apoptosis. This protein is induced in a variety of different EBV infected lymphocyte cell lines during lytic replication and also in Burkitt lymphoma cell lines during latency (Babcock *et al.*, 2000).

From distinct B lymphocyte subsets, tumor development is a complex mechanism involving host factors, alterations in tumor clones and viral input. The decreased immunosurveillance of the virus in HIV infected individuals with CD4 levels less than or equal to 50 cells/ $\mu$ l leads to lowered EBV specific cytolytic T lymphocytes, antigen stimulation and selection of B lymphocytes during generalized lymphadenopathy (due to B lymphocyte hyperplasia). All this results in oligoclonal expansion due to antigenic stimulation and therefore wide monoclonal selection. Dysregulation of the cytokine loop through autocrine IL-6 release from transformed B lymphocytes leads to growth and replication and HIV infected endothelial cells have increased adhesion molecules and

cytokines causing adhesion of lymphoma cells to endothelial cells.(Emilie and Zou, 1997)

## **2.5 Serological methods for EBV diagnosis**

Epstein Barr Virus infected B lymphocytes express a variety of viral encoded antigens. Included among these are some of diagnostic interest like viral capsid antigen (VCA) found in the cytoplasm, early antigen (EA which is a complex of two components, EA-D (diffuse) found in both the nucleus and cytoplasm of B cells and EA-R (restricted) found in the cytoplasm only) and the Epstein – Barr nuclear antigen (EBNA found in the nucleus of all EBV infected cells and EBNA –1 a synthetic peptide (p62) fusion polypeptide derived from a segment of EBNA (Middeldorp & Herbrink, 1988).

The key concern of EBV diagnostics is the accurate distinction of a primary infection from a past infection or seronegative status. Reactivation of EBV and subsequent viral replication is a common phenomenon but is normally clinically silent in immunocompetent individuals. However EBV reactivation causes considerable problems in immunosuppressed patients (Gartner, 2000). Diagnosis of primary EBV infection is based on testing for IgG and IgM antibodies to viral capsid antigens (VCA) and IgG antibodies to EBV nuclear antigens (EBNA) especially EBNA-1 (Middeldorp & Herbrink, 1988). Although the gold standard in EBV diagnostics is the indirect immunofluorescence assay (IFA), the enzyme immunoassay (ELISA) is often used in routine diagnostics because of its reliability in high throughput analyses. Laboratory diagnosis of EBV infection is based on serological tests to detect both specific antibodies to EBV antigens (Henle & Henle, 1981; 1982; De The, 1982; Purtilo, 1985) and heterophile antibodies (Paul & Bunnell, 1932; Davidsohn, 1969). At clinical onset the humoral response to EBV infection is characterized by the presence of circulating antiviral capsid antigen (VCA) IgM antibodies (Okano *et al.*, 1988).

## 2.6 Research gap

Serological EBV reactivation has been studied in detail by using parameters such as antibodies to early antigens, VCA IgA or the EBNA – 1 IgG/EBNA-2 IgG ratio and other parameters (Winkelsprecht *et al.*, 1996). However since no clinically relevant disease has been linked to EBV reactivation in immunocompetent individuals these parameters are only of limited value for the key concerns of routine diagnosis. In AIDS however EBV reactivation plays a major role in lymphomas even though serological diagnosis of EBV reactivation failed to correlate with EBV viral load in immunosuppressed individuals (Gartner *et al.*, 2000). The distribution of EBV reactivation phase proteins during HIV disease has not been documented in literature.

This study was based on the connection between EBV reactivation stage, advanced clinical HIV disease and the possibility that EBV infection dynamics at this stage lead to the expression of certain proteins that could be markers of the lymphoproliferative process that often occurs in HIV clinical stage 4 (Rezk & Weiss. 2007). The widespread use of ARVs is leading to aging of the immune system with an increase in neoplasms of lymphoreticular tissues (Sasco *et al.*, 2010). Previous work has shown that EBV viral loads may have poor diagnostic value for defining HIV patients at risk of developing EBV associated disease even though there is evidence of impaired EBV immunological control during HIV infection (Stevens *et al.*, 2002). Epstein Barr Virus serological diagnosis has failed to correlate with EBV viral load in immunosuppressed individuals (Grey, 1989). Serology is currently used to determine acute and chronic EBV infections (Gulley & Tang., 2008). Clinical EBV-related cancer is typically associated with high serological titres of antibodies against EA and IgG VCA with low EBNA titres. These findings should be interpreted cautiously as similar serology is found in autoimmune disease and other reactive disorders. Furthermore, with a dysfunctional immune system such as in AIDS, serology is not reliable (de Sanjose *et al.*, 2007).



### 3.1 Study site

A map of Kenya with major cities labeled: Kakuma, Marsabit, Migingo, Kisumu, Nakuru, Denderuone, Migori, Nairobi (marked with a yellow star), Kisumu, Kericho, Arusha, Malindi, and Mombasa. The map also shows the Indian Ocean to the east and the Google logo at the bottom left.



### 3.2 Study design and scope

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### **3.3 Study population**

Outpatient newly and previously diagnosed adult HIV+ patients attending the Mbagathi District Hospital HIV Outpatient Department who had been referred for laboratory investigations including CD4 cell counts, haematology and sputum culture for TB among others.

#### **3.3.1 Inclusion criteria**

Human Immunodeficiency Virus positive outpatients above the age of 18 years and at or below World Health Organization (WHO) stage 3 clinical disease who were not enrolled in an ARV treatment program at MDH and met all study ethical considerations were included in the study.

#### **3.3.2 Exclusion criteria**

Those patients who were below 18 years of age, those who were above 18 years of age but who were inpatients and were above WHO HIV stage 3 clinical disease and already enrolled in an ARV treatment program at MDH were excluded from the study. Those who did not consent to participate in the study despite meeting all inclusion criteria were also excluded.

### **3.4 Sampling of patients**

Study subjects who fulfilled all the inclusion criteria were systematically sampled (by selecting every 5<sup>th</sup> HIV+ patient) from outpatient adult HIV+ patients who were undergoing other clinical and laboratory investigations. The skip interval was calculated using the formula:

$$K^{\text{th}} = \text{Total population/sample size} = 500 \text{ HIV patients in a month} / 101 = 4.8 \text{ or } 5$$

### 3.4.1 Sample size calculation

The following sample size formula was used based on EBV prevalence rates of 90 % (Ocheni S *et al* ., 2010)

$$n = \frac{z^2 \alpha / 2 \cdot P(1-P)}{d^2}$$
$$= \frac{1.96^2 / 2 \times (0.9 \times (1-0.9))}{0.05^2}$$

n = minimum sample

$z^2 \alpha / 2$  = Standard normal deviate (1.96) at 95% Confidence interval

P=Prevalence of EBV=90%

d = precision level of 5%

**Thus the minimum sample size was 138 patients.**

However, a sample size of 101 was used due to lack of reagents.

### 3.5 Procedures on patients and samples

Study subjects who fulfilled the inclusion criteria underwent the following:

#### 3.5.1 Administration of a consent form and patient medical history questionnaire

An informed consent form was administered and the study patient had to sign the same (Appendix I ).Thereafter a questionnaire was administered to capture past and present socio-economic and medical history. Personal behavioural habits like smoking, alcohol consumption and chewing *Catha edulis* (khat) were also recorded in the same questionnaire (Appendix II).

### **3.5.2 Clinical examination**

Clinical findings were recorded in a structured form (Appendix III) under the following headings:

**3.5.2.1 Extraoral examination** – to record any peri-oral and cervical lymphadenopathy.

**3.5.2.2 HIV clinical staging** based on WHO (WHO Africa Region WHO/HIV 2005/02) criteria which defined the following HIV clinical stages (table 3.2).

### **3.5.3 Principles of EBV immuno-assays**

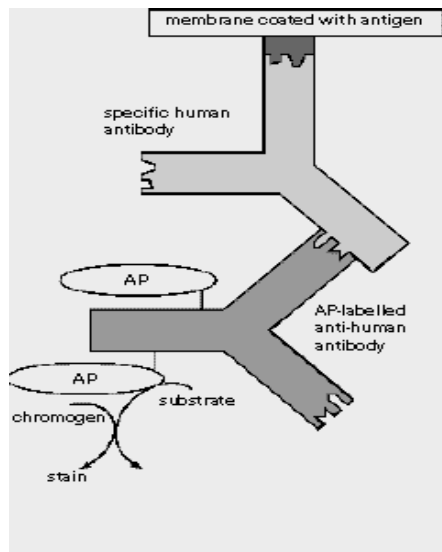
The EBV - Profile 2 immunoassay provided a qualitative in –vitro assay for IgG class antibodies in plasma to five different EBV antigens namely viral capsid antigens (VCA) gp125, p22, p19, Epstein Barr Nuclear Antigen 1 (EBNA – 1) and Early antigen – D (EA-D). The test kit contained test strips coated with thin parallel lines of several purified biochemically characterized antigens used as solid phase. In the first reaction step-diluted patient samples were incubated with the immunoblot strips. In the case of positive samples the specific IgG antibodies (also IgA and IgM) bound to the corresponding antigenic site. To detect the bound antibodies a second incubation was carried out using alkaline phosphatase labeled anti-human IgG (enzyme conjugate) which is capable of promoting a colour reaction in the third step when the bound antibodies were stained with a chromogen/substrate (Nitro blue Tetrazolium 5 Bromo 4 chloro 3 indolylphosphate) solution (Figure 3.3). Thus it was possible to analyze several antibodies next to each other and simultaneously under identical conditions (EBV Profile 2 Euroline ® kit instructions, 2006).

#### **3.5.4 EBV immunoblot methods**

Immunoglobulin G antibodies in whole blood for a range of EBV proteins were qualitatively demonstrated for the present study. These were directed at the following antigens; VCA gp125, VCA p19, EA – D, p22, EBNA1. The solid phase EBV ELISAs were performed on diluted venous blood stored in EDTA vacutainers using Euroline ® (Medizinische Labordiagnostika AG, Lubeck, Germany) test kit instructions. Each kit contained a 16 channel incubation tray, Nitrobluetetrazoliumchloride/5-Bromo-4-chloro-3-indolylphosphate (NBT/BCIP) substrate solution, Blocking Buffer, Universal buffer 10x concentrate, Alkaline phosphatase labeled anti-human goat IgG, IgG Human 100x concentrate positive control and 16 strips, each coated with, VCA gp125, VCA p19, VCA p22, EBNA -1 and EA-D

**Table 3.2: Symptoms defining each HIV clinical stage (WHO Africa Region )**

HIV Clinical Stage	Symptoms
1	Asymptomatic individual with persistent generalised lymphadenopathy.
2	Moderate unexplained weight loss (<10% of presumed or measured body weight) and one or more of the following co-morbidities: Recurrent respiratory tract infections (sinusitis, bronchitis, otitis media, pharyngitis), Herpes zoster, Angular cheilitis, Recurrent oral ulcerations, Papular pruritic eruptions, Seborrhoeic dermatitis, Fungal nail infections of fingers
3	severe weight loss (>10% of presumed or measured body, Unexplained chronic diarrhoea for longer than one month, unexplained persistent fever (intermittent or constant for longer than one month, oral candidiasis, oral hairy leukoplakia, Pulmonary tuberculosis (TB) diagnosed in last two years, severe presumed bacterial infections (like pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteraemia), Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis
4	HIV wasting syndrome, Pneumocystis pneumonia, Recurrent severe or radiological bacterial pneumonia, Chronic herpes simplex infection (orolabial, genital or anorectal of more than one months duration).Oesophageal candidiasis, Extrapulmonary TB, Kaposi Sarcoma, Central nervous system toxoplasmosis, HIV encephalopathy



AP = Alkaline Phosphatase

**Figure 3.2: Principles of the EBV ELISA test from Euroline test kit instructions (2006)**

#### 3.5.4.1 Preparation of reagents

All reagents were brought to room temperature (between 22 – 30<sup>0</sup> C) from 4<sup>0</sup> C for 30 minutes before use. The package with the test strips was then opened. The positive control was diluted 1:100 with ready to use Universal buffer and mixed well. The 10x concentrate enzyme conjugate was diluted 1:10. For 1 test strip 0.15ml anti-human IgG concentrate was diluted with 1.35 ml ready to use universal buffer.

#### **3.5.4.2 Incubation step**

The test strip was put into the incubation channel which was then filled with 1.5 ml blocking buffer and placed on a shaker for 15 minutes. The buffer was then aspirated off and 1.5ml of diluted serum sample (1:100) was pipetted into the incubation channel and the incubation tray was placed on a shaker and incubated for 1 hour. The strips in the channels were then washed by aspirating off the liquid and washing three times for 5 minutes with 1.5 ml universal buffer.

#### **3.5.4.2 Conjugation step**

The buffer was then aspirated off and 1.5ml enzyme conjugate was pipetted into the incubation channel and shaken for 1 hour. The enzyme conjugate was then aspirated off and washed three times for 5 min with 1.5 ml universal buffer.

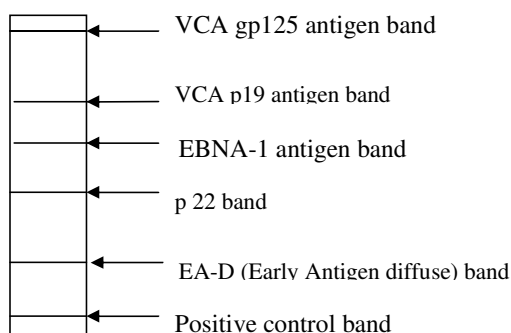
#### **3.5.4.3 Incubation with substrate**

After aspirating off the universal buffer 1.5ml substrate was added into the incubation channel and shaken for 20 minutes. This final reaction was stopped by aspirating off and rinsing three times with 1.5 ml deionized water. The test strips were then read visually. Correct incubation was confirmed by intensive staining of the control band. When the control band showed a weak colour reaction or none at all, the results were considered inconclusive and the test was repeated. The antigen bands are located at exactly defined positions. Positive and negative results were easily and reliably differentiated from each other by strength of colour reaction. The intensity of the antigen bands is an indication of the antibody titer (Figure 3.2 and Plate 3.1). All laboratory parameters were recorded in a standard form (Appendix IV).



### 3.5.5 Evaluation of band intensity

Following Labordiagnostika Medizinische AG EBV ELISA (Euroline) standard form protocol the protein bands were graded from 1 – 4 ranging from the weakest (1) to the strongest (4) in band intensity, using the control band as a 4. A band of 2 and below was considered negative and a band of 3 and above was considered positive.



**Figure 3.3: Schema of EBV test strip from Euroline test kit instructions**

### 3.5.6 Limitations

1. Epstein Barr Virus immunoblots were evaluated visually without the benefit of a digital evaluation since the EuroLine scan computer program which could have characterized the VCA bands into IgM and IgG class antibodies was not locally available (EBV-Profile 2 Euroline test kit instructions, 2006). Immunoglobulin M antibodies are produced in early infections and their presence could affect immunoblot band staining.

2. Immunosuppression is known to depress the antibody expression of B lymphocytes and thus host protein profiles are not a reliable indicator of viral load or viral activity including the presence and concentration of viral proteins (de Sanjose *et al.*, 2007).

3. The sample size should have been adjusted for EBV infection stages because some of the numbers in various infection stages were very small. Gartner *et al.*, 2003 did a similar study using 100 patients in each EBV infection stage. However the cluster analysis addressed this limitation.

### **3.5.7 Assessment of EBV infection status**

The stage of infection was assessed as described by Gartner *et al.*, (2003). Seronegativity or negative infection status was defined by the absence of intense bands of any of the 5 reference proteins. Early stage infection was defined by the presence of intense bands of Anti- gp125 and/or p19 IgG and sometimes Anti-EA-D. Late stage infection was defined by the presence of intense bands of Anti- EBNA1 and Anti- gp125 and/or p19. Reactivated infection was defined by the presence of intense bands of Anti-VCA, Anti EBNA-1, Anti-p22 and Anti – EA-D

## **3.6 Data management**

### **3.6.1 Data collection**

Clinical and laboratory data were recorded at the MDH laboratory and Dental clinic and the EBV ELISA results were recorded at the Yellow Fever laboratory, Centre for Virus Research, KEMRI Headquarters, Nairobi. Data was entered into designed paper data collection forms (Appendices II,III,IV), then transferred to a Microsoft Excel program (Millennium Edition) and verified by checking for data entry errors. Bivariate and multivariate analysis was done by transferring the database to a R (GNU S) computer program version 2.7

### **3.6.2 Data analysis**

Descriptive statistics with standard deviations and confidence intervals for socio-demographic and EBV serological status were calculated using the R (GNU S) version 2.7 computer program. Inferential statistics were done using bivariate and multivariate analysis. Cluster analysis was done to assess P values (an indication of how strongly the cluster is supported by data) via multi-scale bootstrap resampling. Two types of P values were provided, Approximately Unbiased (AU) P values and Bootstrap probability (BP) values. Clusters with AU larger than 95% are strongly supported by data.

### **3.7 Ethical considerations**

Clearance for this study was obtained from both the Scientific Steering Committee and the Ethical Review Committee of the Kenya Medical Research Institute (SSC No 908) (Appendix V). Patients were not coerced into participation while informed consent in writing was a prerequisite for inclusion into the study (Appendix I). All data entries were coded to ensure confidentiality.

## **CHAPTER FOUR**

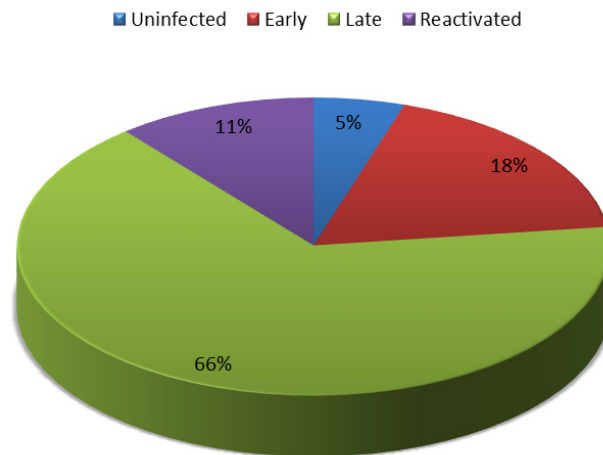
### **RESULTS**

#### **4.1 Characteristics of the study participants**

A total of 101 patients were recruited into the study. They all underwent a clinical head and neck examination, answered a questionnaire and had their blood samples taken for EBV immunoblot assay. The ages of study patients ranged between 20 years and 71 years with a mean of 35.6 years and a standard deviation of 9.7 years. The gender distribution of the sample was sixty-three (62 %) females and thirty eight (38%) males reflecting attendance patterns and a female preponderance of HIV/AIDS in Kenya. The majority were married (55%) while 20% were widowed, 18% were single never married and the balance were divorced or separated. When stratified by monthly income 74 respondents reported an income of less than Kenya Shillings (KES) 5000, while 38 had an income between KES 5001 – 10,000 and 9 reported an income of KES10,001 to 100,000.

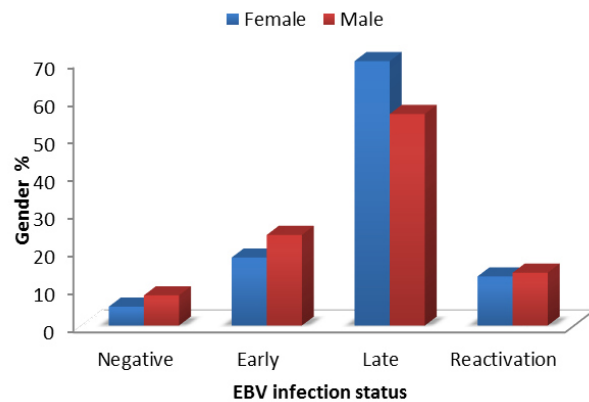
Sixty-four (64%) of the patients reported Nairobi as their residential county with smaller numbers coming from Kajiado, Machakos and other counties. The HIV clinical stage of the patients seen was 6% in Stage 1, 36% in Stage 2 and (58%) in Stage 3.

The prevalence of EBV infection in the study patients was 95% with the modal infection stage being late stage (66%), early infection (18%) and reactivated infection (11%). Only 5% were seronegative or uninfected (Figure 4.1).



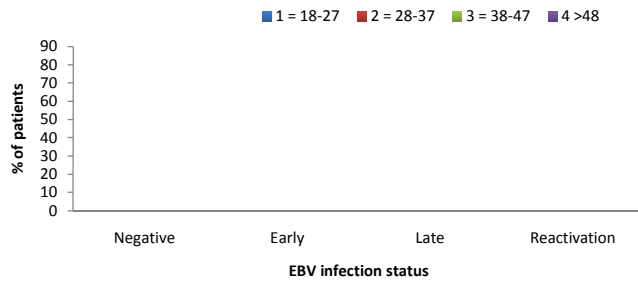
**Figure 4.1: Distribution of study patients by EBV infection stage**

When EBV infection status was stratified by gender, females constituted a higher proportion of the late infections (Figure 4.2). The gender differences in inter and intra EBV infection stage were not statistically significant.



**Figure 4.2: Stratification of each EBV infection stage by gender**

When EBV infection status was stratified by age the modal frequency of early infection was the 28 –37 year age group, the modal frequency of late infection was the 18 – 27 year age group and the modal frequency of reactivated infection was the 38 - 47 age group (Figure 4.3).



**Figure 4.3: Stratification of each EBV infection stage by age group of study patients**

From examination of the confidence intervals, the 18–27 age-group and the over 48 age-group had significant distribution differences between late and early as well as late and reactivated infection stages. Using similar criteria the 28 – 37 year age group had significant distribution differences between late and early infection stages (Table 4.1).

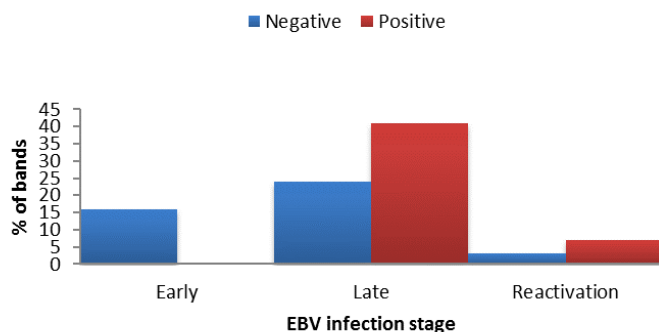
**Table 4.1: Frequencies of age groups in early late and reactivated EBV infection stages**

Age band	% Early	% Late	% Reactivated
18 -27	9.1 (95% CI=1.12 –29.2)	81.8 (95% CI=59.7 –94.8)	4.5 (95% CI=0.1- 22.8)
28 -37	25.0 (95% CI=10.7-44.9)	57.1 (95% CI=37.1- 75.5)	10.7 (95% CI=2.3 – 28.2)
38 - 47	22.2 (95% CI=8.6 –42.2)	55.6 (95% CI= 35.3 – 74.5)	18.5 (95% CI=6.3- 38.1)
48 +	16.7 (95% CI=4.7 –37.4)	62.5 (95% CI= 40.6- 81.2)	16.7 (95% CI=4.7 – 37.3)

#### **4.2 EBV infection status stratified by EBNA bands**

EBV infection stage was stratified by percentage of positive (at or above stage 3 band intensity) and negative EBNA bands (at or below stage 2 band intensity). No EBNA+ bands were seen in early infection however EBNA+ bands were seen in late and reactivated infections (Figure 4.4). The frequency of EBNA+ bands in late infection was 81.6%. (95% CI= 68 – 91 %) while the frequency of EBNA+ bands in reactivated infection was 18.4 %.( 95% CI= 8.8– 32 %). The difference in frequencies between the two infection stages was statistically significant.

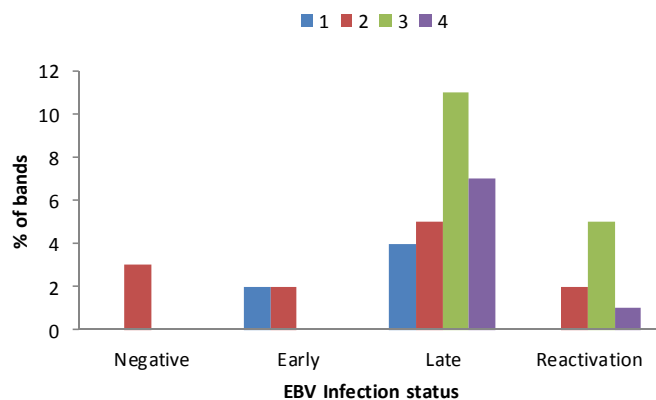




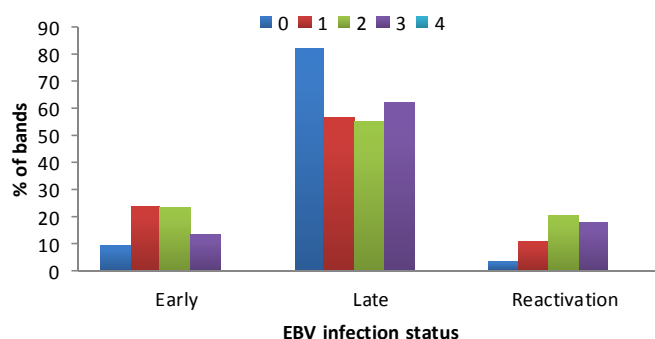
**Figure 4.4: EBV infection stage stratified by percentage of EBNA positive and negative bands in each infection stage**

#### **4.2.1 EBV infection status in HIV clinical stages as stratified by EBNA band intensity**

EBV infection status was plotted against percentage of the four grades of EBNA band intensities in each of HIV clinical stage 3 and early HIV clinical stage (at or below stage 2). Weak EBNA bands (intensity 1 and 2) were more frequently expressed across both HIV clinical stages showing low intensity infection with persistence of EBNA antibodies and/or low grade B lymphocytic expression of EBNA-1 in early to late stage EBV infection. The strong EBNA bands (intensity 3 and 4) were seen only in late and reactivated stage of EBV infection. In early HIV infection the distribution of strong and weak EBNA bands was similar to the distribution of these bands in HIV stage 3 (Figures 4.5 and 4.6).



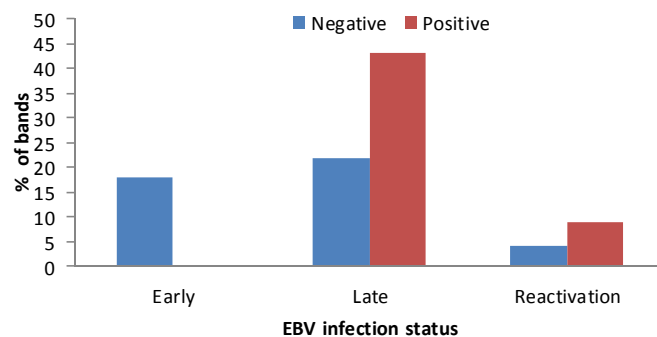
**Figure 4.5: EBV infection status in early clinical HIV stratified by percentage of each EBNA band intensity (graded from 0 -4) in each EBV infection stage**



**Figure 4.6: EBV infection status in HIV clinical stage 3 stratified by percentage of each EBNA band intensity (graded from 0- 4) in each EBV infection stage**

#### **4.3 EBV infection status stratified by p22 bands**

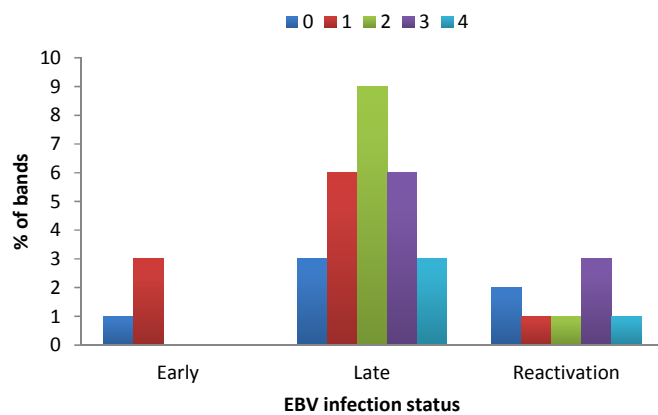
When EBV infection stage was stratified by positive and negative p22 bands, positive bands were seen in late and reactivated infections but not in early EBV infection (Figure 4.7). The frequency of p22+ bands in late infection was 82.4%. (95% CI= 61.9 - 91.6%) while the frequency of p22+ bands in reactivated infection was 17.6% (95% CI= 8.4 - 30.9%). This frequency differential between the two infection stages was statistically significant.



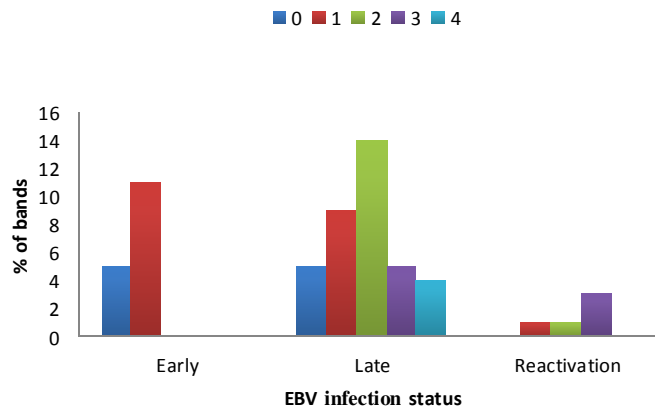
**Figure 4.7: EBV infection status stratified by percentage of positive and negative p22 bands in each infection stage**

#### **4.3.1 EBV infection status in HIV clinical stages stratified by p22 band intensity**

When EBV infection status was stratified by p22 band intensity in early HIV clinical stage and in clinical stage 3, strong bands were only seen in late and reactivated stage with a modal occurrence in late stage EBV infection. Weak p22 bands were seen most frequently in early and late EBV infections (Figures 4.8 and 4.9).



**Figure 4.8: EBV infection status in early HIV clinical stages stratified by percentage of each p22 band intensity (graded from 0 – 4) in each EBV infection stage**

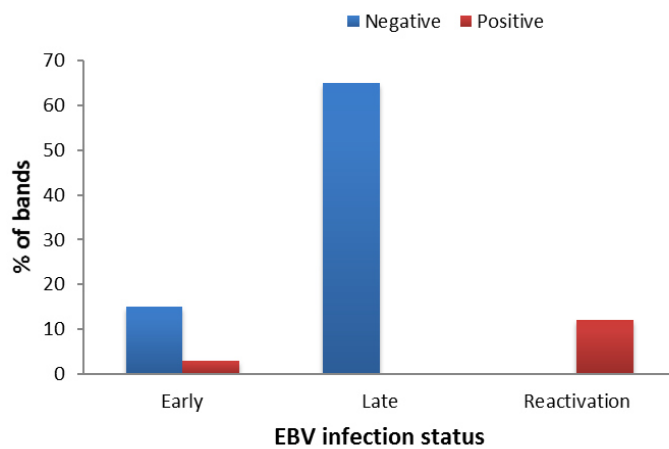


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**figure 4.9: EBV infection status in HIV clinical stage 3 stratified by percentage of each p22 band intensity (graded from 0 – 4) in each EBV infection stage**

#### **4.4 EBV infection status stratified by EA-D bands**

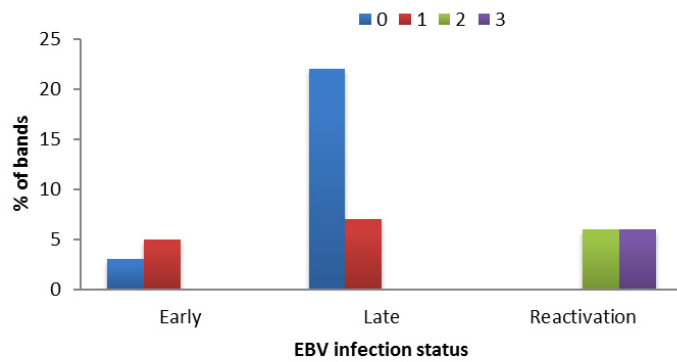
When EBV infection status was stratified by positive and negative EA-D bands, EA-D + bands were seen in early infection and in reactivation (Figure 4.10). The frequency of EA-D+ bands in early infection was 23.1%. (95% CI= 5 – 53%) while the frequency of EA-D + bands in reactivated infection was 76.9% (95% CI= 46.2 – 95%). This frequency differential between the two infection stages is not statistically significant.



**Figure 4.10: EBV infection status stratified by percentage of positive and negative EA -D bands in each infection stage**

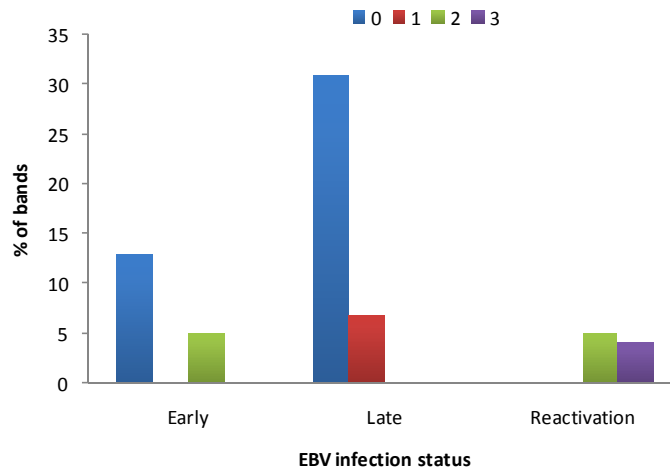
#### **4.4.1. EBV infection status in HIV clinical stages stratified by EA-D band intensity**

Strong bands of EA-D were only seen in reactivated EBV infections and borderline strong bands were seen in early infections when EBV infectious status across early and stage 3 HIV clinical stages were stratified by EA-D band strengths (Figures 4.11 and 4.12).



**Figure 4.11: EBV infection status in early HIV clinical stages stratified by percentage of each EA-D band intensity (graded from 0 – 4) in each EBV infection stage**





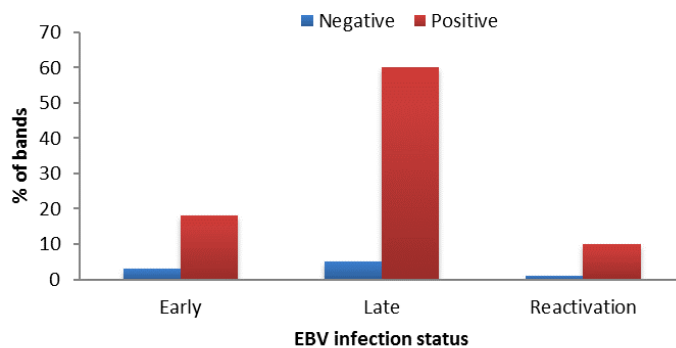
**Figure 4.12: EBV infection status in HIV stage 3 stratified by percentages of each EA-D band intensity (graded from 0 -4) in each EBV infection stage**

#### **4.5 EBV infection status stratified by p19 and gp125 bands**

When EBV infection was stratified by positive and negative p19 bands and against positive and negative gp125 bands, positive bands of both proteins were seen in early, late and reactivation stage of EBV infection with a modal distribution of each in late EBV infection (Figures 4.13 and 4.16).

In early EBV infection the frequency of p19+ bands was 19.3%. (95% CI= 11.7 - 29.1%) while the frequency in late infection was 67.0 % (95% CI= 56 -76.7%). The

frequency of p19+ bands in reactivated infection was 13.7% (95% CI=7.2 -22.6%). gp125 + bands were seen in early, late and reactivation stage of infection (Figure 3.16). The frequency of gp125 + bands in early infection was 14.9 %. (95% CI= 6.2 - 28.3%) while the frequency in late infection was 74.5% (95% CI= 59.7 - 86.1%). The frequency of gp125+ bands in reactivated infection was 10.6% (95% CI=3.5-23.1%).

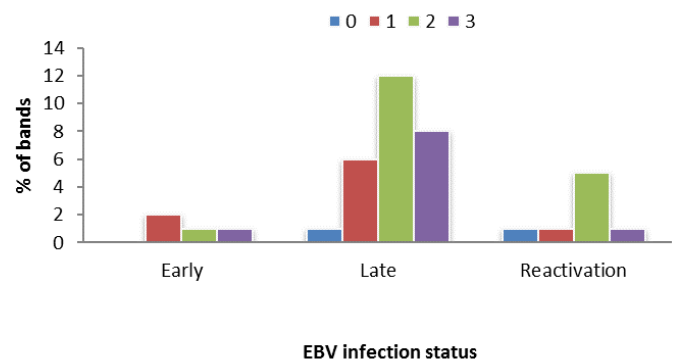


**Figure 4.13: EBV infection status stratified by percentage of positive and negative p19 bands in each EBV infection stage**

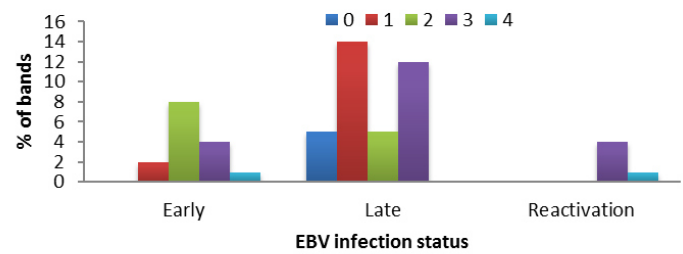
#### **4.5.1 EBV infection status in HIV clinical stages stratified by p19 band intensity**

On stratifying EBV infection status by p19 band intensity in HIV clinical stage 3 and in early HIV clinical stage there was a modal appearance of strong and weak bands in

late EBV infections across both HIV stages with no remarkable difference in reactivated infections across both HIV clinical stages (Figures 4.14 and 4.15) .



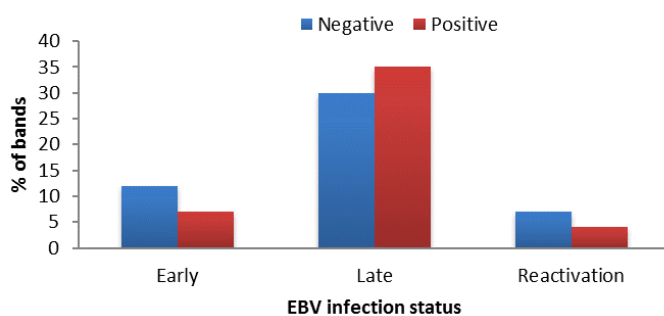
**Figure 4.14: EBV infection status in early clinical HIV stratified by percentage of each p19 band intensity graded from 0-4 in each EBV infection stage**



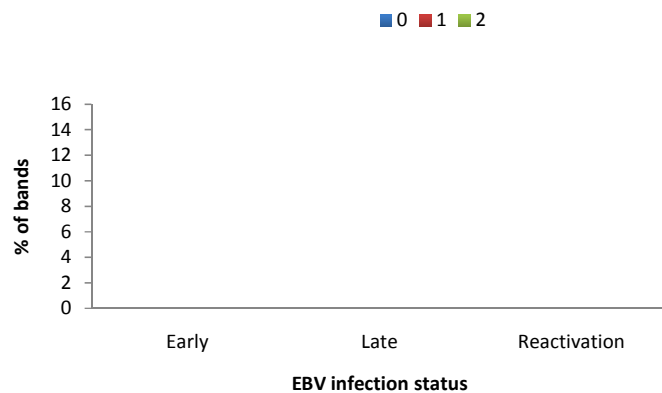
**Figure 4.15: EBV infection status in HIV stage 3 stratified by percentage of each p19 band intensity (graded from 0 – 4) in each EBV infection stage**

#### **4.5.2 EBV infection status in HIV clinical stages stratified by gp125 band intensity**

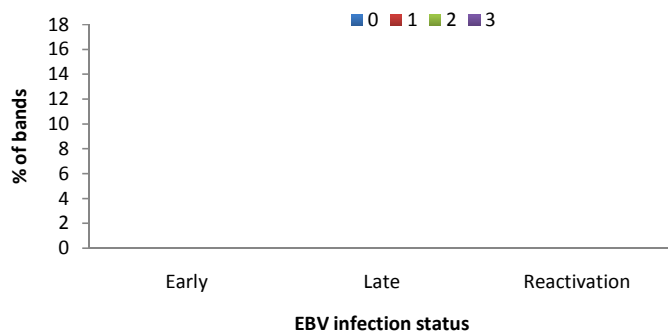
There was no significant difference in percentage of positive and negative bands of gp125 in each of the EBV infection stages even though there was a modal expression of gp125 in late stage EBV infection (Figure 4.16). When EBV infection status was stratified by gp125 band intensity in early HIV clinical stage and HIV clinical stage 3, there was a modal frequency of weak bands in late EBV infection across both HIV clinical stages. Positive bands were only seen in HIV clinical stage 3 (Figures 4.17 and 4.18).



**Figure 4.16: EBV infection status stratified by percentage of positive and negative gp125 bands in each EBV infection stage**



**Figure 4.17: EBV infection stage in early HIV clinical stages stratified by percentage of each gp125 band intensity (graded from 0 – 4) in each EBV infection stage**



**Figure 4.18: EBV infection status in HIV stage 3 stratified by percentage of each gp125 band intensity (graded from 0 – 4) in each EBV infection stage**

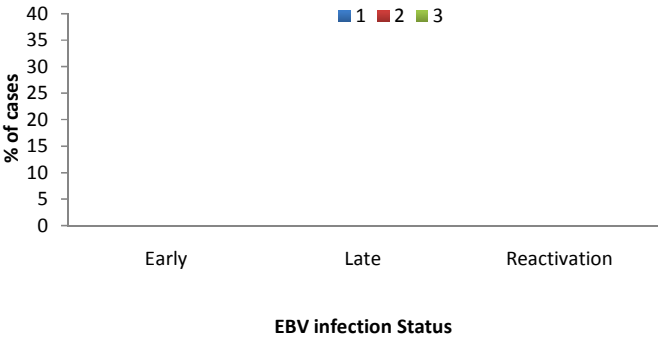
#### **4.6 EBV infection status and HIV clinical stage relationship**

HIV clinical staging was done using the WHO clinical staging system for HIV infected adults (NASCOP, MOH, 2004). Early and late EBV infections had a peak frequency in HIV stage 3 while reactivated infections had a peak frequency in HIV stage 2 (Figure 4.19). In early EBV infection there was a statistical difference between frequencies in early HIV and HIV Stage 3 (Table 4.2).

In late EBV infection there was a marginal statistical difference between frequencies in early HIV and HIV Stage 3. In reactivated EBV infection there was no statistical difference between frequencies in early HIV and HIV Stage 3 (Table 4.2).

**Table 4.2: Frequencies of HIV clinical stages in EBV infection stages**

HIV stage	Early EBV	Late EBV	Reactivated EBV
% HIV Stage 1(95% CI)	0	9.4 (3.5-19.3)	0
% HIV Stage 2(95% CI)	21.1(6.1- 45.6)	32.8 (21.6-45.7)	61.5 (31.6 -86.1)
%HIV Stage 3(95% CI)	78.9 (54.5- 93.9)	57.8 (44.8 - 70.1)	38.5 (13 - 68.4)



**Figure 4.19: EBV infection status stratified by distribution of HIV clinical stage**

#### 4.7 EBV infection status stratified by CD4 counts

When EBV infectious stage was stratified by CD4 counts below and above 200 cells/ $\mu$ l there was no apparent relationship between the two parameters. There frequency differentials of patients with CD4 counts below and above 200 across the three EBV infections stages were not statistically significant (Table 4.3).

Table 4.3: Frequencies of CD4 count levels in EBV infection stages

Infection Status	CD4<200	CD4>200
Early (95% CI)	27.3% (10.7 – 50.2)	20.0% (4.3 – 48.1)
Late (95% CI)	63.6% (40.7 – 82.8)	66.7% (38.4 – 88.2)
Reactivation (95% CI)	9.1% (11.2 – 21.2.2)	13.3% (1.7 – 40.5)

#### 4.8 Bivariate analysis

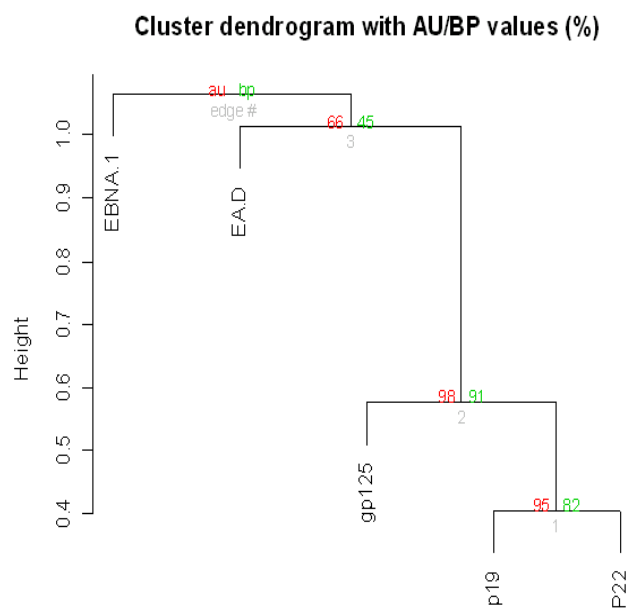
HIV clinical stage 3 was cross tabulated with EBV infection status and a Fishers Exact test done. A relationship between HIV clinical stage 3 and Late and reactivated EBV status was not established. There was a low risk of HIV clinical stage 3 causing advanced (Late and reactivated) EBV infection (OR = 0.60).  $p = 0.34$  (95% CI = 0.19 – 1.76). Equally there was a low risk of HIV clinical stage 3 causing late EBV infection (OR = 0.66) .  $p = 0.76$  (95% CI = 0.14 – 2.56). The risk of HIV clinical stage 3 causing Reactivated EBV infection was low (OR = 0.1).  $p = 0.03$  (95% CI = 0.01– 1.00).



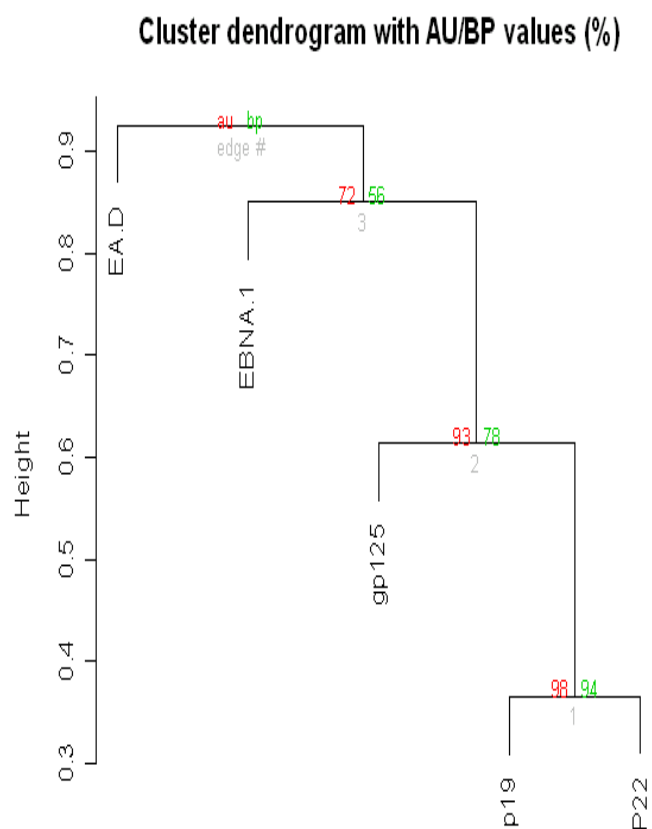
## **4.9 Protein cluster analysis**

### **4.9.1 Protein cluster analysis at various clinical stages of HIV**

Clustering of high intensity bands of the following proteins in HIV clinical stages (1 and 2) and 3, in decreasing order of strength of evidence was demonstrated : (gp125 and p19 and p22), AU P value = 98 and 93 and (p19 and p22), AU P value = 95 and 98. EBNA and EAD clustered closely in both clinical stage (1 and 2) and 3 AU P value = 66 and 72 but EAD was the runt in HIV clinical stage 3 whereas EBNA was the runt in clinical stage (1 and 2) (Figure 4.20 and 4.21).The P value standard error plot revealed that the EBNA – EAD cluster had the highest standard error (0.025) which means that despite the relatively weaker evidence for this cluster it was highly significantly different ( P value = 66 and Standard Error = 0.025) from any other cluster in HIV clinical stage 3 and HIV clinical stage 1 and 2 (Figure 4.22).

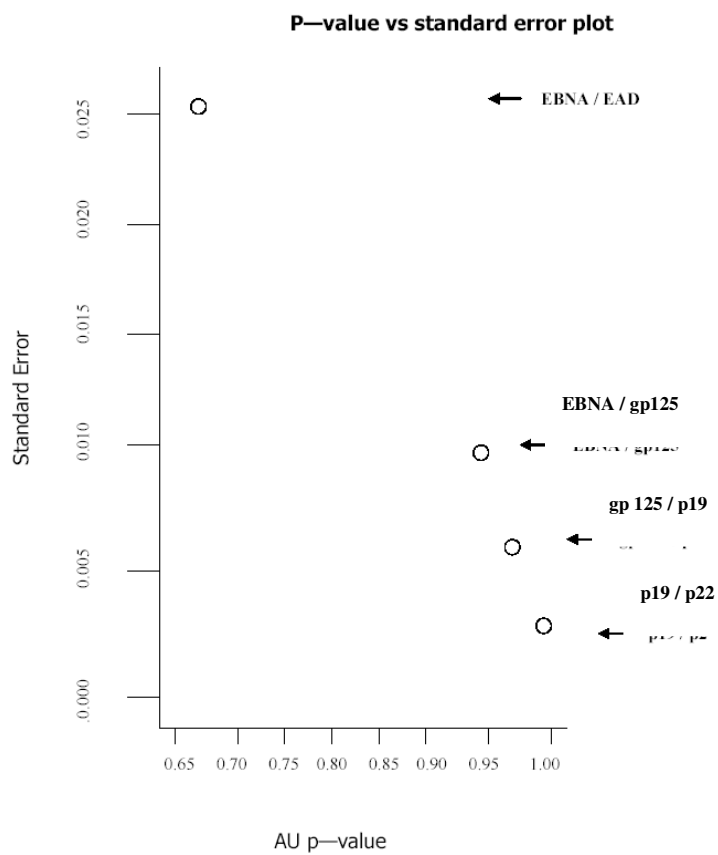


**Figure 4.20: Clustering of high intensity bands in early HIV clinical stages (1 and 2)**



**Figure 4.21: Clustering of high intensity bands in HIV clinical stage 3**

Thus the EBNA /EA-D pair has the lowest AU P value in all HIV clinical stages and the lowest SE (0.025) which is a reflection of the confidence interval (Suzuki *et al.*, 2006). This SE is quite different from the other protein clusters which have a SE range between 0.002 and 0.01. Therefore the EBNA/EA-D cluster is the most statistically significant cluster in HIV clinical stage 3 and in early clinical HIV stages (Figure 4.22).



**Figure 4.22: Approximately Unbiased Probability (AU - P) value versus standard error plot**

## CHAPTER FIVE

### DISCUSSION, CONCLUSIONS AND RECCOMENDATIONS

#### 5.1 Discussion

The basis of this study was that EBV infection is unaffected by HIV infection. Consequently it was expected that EBV antigens and the host antibodies produced in response to them would show no characteristic differences from that seen in the whole spectrum of EBV infection stages in immunocompetent individuals as described by Gartner *et al.*, (2003). As this study proceeded to the analysis of results it became necessary to confine the EBV infection status of interest to advanced infections (Late and Reactivation stage) since EBV reactivation is associated with neoplastic transformation of B lymphocytes and the manifestation of AIDS lymphomas. Additionally the focus on HIV clinical stage shifted to Stage 3 based on the clinical manifestation of lymphomas in HIV clinical stage 4 and the presumption that the molecular events that produce this unfold in HIV Stage 3 (Herida *et al.*, 2003).

It is worth noting that a panel of serological and molecular tests on serum or plasma can screen for Nasopharyngeal carcinoma in high-risk populations, assess prognosis, and monitor disease status over time (Paramita *et al.*, 2007). Although this study did not demonstrate a significant risk of either early or advanced HIV clinical stages causing advanced EBV infection, hierarchical cluster analysis revealed that EA-D and EBNA-1 clustered significantly stronger in HIV clinical stage 3 than in early HIV and compared to any other protein cluster, in the test panel of EBV viral capsid and nuclear proteins at any HIV clinical stage.

### **5.1.1 Distribution of EBV infection stage in the study patients**

This study determined the proportion of study patients with EBV infection as 95% confirming earlier reports that this virus is common in the human population (Crawford *et al.*, 1987). The EBV infection status of the study patients established the modal EBV infection status (66%) as late stage infection with 18% being in early infection, 11% in reactivated infection and 5% being un-infected. The detection of antibodies in immunosuppressed individuals is compromised by the fact that immunosuppression may delay the maturation of the humoral immune response to novel proteins (Gray, 1989). However the fact that 18% of the patients were in early infection was remarkable given the fact that in Kenya unlike in the West, EBV infection is expected to be transmitted early in infancy, from mother to child.

### **5.1. 2 Distribution of EBV infection stage by gender and age**

The difference in gender and age across the EBV infection stages in the study patients was not significant. This shows that the natural history of EBV in these HIV+ individuals is not distributed differently by gender and age in HIV-positive individuals confirming earlier studies (Glaser *et al.*, 2003). This is a reflection of the status quo in immuno-competent individuals.

### **5. 1. 3 Distribution of EBV infection stage by EBNA-1 bands**

EBNA-1 positive bands were significantly more common in late stage and reactivated stage of EBV infection. These results are similar to those of Gartner *et al.*, 2003 which demonstrated similar frequencies of EBNA-1 positive bands in the various EBV infection stages. In both early HIV clinical stages and HIV clinical stage 3, weak EBNA bands were demonstrated in early and late stage EBV infection. Strong bands were only demonstrated in late and reactivated infections indicating increased B lymphocytic expression of anti-EBNA-1 across all stages of HIV infection, this reflecting a

heightened humoral immune response to changing EBV antigen profiles. EBNA+ bands are diagnostic of late and reactivated EBV infections in HIV stage 3.

#### **5.1.4 Distribution of EBV infection stage by p22 bands**

The distribution of p22 positive bands exclusively between late stage EBV infection and reactivated stage was statistically significant. Strong bands were only demonstrated in late and reactivated infections indicating increased B lymphocytic expression of anti-p22 across all stages of HIV infection, this reflecting a heightened humoral immune response to changing EBV antigen profiles. p22+ bands are diagnostic of late and reactivated EBV infections in early and HIV stage 3 patients. This also concurs with the work of Gartner *et al.*, 2003 on distribution of p22+ bands across the EBV infection stages.

#### **5.1.5 Distribution of EBV infection stage by EA-D bands**

The distribution of positive EA-D bands exclusively between early EBV infection and reactivated infection was not statistically significant. Strong bands were only demonstrated in reactivated infections while borderline strong bands were seen in early infection as in the work by Gartner *et al.*, 2003 on frequency of EA-D positive bands.

#### **5.1.6 Distribution of EBV infection stage by p19 and gp125 bands**

The distribution of positive p19 and gp125 bands was noted across all EBV infection stages with statistically significant differences between the modal late stage and the reactivation stage. This is because of the very definition of EBV infectious serology which categorizes infectious stages according to proteins expressed at each stage (Gartner *et al.*, 2003). In HIV clinical stage 3 there is a modal expression of both weak and strong bands of p19 and gp125 in late EBV infection with no remarkable difference in strong bands of both these antibodies in reactivation stage in early HIV or HIV stage

3. From these data both p19 and gp125 cannot on their own, be markers of EBV reactivation in HIV disease.

#### **5.1.7 Frequencies of Anti- EBV antibodies at different EBV infection stages**

The frequency of EBNA+ bands in late EBV infections in the study patients was marginally lower than that of Gartner *et al* (2003) (81.6%: 99%) possibly due to secondary loss of Anti – EBNA antibodies in patients in this series . The frequencies of EA-D in reactivated infection in this study were in total agreement with the reference work at 19% . The frequency of anti p22+ bands in late EBV infections was marginally lower than the reference work (82.4%:99%) possibly due to faster secondary loss of this VCA in the study population (Figure 4.7). The frequency of VCA (calculated using an average of EBNA, p22, p19 and gp125 frequencies) in this series were lower than the reference study (70.75%:100%) possibly for population specific reasons.

The difference in EBNA+ and p22+ band proportions between Late and Reactivation stages of EBV infection was highly statistically significant The difference in p19+ and gp125+ band specific proportions between late and reactivation stage EBV infection was statistically significant.

This study demonstrated the prevalence of EA-D+ bands in early EBV infections in the study patients as drastically lower than Gartner's 2003 work (23.1%: 72%) possibly due to population specific reasons. There was a non-significant frequency differential in EBNA+ bands between early and reactivated EBV infections. Frequencies of gp125 and p19+ bands in early infection were significantly different from those in late stage EBV infection but non significantly different from reactivated infection . These data demonstrate similar protein expression in patients with HIV as with immunocompetent individuals (Gartner *et al* ., 2003).



#### **5.1.8 Anti EBV antibodies in late and reactivated EBV infections**

Late and reactivated EBV infections are characterized by the expression of EBNA and EA- D in immuno-competent patients (Gartner; 2003). This study not surprisingly demonstrated that EBNA+ bands are diagnostic of Late and reactivated EBV infections in HIV stage 3 patients during which gp125+ bands may be seen. P22+ bands are diagnostic of late and reactivated EBV infections in early and HIV stage 3 patients. P19+ bands are not diagnostic of late and reactivated EBV infections in HIV stage 3 patients. EA-D+ bands are diagnostic of reactivated EBV infections in early and clinical stage 3 HIV. Weak bands of all five proteins were seen more frequently in late stage EBV infection in both HIV clinical stage 3 and early HIV clinical stage except in the case of EBNA and p22 which were seen more frequently in early EBV in HIV clinical stage 3. Weak bands are an indication of persistent immunoglobulins produced early on in the EBV natural history or of low grade B lymphocyte production of the same. Immunosuppression has an inhibitory effect on B cells due to low numbers of memory cells in circulation that are expected to assist the T lymphocytes in mounting an immune response to HIV.

#### **5.1.9 HIV clinical stages and EBV relationship from cluster analysis**

EAD and EBNA 1 clustered closely in HIV stage 3 with EAD as the runt in this stage. The evidence for this cluster was slightly stronger (AU = 72%) than the EBNA - EAD cluster in HIV clinical stage 1 and 2 (AU = 66%) where EBNA was the runt. The evidence for EBNA-EAD clustering with gp125 was slightly weaker but very strong in HIV clinical stage 3, (AU 93%) compared to HIV clinical stage 1 and 2 (AU = 98%). The evidence for the clustering of p19 and p22 was slightly stronger in HIV clinical stage 3 (AU = 98%) compared to HIV clinical stage 1 and 2 (AU = 95%). EAD and the Viral capsid antigens gp125, p19 and p22 are lytic proteins. This evidence has demonstrated the clustering of EAD, a lytic protein and EBNA, a nuclear antigen

associated with latency. Both EAD and EBNA are markers of reactivation and therefore this data demonstrates the reactivation of latent EBV infection. However it should be noted that established EBV-related cancer is typically associated with high serological titers against EA and IgG VCA with low EBNA titer. Results should be interpreted with caution since similar patterns are possible in autoimmune disease and other reactive conditions (de Sanjose *et al.*, 2007).

## 5.2 Conclusions

1. 95% of patients had antibodies to some or all of the 5 EBV antigens. Of these, 11% demonstrated early EBV infection antibody profiles, 66% demonstrated late EBV infection antibody profiles and 18% demonstrated EBV reactivation antibody profiles.
2. The proportions of EBV infections stratified by HIV clinical stage were as follows
  - a) Early EBV infections were distributed as follows: HIV stage 1: 0%, HIV stage 2: 21.1% (95% CI = 6.1 - 45.6), HIV stage 3: 78.9% (95% CI = 54.5 - 93.9).
  - b) Late EBV infections were distributed as follows: HIV stage 1: 9.4% (95% CI = 3.5 - 19.3), HIV stage 2: 32.8 % (95% CI = 21.6 - 45.7), HIV stage 3: 57.8 % (95% CI = 44.8 - 70.1). The distribution of late EBV infections across HIV stage 2 and 3 was not statistically significant.
  - c) Reactivated EBV infections were distributed as follows: HIV stage 1: 0 %, HIV stage 2: 61.5 % (95% CI = 6.1 - 45.6), HIV stage 3 :78.9% (95% CI = 54.5 - 93.9). The distribution of reactivated EBV infections across HIV stage 2 and 3 was statistically significant.

3. The distribution of late and reactivated EBV infections across HIV stage 2 was not significantly different. The distribution of late and reactivated EBV infections across HIV stage 3 was also not significantly different. Thus advancing HIV clinical stage has no association with EBV reactivation.
- 4.. EAD and EBNA1 clustered closely in HIV stage 3 and in reverse order in HIV stage 1 and 2.
5. Evidence from cluster analysis indicates that the EBNA/EA-D cluster is statistically significantly different from other protein clusters in early and HIV clinical stage 3 HIV disease.
6. Of all EBV infectious proteins EBNA1/EAD are the strongest candidates for EBV associated tumor markers in HIV

### **5.3 Recommendations**

- i. Arising from this study Solid phase EBV immunoblot tests need to be developed in which two bands for EBNA and EA-D proteins are present in addition to gp125, p19 and p22 and these could be used to predict reactivation and possibly neoplastic transformation in HIV patients, irrespective of their clinical stage as currently defined.
- ii. Further epidemiological studies will have to be done to study EBV infection serology while adjusting for immune system dysfunction in HIV disease so that more evidence can be established for the utility of EBNA and EA-D as early protein markers of HIV lymphoproliferative disease. Emerging technologies such as gene expression profiling and proteomics will identify patterns of viral and human gene expression correlating with diagnosis, prognosis, and outcome in response to therapy. A coordinated effort by basic

scientists and clinical investigators will improve our arsenal of laboratory methods and better define their clinical utility (Gulley *et al.*, 2008). Refined ELISA tests should be developed for early diagnosis of HIV associated lymphomas

- iii. A longitudinal study to trace the expression of all five proteins and in particular EBNA1 and EAD should be conducted in HIV patients

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## **APPENDICES**

### **Appendix I: informed Consent Form**

#### **1 Information to study participants**

Infection with Human immunodeficiency virus (HIV) and Epstein-Barr (EBV) results in the development of lymphomas in some patients. This is a cancer of lymphatic tissue in several body sites. Individuals infected with EBV are at risk of development of lymphomas due to interaction between HIV, EBV and the human body. However the factors that predispose to development of lymphomas are not well understood. Your participation in the study will enable us to understand better ways of preventing lymphomas from developing and or developing laboratory tests for early detection of lymphomas. The Principal investigator in this project is Dr Melvin D'lima of CPHR, KEMRI.

Participation in the study is voluntary. You may refuse to undergo any procedure you find undesirable. No risks are foreseen and you will be provided treatment for any oral mucosal condition found.

## **2 Clinical and laboratory procedures**

You will be involved in a clinical study, which will involve requests on your past and present medical history, a clinical examination of your head and neck. Five milliliters of blood will also be drawn for laboratory investigations.

## **3 Precautions**

The procedures are to investigate your clinical condition and all precautions are undertaken to ensure that your safety is maintained. Except for the collection of blood and a biopsy where necessary, the procedures are non-invasive.

## **4 Benefits of the study**

You will be treated if any soft tissue lesions are found in the mouth. Information obtained from this study may assist in developing ways of reducing the risk of development of lymphomata in HIV infection.

## **5 Confidentiality**

All records obtained in this clinical study will be kept confidential.

Kindly address any queries to Dr Melvin D'lima at CPHR, KEMRI P.O. Box 20752 00200 KNH, Nairobi, Tel +254-20-2725016/7 Cell +254720297433 Or The Chairman, KEMRI/National Ethical Review Committee, P.O. Box 54840 Nairobi, Tel. 254-2-2722541 before signing this document.

I the undersigned have understood the above information, which has been fully explained to me by the Principal investigator

Patient ..... Signature ..... Date  
.....

## **Appendix II : Patient Questionnaire**

1. Serial # \_\_\_\_\_
2. Age \_\_\_\_\_
  - a) 18-29 \_\_\_\_\_
  - b) 30-49 \_\_\_\_\_
  - c) 50-69 \_\_\_\_\_
  - d) 70+ (specify) \_\_\_\_\_
3. Birth district \_\_\_\_\_ District of Residence \_\_\_\_\_
4. Sex
  - a) Male \_\_\_\_\_
  - b) Female \_\_\_\_\_
- 5. Marital status**
  - a) Single unmarried \_\_\_\_\_
  - b) Divorced \_\_\_\_\_
  - c) Separated \_\_\_\_\_
  - d) Widowed \_\_\_\_\_
  - e) Married \_\_\_\_\_
- 6. Monthly income (KESs)**
  - a) Less than 1000 \_\_\_\_\_
  - b) 1000-2000 \_\_\_\_\_
  - c) 2000 – 5000 \_\_\_\_\_

- d) 5000 – 10000 \_\_\_\_\_
- e) 20000 – 50000 \_\_\_\_\_
- f) 50000 – 100000 \_\_\_\_\_
- g) Other( specify) \_\_\_\_\_

**Cooking fuel**

- a) Wood \_\_\_\_\_
- b) Charcoal \_\_\_\_\_
- c) Kerosene \_\_\_\_\_
- d) Gas \_\_\_\_\_
- e) Electricity \_\_\_\_\_
- f) Other (Specify) \_\_\_\_\_

**. Alcohol consumption by type and amount per week**

- a) Beer \_\_\_\_\_
- b) Spirits \_\_\_\_\_
- c) Busaa \_\_\_\_\_
- d) Chang'aa \_\_\_\_\_
- e) Muratina \_\_\_\_\_
- f) Other (specify) \_\_\_\_\_
- g) Amount per week glasses/bottles \_\_\_\_\_

**9. Tobacco consumption by type and amount per week**

- a) Filtered \_\_\_\_\_
- b) Unfiltered \_\_\_\_\_

c) Chewing \_\_\_\_\_

10. Khat usage

a) Yes \_\_\_\_\_

b) No \_\_\_\_\_

c) Frequency per week \_\_\_\_\_

11. Time since ISD diagnosis \_\_\_\_\_ months/years

**12. History of HAART use**

a) Regimen \_\_\_\_\_

b) Duration \_\_\_\_\_

### **Appendix III: Clinical Data Form**

#### **1. Extra-oral exam**

Lymphadenopathy, Yes \_\_\_\_ (1), No \_\_\_\_ (2), Site \_\_\_\_

Parotomegaly, Yes \_\_\_\_ (1), No \_\_\_\_ (2), Site \_\_\_\_

#### **2. HIV clinical stage**

a) Primary infection \_\_\_\_\_

b) Clinical stage 1 \_\_\_\_\_

c) Clinical stage 2 \_\_\_\_\_

d) Clinical stage 3 \_\_\_\_\_

e) Clinical stage 4 \_\_\_\_\_

#### Appendix IV: Laboratory Data Form

1. Serial # \_\_\_\_\_
2. Date \_\_\_\_\_
3. Name \_\_\_\_\_
4. ID# \_\_\_\_\_
5. Postal Address \_\_\_\_\_
6. Physical address \_\_\_\_\_
- Age \_\_\_\_\_

#### HIV status

- 1a. Viral Load \_\_\_\_\_ copies/µlitre      1b. ELISA \_\_\_\_\_
- 1c. CD8 cell count \_\_\_\_\_ cells/µlitre      1d. CD4 Cell count \_\_\_\_\_
- copies/µlitre \_\_\_\_\_

#### EBV serology


Antibody	Band color (1 -4)
VCAP125	
VCAP19	
EA – D	
p22	
EBNA – 1	

#### TB

- 4a. Sputum (Acid fast bacilli) \_\_\_\_\_      4b. CXR \_\_\_\_\_



## Appendix V: KEMRI ERC Approval

  
**KENYA MEDICAL RESEARCH INSTITUTE**

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

KEMRI/RES/7/3/1 8<sup>th</sup> March 2005

Dr. P. Wanzala  
CPHR,  
NAIROBI

---

Thro' Director,  
CPHR,  
NAIROBI

Dear Madam,

RE: SSC Protocol No. 908 – Oral shedding of Epstein-Barr virus in HIV infection, by  
Peter Wanzala (CPHR)

---

Refer to your letter dated 7<sup>th</sup> March 2005.

We acknowledge receipt of the revised consent form and therefore grant the protocol approval.

You may proceed with your study.

*ROTHA...*  
R. C. M. KITHINJI,  
FOR: SECRETARY,  
KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE

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In Search of Better Health

**Appendix VI: Authorization letter from Principal Investigator**



Centre for Public Health Research, KEMRI  
P. O. Box 20752  
00202, Nairobi  
Tel: 020 2725017/8  
October 30, 2015

Prof Z Nganga  
The Director  
ITROMID  
JKUAT  
Juja

Dear Prof Nganga

**Re: MSc PROJECT - DISTRIBUTION OF EBV ANTIBODIES IN HIV POSITIVE INDIVIDUALS AT VARIOUS STAGES OF HIV/AIDS DISEASE AT MBAGATHI DISTRICT HOSPITAL, NAIROBI COUNTY - A STUDY CONDUCTED IN 2006.**

This is to confirm that the above titled MSc student project conducted by Melvin Edward D'lima (TM310/0024-2004) was part of my ongoing approved project, "Oral shedding of Epstein Barr virus in HIV infection" (KEMRI SSC No 908 - approved March 08 2005) which involved salivary and seroprevalence of whole Epstein Barr virus and viral antibodies.

Yours faithfully

A handwritten signature in dark ink, which appears to read "Wanzala", is written over a horizontal line.

Dr Peter Wanzala, PhD  
Principal Investigator  
CPHR-KEMRI

**Appendix VII: Publication arising from this thesis**

November 27, 2014 HIV/AIDS, Volume27\_4

**Clustering of anti-EBV antibodies in patients at various clinical stages of HIV infection, as evidence for potential tumor markers for AIDS associated lymphoproliferative disorders.**

Melvin Edward D'lima, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology

Rebecca Waihenya,  
Jomo Kenyatta University of Agriculture and Technology

Newell Johnson,  
Griffith University, Australia

Peter Wanzala, KEMRI

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Sources of financial support, if any

Dr Peter Wanzala

## Summary

Co-infection of EBV and HIV alters the EBV and human host cell life cycle and is associated with lymphoproliferative disorders. Changing EBV infection stages are characterized by expression of various viral capsid and nuclear antigens. This cross sectional qualitative study describes the clustering of anti EBV antibodies in HIV positive patients. A total of 101 adult subjects were randomly selected from HIV seropositive outpatients at Mbagathi District Hospital, Nairobi. Citrated venous whole blood was used for EBV antibody assays by solid phase ELISA using EBV Profile 2™ kits. HIV clinical staging of study patients demonstrated 5.9% in Stage 1, 35.6% in Stage 2 and 57.8% in Stage 3. EBV infection staging, based on antibody profiles, showed 18% in early infection, 66% in late infection, 11% in reactivated infection, with 5% EBV seronegative. Cluster analysis of high intensity protein bands demonstrated EBNA1/EAD as the weakest cluster pair by approximately unbiased or Bootstrap probability values in all HIV clinical stages but these were highly significantly different from any other cluster. EBNA and EAD appear to be the strongest candidate biomarkers of EBV reactivation in this population.

Comment [p1]: ??

## Introduction

In AIDS, altered EBV latency plays a major role in lymphomas even though serological diagnosis of EBV reactivation failed to correlate with EBV viral load in immunosuppressed individuals.<sup>1</sup> The distribution of EBV reactivation phase proteins during HIV disease has not been documented in literature.

This study was based on the nexus between EBV reactivation stage, advanced clinical HIV disease and the possibility that EBV infection dynamics at this stage lead to the

expression of certain proteins that could be markers of the lymphoproliferative process that often manifests in HIV clinical stage 4.<sup>2</sup> The widespread use of ARVs is leading to aging of the immune system with an increase in neoplasms of lymphoreticular tissues.<sup>3</sup> Previous work has shown that EBV viral loads may have poor diagnostic value for defining HIV patients at risk of developing EBV associated disease even though there is evidence of impaired EBV immunological control during HIV infection.<sup>4</sup> EBV serological diagnosis has failed to correlate with EBV viral load in immunosuppressed individuals.<sup>5</sup> Serology is currently used to determine acute and chronic EBV infections.<sup>6</sup> However clinical EBV-related cancer is typically associated with high serological titres against EA and IgG VCA with low EBNA titres. These findings should be interpreted cautiously as similar serology is found in autoimmune disease and other reactive disorders. Furthermore with a dysfunctional immune system such as in AIDS, serology is not reliable.<sup>7</sup>

Whilst advanced clinical HIV infection is associated with a number of EBV related lymphoproliferative disorders, the altered humoral response arising from HIV immunosuppression, to antigenic challenge in general likely renders standard methods, or at least their interpretation, inconclusive. Therefore EBV serology has not been used to diagnose and monitor HIV associated lymphoproliferative disorders. In advanced centres these are monitored by Real Time Polymerase Chain Reactions and other genomic studies. These techniques are not economically viable for routine diagnostics in less equipped laboratories. However, EBV infection proteomic dynamics are being altered by concomitant HIV infection. Therefore, it is plausible that a shift from latent to lytic EBV protein profiles will occur. This will manifest in reactivation of latent EBV infection in HIV disease and potentially mark the start of tumorigenesis.

Currently there are no serological markers for the risk of EBV-induced lymphoproliferative disorders in the course of HIV infection.

Comment [p2]: ref

## **Materials and methods.**

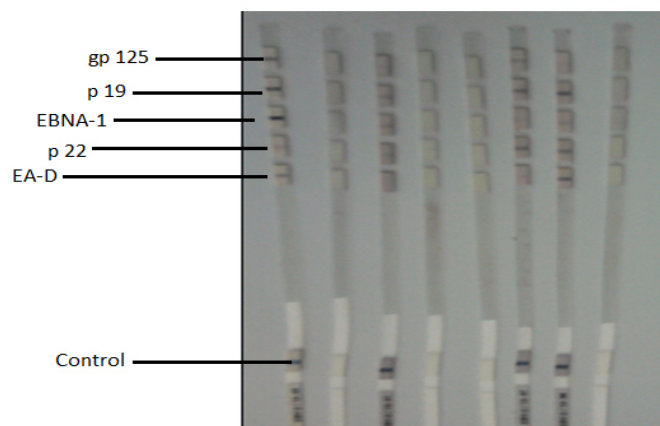
The cross sectional study was undertaken among newly and previously diagnosed adult HIV positive patients attending the Mbagathi District Hospital (MDH) Outpatient Department in Nairobi County between January and December 2006. Clearance for this study was obtained from both the Scientific Steering Committee and the Ethical Review Committee of the Kenya Medical Research Institute (SSC No 908). Patients were not coerced into participation while informed consent in writing was a prerequisite for inclusion into the study. All data entries were coded to ensure confidentiality.

HIV+ outpatients above the age of 18 years and at or below World Health Organization (WHO) stage 3 clinical disease who were not enrolled in an ARV treatment program at MDH were included in the study on condition that informed consent for inclusion in the study was given. A total of 101 patients were included in the study .Every 5th patient who met the inclusion criteria was selected for inclusion in the study using a skip interval calculation

A patient interview questionnaire was administered to all study patients to capture socio-economic and medical history from patient case notes, history taking and recording. An extraoral and intraoral examination was conducted on soft and hard tissues. The patients were classified by HIV clinical stage using presenting signs and symptoms based on WHO Africa Region WHO/HIV 2005/02 criteria.<sup>8</sup>

A qualitative solid phase ELISA (EBV-Profile 2 Euroline ® from Medizinische Labordiagnostika AG, Lubeck Germany <sup>9</sup> was carried out on diluted sample whole blood. This test is capable of determining the presence of IgG class antibodies to five different EBV antigens namely viral capsid antigens (VCA) gp125, p22, p19, Epstein Barr Nuclear Antigen 1 (EBNA – 1) and Early antigen – D (EA-D). The basis of this test is the promotion of a colour reaction by anti EBV antibodies binding to a series of 5

antigen bands on a single test strip incubated under similar test conditions. Using manufacturer's instructions the protein bands were visually graded by band intensity from 1 – 4 ranging from the weakest (1) to the strongest (4), using the control band as a 4. A band of 2 and below was considered negative and a band of 3 and above was considered positive (Plate 1).



**Plate 1: Sample of test strips after incubation**

The stage of EBV infection was assessed as described by Gartner *et al.*<sup>10</sup> Seronegativity or negative infection status was defined by the absence of strong bands of any of the 5 reference proteins. Early stage infection was defined by the presence of strong bands of Anti- gp125 and/or p19 IgG and sometimes Anti-EA-D. Late stage infection was defined by the presence of strong bands of Anti- EBNA1 and Anti- gp125 and/or p19.

Reactivated infection was defined by the presence of strong bands of Anti-VCA, Anti EBNA-1, Anti-p22 and Anti – EA-D

Secondary laboratory data collected, included, results of HIV rapid test using Unigold™ (Trinity Biotech PLC, Bray, Ireland) and confirmed with Determine™ (Dainabot Company, Tokyo, Japan) and CD4 counts using a FACS counter (Becton – Dickinson, Belgium).

Data was entered and stored in a Microsoft Excel program. This database was then transferred to a R (GNU S) computer program version 2.7. Cluster analysis was then performed using the latter program.

Comment [p3]: ref

## Results

The ages of study patients ranged between 20 years and 71 years with a mean of 35.6 years and a standard deviation of 9.7 years. The gender distribution of the sample was sixty-three (62 %) females and thirty eight (38%) males reflecting attendance patterns and a female preponderance of HIV/AIDS in Kenya. The majority were married (55%) while 20% were widowed, 18% were single never married and the balance were divorced or separated. Based on the WHO clinical staging system for HIV infected adults, <sup>8</sup> the distribution of patients seen was 6% in Stage 1, 36% in Stage 2 and (58%) in Stage 3.

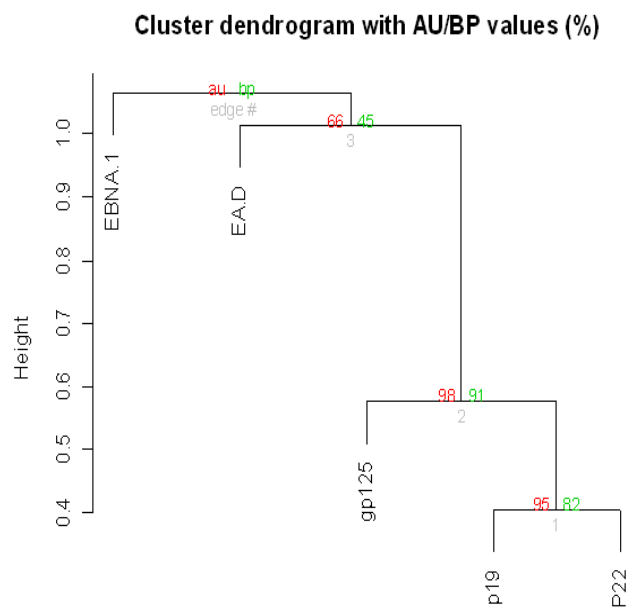
Comment [p4]: rest

The prevalence of EBV infection in the study patients was 95% with the modal infection stage being late stage (66%), early infection (18%) and reactivated infection (11%). Only 5% were seronegative or uninfected

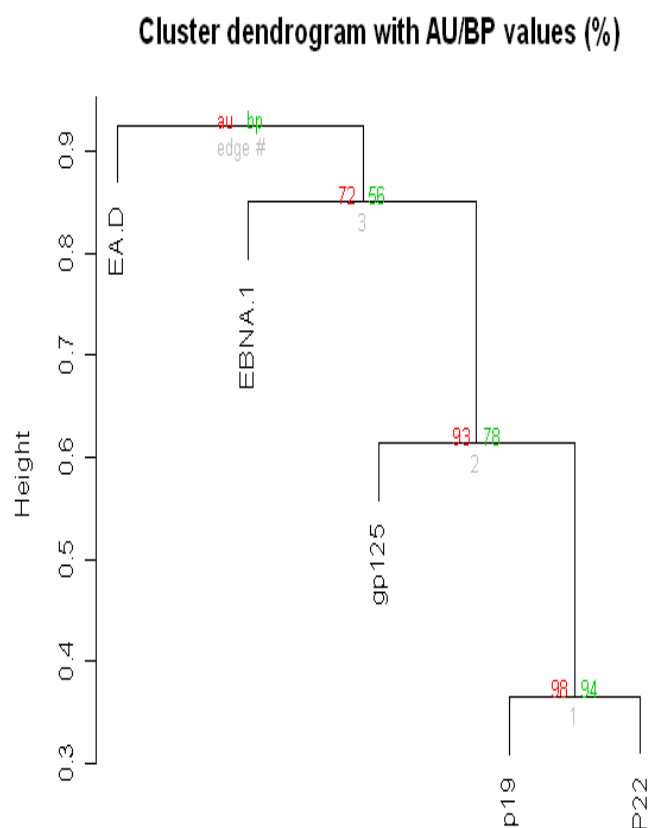
When dendrograms of high intensity antigen-antibody bands were plotted, and the Approximate unbiased P values were compared, the data demonstrated clustering of the following proteins in decreasing order of strength of evidence; gp125 and p19 and



p22) , (p19 and p22) clustered in both HIV (clinical stage 1 and 2) and in clinical stage 3 and EBNA and EAD clustered closely in both clinical stages but EAD was the runt in HIV clinical stage 3 whereas EBNA was the runt in clinical stage 1 and 2 (Figure 1 and 2).The P value standard error plot revealed that the EBNA – EAD cluster had the highest standard error which means that despite the relatively weaker evidence for this cluster it was highly significantly different from any other cluster in HIV clinical stage 3 and HIV clinical stage 1 and 2.(Figure 3)



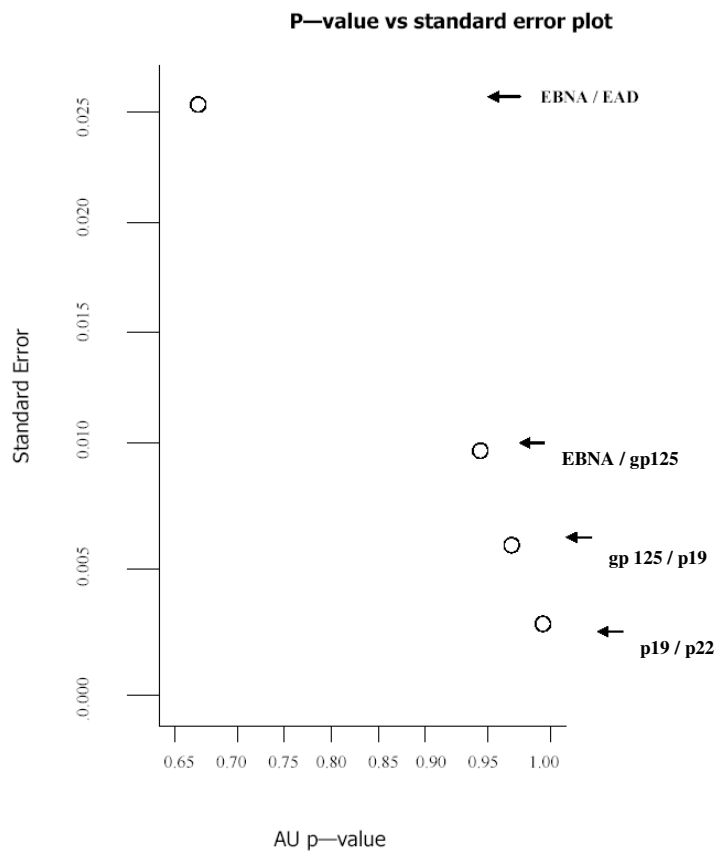
**Figure 1: Clustering of high intensity bands in early HIV clinical stages**



**Figure 2: Clustering of high intensity bands in HIV clinical stage 3**

Standard error is a measure of the confidence interval.<sup>11</sup> Thus the EBNA /EA-D pair has the highest AU P value and the lowest SE from the Confidence interval it has no overlap with the other clusters. It is therefore the most statistically significant cluster in

HIV clinical stage 3 and in early clinical HIV stages (Figure 3).



**Figure 3: P value versus standard error plot**

### Discussion

The limitation of EBV immunoblot evaluation visually was recognized. A digital evaluation of band intensity was not possible since the EUROLIne scan computer

program which could have characterized the VCA bands into IgM and IgG class antibodies was not locally available (EBV-Profile 2 Euroline test kit instructions,2006).<sup>9</sup> Thus the presence of IgM antibodies produced in early infections could affect immunoblot band staining.

Another recognized limitation was that immunosuppression is known to depress the antibody expression of B lymphocytes and thus host protein profiles are not a reliable indicator of viral load or viral activity including the presence and concentration of viral proteins.<sup>7</sup>

These limitations notwithstanding, EAD and EBNA1 clustered closely in HIV stage 3 and in reverse order in HIV stage 1 and 2. Evidence from cluster analysis indicates that the EBNA/EA-D cluster is statistically significantly different from other protein clusters in early and HIV clinical stage 3 HIV disease.

Of all EBV infectious proteins EBNA1/EAD are the strongest candidates for EBV associated tumor markers in HIV.

### **Acknowledgements**

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appreciation for permission to conduct patient examination, data and sample collection.

Dr Peter Wanzala generously financed the purchase of test kits

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[*Afr J Health Sci.* 2014; 27(4),411-417]