Molecular Characterisation of Epstein-Barr Virus in Lymphomas Diagnosed at the University Teaching Hospital, Lusaka

By

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A Dissertation Submitted to the University of Zambia in Partial Fulfilment of the Requirements for the Degree of Master of Science in Clinical Pathology

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Declaration

I, Doris K. Kafita, declare that this is my own work. It is being submitted for the Degree of Master of Science in Clinical Pathology at the University of Zambia, Lusaka. It has not been submitted for any degree at this or any other university.

_____ 2nd day of November, 2015

Doris K. Kafita

Certificate of Approval

Dissertation Title: Molecular Characterisation of Epstein-Barr Virus in Lymphomas Diagnosed at the University Teaching Hospital, Lusaka

This dissertation by **Doris K. Kafita** has been approved in partial fulfilment of the requirements for the degree of Master of Science in Clinical Pathology at the University of Zambia.

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Abstract

Epstein-Barr virus (EBV) is a ubiquitous virus that infects more than 90% of the world's population, and is implicated in the initiation and progression of lymphomas. In Zambia lymphomas are among the most commonly diagnosed malignancies in both men and women. However, the diagnosis of lymphomas is not linked to any aetiological agents since it is mainly based on clinical suspicion and morphological appearance of tissue on microscopy. This poses a risk of misdiagnosis, resulting in improper patient care as the probable cause of the disease is not targeted during diagnosis. Therefore, the objective of this study was to characterise Epstein-Barr virus detected in lymphomas diagnosed at the University Teaching Hospital in Lusaka. One hundred and fifty suspected archival formalin-fixed paraffin embedded lymphoma tissues stored over a 4 year period from January 2011 to December 2014 in the Histopathology Laboratory were analysed. It involved tissue processing, DNA extraction, molecular detection and subtyping of the EBV using Polymerase Chain Reaction and DNA sequencing. Most commonly diagnosed lymphomas were non-Hodgkin's lymphoma (NHL) (80%) followed by Hodgkin's lymphoma (HL) (20%). EBV was detected in 51.8% of the cases, 54.5% of which were associated with NHL cases and 40.9% with HL cases. Subtyping of the virus showed that NHL harboured 81.3% and 18.8% of EBV subtype 1 and type 2, respectively. HL cases harboured 55.6% EBV subtype 1 and 44.4% EBV subtype 2. One of the cases harboured both subtypes of the virus. EBV subtype 1 strains showed genetic diversity. These findings indicate that EBV is closely associated with lymphomas. Therefore, targeting of EBV for identifying new therapy targets in EBV-positive lymphomas should be of interest as this may lead to the improvement of current lymphoma diagnosis.

Dedication

This work is dedicated to my parents, Wellington and Medwin Kafita, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

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List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
BART	BamHI A Rightward Transcript
BL	Burkitt's Lymphoma
CHL	Classical Hodgkin Lymphoma
CST	Complementary Strand Transccript
CTL	Cytotoxic T Lymphocyte
DLBCL	Diffuse Large B cell Lymphoma
DNA	Deoxyribonucleic Acid
EBNA	Epstein-Barr Nuclear Antigen
EBV	Epstein-Barr Virus
EBER	Epstein-Barr Encoded RNA
FFPE	Formalin Fixed Paraffin Embedded
H and E	Haematoxylin and Eosin
HIV	Human Immunodeficiency Virus
HL	Hodgkin Lymphoma
HRS	Hodgkin Reed Sternberg
ISH	*In Situ* Hybridisation
LMP	Latent Membrane Protein
MALT	Mucosa Associated Lymphoid Tissue
MC	Mixed Cellularity
MCHL	Mixed Cellularity Hodgkin Lymphoma
NHL	Non Hodgkin Lymphoma

NPC	Nasopharyngeal Carcinomas
NSHL	Nodular Sclerosis Hodgkin Lymphoma
NS	Nodular Sclerosis
PCR	Polymerase Chain Reaction
PET	Paraffin Embedded Tumour
PTLD	Post Transplant Lymphoproliferative Disorder
RNA	Ribonucleic Acid
USA	United States of America
UTH	University Teaching Hospital

Chapter 1

Introduction

1.1 Background

Epstein-Barr virus (EBV) is an oncogenic lymphotropic virus that belongs to the γ-herpesvirus family (Schafer *et al*, 2015). It is a highly successful ubiquitous virus that infects more than 90% of the world's population, and is associated with several diseases whose incidence differs dramatically in different parts of the world (Rickinson and Kieff, 2007; Cohen, 2015). It was the first human tumour virus to be identified (Young and Rickinson, 2004). While infection of EBV is ubiquitous, tumorigenesis only occurs in a small fraction of the infected population, suggesting that the tumorigenic transformation of human cells by EBV involves complex virus-host interactions and other additional co-factors. A compromised host immune condition and a chronic inflammatory microenvironment probably play major roles in mediating the pathogenic actions of EBV in human malignancies (Rickinson, 2014).

EBV is aetiologically linked to multiple malignancies that include nasopharyngeal carcinoma (NPC) in Southern Chinese people, a high incidence of Burkitt's lymphoma (BL) in sub-Saharan Africa and a high incidence of infectious mononucleosis in teenagers and young adults in western countries (Kimura *et* al, 2013; Tsao *et al*, 2015; Balfour *et al*, 2015). Each of these exceptional geographical or demographic differences in disease incidence may be accounted for by other cofactors but there has long been interest in the possibility that genetic variation in the EBV in different parts of the world might play a role (Tzellos and Farrell, 2012).

EBV is present within the tumour cells in about half of all Acquired Immunodeficiency Syndrome (AIDS)-related non-Hodgkin's lymphomas (NHL), and in nearly all AIDS-related Hodgkin and primary brain lymphomas (Fan *et al,* 2005; Iliyasu *et al*, 2014). Other conditions associated with EBV include Hodgkin's lymphomas (HL) and some diffuse large B-cell lymphomas (DLBCL) (Hassan *et al*, 2006; Carbone *et al*, 2008; Nguyen-Van *et al*, 2011; Ishtiaq *et al,* 2013; Abadi *et al,* 2013). AIDS patients are at greater risk of developing aggressive lymphomas, and in most cases EBV has been associated with the pathogenesis of these tumours (Fan *et al*, 2005; Roschewski, 2012). Older adults and children who are EBV-positive have a poor prognosis, possibly reflecting a poor immune status, which in turn means that these patients may tolerate the disease and its treatment less effective (Keegan *et* al, 2005; Park *et al*, 2007; Saeed, 2009). Thus, exploitation of EBV association for clinical purposes and therapeutic interventions is of interest (Hassan *et al*, 2006).

1.2 Statement of the Problem

The Human Immunodeficiency Virus (HIV)-associated immunosuppression has led to an increase in the incidence of malignant lymphomas (Biggar *et al*, 2006; Grogg *et al*, 2007). In Zambia lymphomas are among the most commonly diagnosed malignancies in both men and women, accounting for 6.3% and 5%, respectively (Bowa *et al*, 2009). However, their diagnosis is not linked to any aetiological agent as the diagnosis of lymphomas is mainly based on clinical suspicion and morphological appearance of tissue on microscopy. This means poor prognosis for EBV-positive lymphoma patients since the causative agent is not targeted during diagnosis. Studies conducted in other countries have demonstrated that EBV is aetiologically linked to lymphomas (Audouin *et al*, 2010; Abadi *et al,* 2013).

Many studies have demonstrated that there is substantial geographical variation in EBV strains circulating in infected populations. These findings demonstrate the need to clarify what wild type EBV is, and how it varies in different parts of the world so that disease specific variation can be identified. Unfortunately, in Zambia, no such studies have been conducted to provide information on the detection and genotypic distribution of EBV strains in lymphomas or other EBV-associated disease.

1.3 Justification of the Study

Malignant diseases have always presented a serious problem to medical practitioners as there are very few treatment options available (Peh *et al,* 2003). Demonstration of the presence of viral DNA sequences in tissue samples is one way of proving the presence of the viral causes of lymphomas (Mc Laughlin *et al*, 2008; Tang *et al*, 2012).

Taking into account that conventional diagnostic tools are limited in their capacity of detecting the presence of infectious agents in tissue sections, molecular tools such as the Polymerase Chain Reaction (PCR), offer an opportunity for rapid and accurate EBV detection and typing in patients at risk of developing or have lymphoproliferative disorders. The detection and genotyping of EBV strains offer prospects for understanding whether naturally occurring sequence differences in the virus affect infection or EBV associated diseases. This is also important for informing the design and development of future EBV vaccines. To our knowledge this is the first study of its kind to be conducted in Zambia.

1.4 Literature Review

1.4.1 EBV Basic Biology

EBV or human herpes virus type 4 is a double-stranded, 170kb DNA virus packaged within an icosahedral capsid surrounded by an envelope belonging to the human gammaherpes virus subfamily (Peh *et al*, 2003; Jalouli, 2010). The viral genome exists in linear form in mature virions and in circular episomal form in latently infected cells, and encodes more than 85 genes (Thompson and Kurzrock 2004; Korcum *et al*, 2006). Two types of EBV have been identified, EBV type 1 and EBV type 2 (also referred to as EBV type A and EBV type B, respectively) based on the DNA sequence divergence within the EBV nuclear antigens (EBNA-2,-3a,-3b,-3c) and the EBV-encoded RNAs (EBER) region (Correa *et al*, 2004; Dolan *et al*, 2006; Odumade *et al*, 2011; Ibrahim *et al*, 2013; Palma *et al*, 2013). The biological differences between type 1 and type 2 appear to be mainly caused by the divergence within the EBNA-2 protein. The type 2 strain is less efficient at transforming lymphocytes than the type 1 strain and shows other differences in cell culture (Mendes *et al*, 2008; Tzellos and Farrell, 2012). Usually, EBV type 1 is the predominant type in lymphatic cells of EBV-infected cells. In human disease it now appears that the type 2 strain occurs preferentially in lymphoproliferative lesions that develop in HIV patients (Tinguely *et al*, 2000).

EBV-1 is detected more frequently in most populations, while EBV-2 is nearly as prevalent as EBV-1 in New Guinea, as well as in equatorial Africa (Thompson and Kurzrock, 2004; Correa *et al*, 2004). Persistent infection with more than one EBV type or strain is usually seen, particularly in immunocompromised patients. In the oropharynx and lymphocytes of these patients, intertypic recombinants have been identified and rarely from healthy people (Aguirre *et al*, 1999; Mendes *et al*, 2008).

Upon infection, EBV passes through the oropharyngeal epithelium to B lymphocytes. It infects resting B cells and turns them into continuously proliferating lymphoblastoid cell lines that express nine latency-associated viral proteins, including six nuclear antigens (Epstein-Barr nuclear antigen (EBNA)-1, -2, -3A, -3B, -3C and -LP) and three membrane proteins (latent membrane protein (LMP)- 1, -2A and -2B) (Ocheni *et al*, 2010). Resting EBV-infected B cells with limited antigen presentation persist at a frequency of 1 in 1×10^5-10^6 cells and constitute the long-term viral reservoir (Hassan *et al*, 2006; Mendes *et al*, 2008). Intermittently, these resting B cells enter the lytic cycle and lyse, releasing virions back into the saliva while also infecting more host B lymphocytes. Once the virus has colonized the B-lymphoid compartment, reactivation from latency can occur at any mucosal site where B cells reside (Thompson and Kurzrock, 2004).

In immunocompetent hosts, the virus is latent in B cells of the peripheral blood and is not associated with disease (Souza *et al*, 2005). However, in immunocompromised patients, immune surveillance to the virus is often impaired which leads to reactivation of the virus or switching between the stages of latency and active infection and subsequent expression of proteins of latency. Therefore, a larger number of B cells are infected with EBV which may lead to malignancy (Gottschalk *et al*, 2005; Davies *et al*, 2010; Michelow *et al*, 2012; Palma *et al*, 2013). Persons with AIDS have a 60-fold increased risk of developing lymphoma, compared with the general population, and virtually all Hodgkin and non-Hodgkin lymphomas that occur in the late stages of HIV infection are EBV positive (Carbone 2003; Calattini *et al*, 2010).

1.4.2 EBV-Encoded Latent Proteins and RNAs

In order to maintain the integrity of viral genome and evade the host immune system, up to nine EBV-encoded proteins are expressed in B cells which are latently infected (Navari *et al*, 2014). These are the EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA-LP) and the latent membrane proteins (LMP1, LMP2A and LMP2B) (Grywalska and Rolinski, 2015).

Table 1.1 EBV Products and Functions

Protein	Function
EBNA1	Essential for immortalisation of cell, replicates EBV genome, segregates viral episomes at mitosis
EBNA2	Transcriptional coactivator that upregulates expression of viral and cellular genes (especially c-myc), essential for EBV immortalisation of cell, one of the first viral proteins produced during EBV infection
EBNA3	
3A	Essential for EBV immortalisation of cell, interacts with CBF1
3B	3B Not essential for EBV immortalisation of cell, interacts with CBF1, function remains largely unknown
3C	3C Essential for EBV immortalisation of cell, overcomes retinoblastoma protein (pRB) checkpoint in cell cycle, interacts with CBF1, increases production of LMP1
EBNA-LP	Interacts with EBNA2 to inactivate p53 and Rb, interacts with transcription factors in notch signalling pathway, one of first viral proteins produced during EBV infection, redistributes EBNA3A in nucleus, and contributes to EBV immortalisation of cell
LMP1	Mimics CD40 ligand binding signal, elevates levels of bcl-2 and a20, acts as a constitutively active receptor, essential for EBV immortalisation of cell
LMP2A and B	Drives EBV into latency. May play a role in oncogenesis of Hodgkin's disease and nasopharyngeal carcinomas
EBER1 and 2	Forms complexes with L22, associates with PKR, not essential for EBV immortalisation of cell
CSTs or BARTs	Complementary strand transcripts encoded at high levels in nasopharyngeal carcinomas. Potential protein products may modify Notch signalling

Adapted from Thompson and Kurzrock (2004)

Furthermore, two types of EBV-encoded non-translated RNA are transcribed in latently infected B cells, EBERs (EBV-encoded RNAs) and BARTs (BamHI A rightward transcript) (Kieff *et* al,

2010). The genes that are important in lymphoma development —EBNA1, EBNA2, LMP1, LMP2 and EBERs — their functions are described in Table 1.1.

1.4.3 EBV Infection and lymphomas

EBV is the aetiological agent for infectious mononucleosis and is one of the most common viruses that are transmitted through the oral transfer of saliva (Slots *et al*, 2006; Irshaid *et al*, 2010). It is ubiquitous and establishes a life-long persistent asymptomatic infection in more than 90% of the population worldwide (Peh *et al*, 2003; Jalouli, 2010; Ocheni *et al*, 2010; Zhang *et al*, 2010). It is the most prolific viral contributor to the development of human lymphomas. In cases of immunodeficiency, such as AIDS, organ transplants, and genetic immune disorders, EBV can reactivate, causing abnormal proliferation of lymphocytes and the potential for several different lymphomas (Thompson and Kurzrock, 2004; Norzuriza *et al*, 2008).

The tumorigenic potential of EBV was first observed in Burkitt's lymphoma, a special type of childhood cancer common among African children. Co-factors are believed to be involved in the pathogenesis of Burkitt's lymphoma (Thorley-Lawson and Allday, 2008). The incidence of Burkitt's lymphoma is closely associated with malaria infection, though the exact contribution of malaria infection to Burkitt's lymphoma remains undefined (Orem *et al*, 2007). The chronic inflammation associated with malaria infection may promote clonal expansion of EBV-infected B cells. Malaria infection may also compromise the host immune system through unknown processes and provide a permissive environment for EBV-infected B cells to evolve into Burkitt's lymphoma cells (Moormann *et al*, 2011). Translocation of c-myc in infected B cells plays a key role in the aetiology of Burkitt's lymphoma. EBV was identified directly under electron microscopic observation of cell lines established from Burkitt's lymphoma. The ability

of EBV to induce proliferation in infected B cells was later demonstrated by culturing peripheral B cells with filtered supernatant harvested from Burkitt's lymphoma cells, where clusters of proliferative clones of EBV-infected B cells could be readily demonstrated (Gutzeit *et al*, 2014).

The ability of EBV to transform and immortalize human B cells strongly implicates the tumorigenic potential of the virus (Kumar *et al*., 2004; Fatima *et al*, 2011; Iliyasu *et al*, 2014). In addition to Burkitt's lymphoma, EBV infection was later observed in other human malignancies, including haematological and lymphatic tumours, such as Hodgkin's disease, T cell lymphoma, and Natural Killer (NK) cell lymphoma, and epithelial cancers, such as nasopharyngeal and gastric carcinomas (Young and Rickinson, 2004; Tsao *et al*, 2015).

1.4.4 Epidemiology of Lymphomas

Lymphomas are a group of malignant neoplasms of the immune system that originate from lymph nodes and extranodal lymphatic system (Zhang *et al*, 2010; Abdelmageed *et al*, 2014). They are broadly divided into Hodgkin's lymphomas (HL) which account for about 10% of all lymphomas, and non-Hodgkin's lymphomas (NHL), which account for 90% (Smedby *et al*, 2006; Shankland *et al*, 2012).

1.4.4.1 Non-Hodgkin's Lymphoma

NHL is not a single cancer but a wide group of cancers, each with a distinct geographical distribution (Yeole *et al*, 2008). Worldwide, NHL is the 8th most commonly diagnosed cancer in men, and is ranked 11th in women (Sharma *et al*, 2014). The disease accounts for about 5.1% of all cancer cases and 2.7% of all cancer deaths (Boffetta, 2011). The most common NHL subtypes in Western countries are diffuse large B-cell lymphoma and follicular lymphoma,

which account for about 30% and 20% of the cases, respectively. All the other subtypes of NHL have a frequency of less than 10% (Smedby *et al,* 2006; Alexander *et al*, 2007). In the United States, it is the fifth most commonly occurring cancer in both men and women, and there are approximately 19 new cases per 100 000 persons each year (Fisher *et al*, 2004). The incidence of Burkitt's lymphoma appears to vary widely with geographical locations and climate and estimated to be 50 times higher in Africa than in the United States of America (USA) (Blum *et al*, 2004; Ferry, 2006).

NHL appears to be common in developing countries where a combination of environmental, infectious and genetic factors affect the development of these disorders (Ishtiaq *et al*, 2013). The highest NHL incidence and mortality rates are seen in Eastern Africa, with estimated incidence rates over 7.5 per 100.000 and mortality rates over 5.7 per 100.000 (Ferlay *et al*, 2004; Orem *et al,* 2007). The burden of HIV-associated NHL is currently estimated at about 15,000 per year in the equatorial belt of Africa (Orem *et al,* 2004; Naresh *et al,* 2011). The incidence of NHL in Uganda has increased, 6.7% annually in men and 11% annually in women since the beginning of HIV pandemic (Parkin *et al*, 2010; Ulrickson *et al,* 2012). A study in north western Tanzania reported an incidence of Endemic Burkitt's lymphoma of 4.2 per 100, 000 (Kabyemera *et al*, 2013). A recent review at the University Teaching Hospital in Lusaka, Zambia showed that NHL ranked 5th, accounting for about 6.3% of all malignancies detected (Bowa *et al*, 2009).

1.4.4.2 Hodgkin's Lymphoma

HL is the most common cancer in adolescents and young adults and affects about 62,000 persons/year and causes 25,000 deaths/year (Parkin *et al*, 2001). It accounts for about 1% of all cancers and 30% of lymphoid malignancies worldwide (Swerdlow *et al*, 2008; Grywalska *et al*,

2013). HL is the third most common cancer in people aged 15-29 years, and the sixth most common diagnosed cancer in children under 14 years (Yung and Linch, 2003; Hoffbrand *et al*, 2011). In the Western world, its annual incidence rate is about 3-4 new cases per 100,000 persons (Gibcus *et al*, 2009; Kuppers *et al*, 2012). The annual age adjusted incidence rates of HL are 2.4 and 2.8 per 100,000 in the United Kingdom and the United States, respectively (Altekruse *et al*, 2010; Maggioncalda *et al*, 2010). The most common subtype among the young adults is nodular-sclerosis (NS) (Cartwright *et al*, 2004). The frequency of mixed cellularity (MC) increases with age, while that of nodular sclerosis (NS) reaches a plateau in the group more than 30 years of age (Mozaheb, 2013). One study in the USA, from 2000-2007, 16 710 cases of HL showed that Asians and Blacks had low incidences. The bimodal pattern of incidence also was less prominent for black males, with Asians and Blacks presenting at a mean age of 38 years compared to 42 years for whites (Pareen *et al*, 2011).

In Africa, studies on prevalence of HL have reported percentages ranging from 12.5%-30% of lymphoid malignancies in Nigeria, Egypt and Sudan (Adelusola *et al*, 2009; Audouin *et al*, 2010; Iliyasu *et al*, 2014; Abdelmageed *et al*, 2014). In South Africa, from 1992-2001, the average number of new patients diagnosed with HL was 12/year. However, a rise in HIV-associated HL has been noted in the latter 10 year period (2002 – 2011) to 18/year, representing a 52% increase (Patel, 2012). A study done in Zambia to determine the distribution of cancers in Zambia reported HL to account for 0.3% of all cancers (Zyaambo *et al*, 2013).

1.4.5 Clinical Presentation of Lymphomas

1.4.5.1 Non-Hodgkin's Lymphomas

Generally, the clinical presentation of NHL differs depending on the type of lymphomas and the area of the body which is affected. Aggressive lymphomas mainly present acutely or sub-acutely with a mass which grows rapidly and often accompanied with systemic B symptoms (i.e. fever, night sweats and weight loss) (Hingorjo and Syed, 2008). Examples of lymphomas with high aggressive presentation include diffuse large B cell lymphoma, Burkitt's lymphoma, adult T-cell lymphoma and precursor B and T lymphoblastic lymphoma (Hingorjo and Syed, 2008; Duncan, 2014). Indolent lymphomas are usually insidious, presenting only with slow growing lymphadenopathy, hepatomegaly, splenomegaly or cytopenias. Examples include follicular lymphoma, chronic lymphocytic lymphomas and splenic marginal zone lymphomas (Thieblemont, 2005; Salles, 2007).

Patients who are over the age of 50 without any known immunodeficiency, organ transplantation or previously diagnosed with lymphomas have frequent extranodal involvement (e.g. stomach, lung, tonsils and skin) and poor prognosis (Menon *et al*, 2012). AIDS-related NHL is characterized by higher grade (40–60%), extranodal disease (80%), advanced clinical stage (60–70%) often presenting with B symptoms (i.e., unexplained fever, night sweats, and weight loss in excess of 10% of normal body weight), and shortened survival (median 7-8 months) when compared with lymphomas in HIV-seronegative patients (Otieno *et al*, 2002; Mwamba *et al*, 2012).

1.4.5.2 Hodgkin's Lymphomas

The most common manifestation of HL, especially in younger patients, is the development of persistent, painless, and firm but not hard, supradiaphragmatic lymphadenopathy, usually in the neck or supraclavicular fossa. In older patients, retroperitoneal lymphadenopathy may present as an abdominal mass with back pain (Connors, 2009; Duncan, 2014). Rare cases of isolated involvement at other extranodal sites, such as skin, brain, gastrointestinal tract, or musculoskeletal tissue have been reported which are quite exceptional and constitute less than 1% of Hodgkin's lymphoma presentations (Swerdlow *et al*, 2008).

Other manifestations of HL other than a mass lesion are the three 'B symptoms' night sweats, unexplained fever and weight loss in approximately 25% of patients which are associated with poorer prognosis (Fraga and Forteza, 2007; Follows *et al*, 2014). HIV-positive CHL is characterized by a more aggressive clinical presentation with the presence of B symptoms, and frequent advanced stages (García-Cosío *et al*, 2008).

1.4.6 Diagnosis of Lymphomas

Diagnosis of lymphomas is both clinical and laboratory. If signs and symptoms suggest that a person might have lymphoma, medical history and physical examinations are conducted. Symptoms of lymphomas are not specific enough to say for sure if they are being caused by cancer. Due to the varied clinical picture, many patients are misdiagnosed and treated for diseases like tuberculosis or systemic lupus erythematosus (Hingorjo and Syed, 2008). Therefore, the diagnosis of lymphomas depends on the histopathological findings on biopsy of an enlarged lymph node (Hingorjo and Syed, 2008).

There are several diagnostic techniques which have been developed to aid the accurate diagnosis of lymphomas in pathology laboratories, and these include:

1.4.6.1 Histological Detection

Histological diagnosis of lymphomas is the most popular approach for the diagnosis of lymphomas, mainly utilises the Haematoxylin and Eosin (H and E) technique. It is an ideal method for recognising various tissue types and morphological changes that form the basis of contemporary cancer diagnosis, including lymphomas (Fischer *et al,* 2008; Jadali *et al,* 2008; Hassan *et al,* 2006). The histological appearance varies according to the type of lymphoma. For example, HL is characterised by the presence of malignant Reed-Sternberg cells, which are giant malignant cells characteristic of HL, having large, abundant cytoplasm, double or multiple nuclei, with prominent nucleoli surrounded by distinctive clear zone which together give an owl's eye appearance to the cell in an appropriate background of non-neoplastic inflammatory cells (Hingorjo and Syed, 2008; Adelusola *et al,* 2009). Burkitt's lymphoma is composed of monomorphic medium size B-cells with basophilic cytoplasm and numerous mitotic figures and a starry sky appearance (Chuang *et al,* 2007; Olaniyi, 2012). However, this technique cannot detect biological causes of the disease such as viruses.

1.4.6.2 Immunohistochemical Staining Techniques

Immunohistochemical techniques are important for detecting cell or tissue antigens ranging from amino acids and proteins to infectious agents and specific cellular populations (Matos *et al,* 2010). Detection of viral proteins can be achieved by immunohistochemical stains of paraffin sections by targeting viral proteins such as Latent Membrane Protein 1 (LMP1) (Ishtiaq *et al,* 2013). This technique has been used to detect EBV in Post-Transplant Lymphoproliferative

Disorder (PTLD), HL, and in infectious mononucleosis cases (Ishtiaq *et al*, 2013). However, it has disadvantages such as cross-reactivity and background staining that may lead to false interpretation of results (Ramos-Vara, 2011).

1.4.6.3 *In Situ* Hybridisation

In Situ Hybridisation (ISH) for Epstein-Barr virus (EBV)-encoded RNA (EBER) is considered the "gold standard" for detecting and localizing latent EBV in biopsy samples (Fan *et al*, 2000; Gulley and Tang, 2008). Because EBER transcripts are naturally amplified, they represent a reliable target for detecting and localizing EBV in tissue sections by *in situ* hybridisation (Mwakigonja *et al*, 2010; Tang *et al*, 2010). EBER ISH can be accomplished on paraffin sections or on cytology preparations (Gulley, 2001; Song *et al*, 2015). In biopsies where the differential diagnosis includes infectious mononucleosis, HL, and/or NHL, EBER hybridization is often helpful in making the correct diagnosis (Qi *et al*, 2013; Iliyasu *et al*, 2014). In EBV-related Hodgkin's disease, EBER is largely restricted to Reed-Sternberg cells and mononuclear variants; whereas infectious mononucleosis is characterized by a mixture of small and large EBER-positive cells including immunoblasts rimming necrotic zones (Gulley, 2001; Gru *et al*, 2013). However, the major disadvantage of this technique is the non-specificity of labelling that may lead to false positive results. The technique does not also offer information on translational and post-translational modification of the gene of interest (Segalés *et al*, 1999; Kumar *et al*, 2010).

1.4.6.4 Molecular Detection

Polymerase Chain Reaction (PCR) amplification is a useful diagnostic method for detecting EBV infection (Klumb *et al,* 2004; Hassan *et al,* 2006; Kabyemera *et al,* 2013). PCR amplification of specific EBV genome sequences is a rapid, sensitive and specific method for identifying viral DNA. PCR makes it possible to detect minimal amount of viral DNA in tissues and smears. Furthermore, this method makes it possible to study biopsy samples in paraffin wax blocks and permits retrospective studies (Dias *et al,* 2009). PCR amplification of EBV DNA is accomplished using primers spanning conserved EBV sequences, whereas strain typing relies on amplification of polymorphic regions of the viral genome (Gulley, 2001). However, this technique has some limitations such as mutations in the fragment generated since the DNA polymerase used in the PCR reaction is prone to errors and the specificity of the generated PCR product may be altered by nonspecific binding of the primers to other sequences on the template DNA (Garibyan and Avashia, 2013).

1.4.7 Treatment of EBV-Associated Lymphomas

Most EBV-associated tumours respond poorly to intensive chemotherapy regimens or have a significant relapse rate, and the presence of the EBV genome within these tumours raises the possibility of developing strategies directed at viral targets (Israel *et al,* 2003; Heslop *et al,* 2005). Approaches under evaluation include immunotherapy approaches, interferon, and small molecules targeting aspects of virus biology (Israel *et al*, 2003).

Despite the identification of clinical prognostic factors and optimal use of primary and secondary treatments, classical Hodgkin's lymphoma (CHL) remains fatal for more than 15% of patients (Yung and Linch, 2003; Carbone *et al*, 2008). Therefore, several phase 1 clinical trials

are being done in patients with CHL using both polyclonal EBV Cytotoxic T Lymphocytes (CTLs) and CTLs enriched in precursors targeting LMP-2 (Carbone *et al*, 2008). One therapeutic strategy that takes advantage of the EBV genome in tumour cells involves induction of the lytic form of EBV infection in tumour cells, followed by administration of nucleoside analogue ganciclovir (Feng *et al*, 2004).

1.5 Research Questions

What proportion of lymphomas diagnosed at the University Teaching Hospital in Lusaka harbour EBV DNA sequences? What are the subtypes of EBV detected in lymphomas at the University Teaching Hospital in Lusaka?

1.6 Objectives

1.6.1 General Objective

To characterise Epstein-Barr virus detected in lymphomas diagnosed at the University Teaching Hospital in Lusaka by using molecular tools.

1.6.2 Specific Objectives

1.6.2.1 To determine the commonly diagnosed lymphomas at the University Teaching Hospital

1.6.2.2 To detect the presence of EBV and its subtypes in lymphoma tissues at the University Teaching Hospital.

Chapter 2

Materials and Methods

2.1 Study Design

This was a laboratory-based retrospective cross-sectional study on archival formalin-fixed paraffin embedded (FFPE) lymphoma tissue and histopathology data stored over a 4 year period from January 2011 to December 2014 in the Histopathology Laboratory of the University Teaching Hospital, in Lusaka.

2.2 Study Site

The study was conducted at the University Teaching Hospital, Department of Pathology and Microbiology, Histopathology Laboratory, in Lusaka, Zambia. The University Teaching Hospital is a tertiary referral and teaching hospital with a bed capacity of about 1,664. The hospital has 7 clinical departments: Gynaecology and Obstetrics, Surgery, Medicine, Paediatrics and Child Health, Outpatient and Pathology and Microbiology. It is the biggest reference hospital and the centre for all histopathology diagnostic work in Zambia.

2.3 Sampling Frame

One hundred and fifty archival formalin-fixed paraffin embedded (FFPE) lymphoma tissue stored over a 4 year period from January 2011 to December 2014 were analysed. The blocks included cases from all age groups which ranged from 9 months to 85 years old. The tissue blocks were from different anatomical sites (anal tissue, right parotid tumour, inguinal lymph node, cervical lymph node, nose, ulcerated skin, etc.) obtained from 86 males and 64 females patients.

2.3.1 Inclusion Criteria

All archival paraffin wax-embedded tissue blocks of lymphomas processed in the Histopathology Laboratory from January 2011 to December 2014 were included in this study.

2.3.2 Exclusion Criteria

Tissue blocks other than those from suspected lymphoma cases or those whose data on sex and age were missing were excluded from the study.

2.4 Sample Size Determination

Prevalence of EBV in lymphomas at the UTH is unknown. Since the prevalence is unknown, a conservative estimate of 50% was used. In order to estimate the prevalence within 5% (or 0.05) and considering 95% confidence level, a minimum sample size of 150 was analysed, as shown by the calculation:

$n = \frac{p(1-p)}{e^2}$, where $p = 50\%$ (or 0.5) is the sample proportion and

$$e^2 = \left(\frac{0.05}{1.96}\right)^2 \approx 0.000625$$

n = 400

Applying correction for finite population size formula (Dell *et al*, 2002), the new sample size will be:

$$\text{New ss} = \frac{\text{n}}{1+\frac{n-1}{pop}}$$

Where, ss= sample size; pop = population,

We calculate the new $n = \frac{n}{1 + \frac{n-1}{N}}, = \frac{400}{1 + \frac{400-1}{240}} = 150$

n= sample size 400 as calculated above, N=Total number of lymphoma blocks stored from January 2011 to December 2014.

Therefore, the minimum sample size (n) was 150

To obtain the sample size calculated, cases to be analysed were selected by systematic sampling. First a list of all lymphoma cases stored over a 4 year period was made. The k^{th} in the sampling frame was calculated by dividing 240, the total number of cases (K) by the sample size, 150. Selection of the first case from the first K case was done by using simple random sampling. Then every k^{th} case on the list was selected (Castillo, 2009).

2.5. Determination of the Commonly Diagnosed Lymphomas

2.5.1 Paraffin Block Retrieval

One hundred and fifty formalin-fixed paraffin embedded (FFPE) lymphoma blocks were retrieved from the UTH Histopathology Laboratory specimen archive. The blocks included cases from all age groups and from different anatomical sites. All the blocks contained tissues which were previously diagnosed as lymphomas based on clinical appearance and H and E staining. Each tissue block was given a new identification code and number for easy identification.

2.5.2 Section Cutting

Sections of tissue were cut at 6µm on a Shandon Finesse 325 microtome (Thermo Scientific-Shandon, California, USA). Briefly, tissue blocks were first placed face down on an ice-cold plate for 20 minutes and cut on a microtome to generate serial sections. Separate blades were used to cut each tissue to avoid contamination during PCR amplification of the viral targets. Each ribbon were picked by forceps and transferred to a water bath set at 37°C in order to float out the sections. The ribbons were laid on the water bath to allow the sections to stretch for a few seconds. The sections were then carefully separated and each section was picked on a glass slide at an angle. The slide sections were allowed to drain for a few minutes before transferring them to a hot plate for drying.

2.5.3 Haematoxylin and Eosin Staining

Before Haematoxylin and Eosin **(**H and E) staining, sections were taken to water by first deparaffinising the sections in two changes of xylene (2 minutes each), washed in two changes of absolute alcohol (2 minutes each), 2 minutes in 95% alcohol, 2 minutes in 90% alcohol, 2 minutes in 80% alcohol, 2 minutes in 70% alcohol and then in water for 2 minutes. The slides were stained with Ehrlich's Haematoxylin solution (Hopkin and Williams, Birmingham, England) for 25 minutes. After staining, the slides were washed in running tap water for 5 minutes and then differentiated in 1% acid-alcohol (1% hydrochloric acid in 70% alcohol) for 15-30 seconds. The slides were further blued in running tap water for 10 minutes and counter stained in 1% aqueous eosin for 5 minutes. Finally, the slides were rinsed in water, and dehydrated in 70%, 80%, 90%, 95% alcohol and in two changes of absolute alcohol for 2 minutes at each stage. The slides were then cleared in 2 changes of xylene for 2 minutes in each

jar, cleared in DPX (Electron Microscopy Sciences, Hatfield, PA, USA), covered with a cover-slip and allowed to dry for 2 hours.

2.5.4 Microscopic Examination

The slides were observed under the microscope to confirm the diagnosis of lymphomas by a qualified histopathologist. In NHL reactive background cells were not seen as in HL. Monomorphic medium sized B cells with basophilic cytoplasm and numerous mitotic figures were seen. In some cases, the tumour cells exhibited a high mitotic rate and a high degree of apoptosis with diffuse sheets and starry-sky pattern imparted by numerous benign histiocytes with phagocytosis of apoptotic cellular debris. Identification of CHL was mainly based on the identification of Reed-Sternberg cells which had two or more nuclear lobes; prominent, mirror image, eosinophilic, inclusion-like nucleoli; and abundant amphophilic cytoplasm and their variants residing in an infiltrate containing a variable mixture of non-neoplastic small lymphocytes, eosinophils, neutrophils, histiocytes, plasma cells, fibroblasts and collagen fibers.

2.6 Detection of EBV and its Subtypes in Lymphomas

2.6.1 Section Cutting

Tissue sections were cut as described under Section 2.5.2. New blades were used to cut each tissue to avoid contamination during PCR amplification of the viral targets. The sections were then transferred to a separate sterile 1.5ml microfuge tube until required for DNA extraction. Gloves were used at all stages during tissue manipulation.

2.6.2 DNA Extraction

Up to 30mg of tissue sections was placed in a sterile 1.5ml microfuge tube. DNA was then extracted using the EZNA Tissue DNA Extraction Kit (Omega Bio-Tek Inc, Norcross, Georgia, USA) for paraffin-embedded tissue according to the manufacturer's protocol. The DNA was eluted in 50µl volumes, and then stored at -20^{o}C until required.

2.6.3. Detection of EBV DNA Sequences

In order to ensure DNA integrity and absence of PCR amplification inhibitors, all DNA samples were tested with primers to amplify a fragment of the human β-actin gene. This acted as an internal control. A final volume of 25µl was used, containing 2µl bacterial DNA template, primer (Forward primer: 5'-GCCATGTACGTTGCTATCC-3' and Reverse primer: 5'-CCGCGCTCGGTGAGGATC-3') and 2X PCR Master Mix (Thermo Scientific, Hanover, MD, USA), with a final $MgCl_2$ concentration of 3mM. After initial denaturation step of 5 min, at 94°C, 35 cycles of amplification were performed. Cycles consisted of denaturation at 95°C for 30sec, Annealing at 55°C for 1 min, Extension at 72°C for 1min and final elongation at 72°C for 10 min yielding a final product of 200bp. Amplification reaction was carried on a GeneAmp System 2700 PCR thermocycler (Applied Biosystems, Foster City, CA, USA). After PCR assay, the 5µl amplified products were subjected to Electrophoresis on a Tris-Borate-EDTA (TBE) agarose gel (wt/vol) (100V) containing 1µl ethidium bromide (10 mg/ml). A 50bp ladder (Thermo Scientific, Hanover, MD, USA) was used as a molecular weight standard and all gels were visualised using a Biotop Biosens SC-645 Gel Documentation System (Biotech Co. Ltd, Shanghai, China).

EBV detection and typing was determined by PCR with specific primers for EBNA3C gene. The sequences and positions of these primers are as follows: EBVD-F, 5'-AGAAGGGGAGCGTGT GTTGT-3' (B95-8 coordinate 87651-87670); EBVD-R, 5'-GGCTCGTTTTTGACGTCGGC-3' (B95-8 coordinate 87803-87784), which yield an amplification product of 153bp for EBV-1 and a product of 246bp for EBV-2. PCR was performed in 25µl using 2.5µl of 5pM of the forward and reverse primers, 12.5µl 2X PCR Master Mix (Thermo Scientific, Hanover, MD, USA), with a final $MgCl_2$ concentration of 3mM, 2ul genomic DNA. The reaction mixture was initially denatured at 95°C for 5 min followed by 35 cycles including denaturation at 95°C for 45s, annealing at 56°C for 45s, extension at 72°C for 1min, and finally elongation at 72°C for 10min. Electrophoresis of 5µl of the PCR product was performed on a Tris-Borate-EDTA (TBE) agarose gel (wt/vol) (100V) containing 1µl ethidium bromide (10 mg/ml). A 50bp ladder (Thermo Scientific, Hanover, MD, USA) was used as a molecular weight standard and all gels were visualised using a Biotop Biosens SC-645 Gel Documentation System (Biotech Co. Ltd, Shanghai, China)

2.6.4 PCR Subtyping of EBV Targeting the EBNA-2 Gene

All those identified as EBV-1 and EBV-2 where then subjected to PCR by amplifying a portion of the EBNA-2 gene. Either with EBNA-2 primers specific for EBV type 1 EBVD1F (5'-TCTTGATAGGGATCCGCTAGGATA-3', nucleotide positions 48839-48862: and EBVD1R (5'-ACCGTGGTTCTGGACTATCTGGATC-3', nucleotide positions 49335-49311) or with EBNA-2 primers specific for EBV type 2 EBVD2F (5'-ACTGGATATGAATCCCCTGGGCAG-3', nucleotide positions 48766-48789 and EBVD2R (5'-GAGTCCTGTACTATCAGAACTACAATG-3', nucleotide positions 49231-49205). The amplified products had a size of 497bp (EBV type-l sequences) or 466bp (EBV type-2

sequences), respectively. Amplification conditions (in 50µl assays) were: Samples were processed for 35 cycles on a GeneAmp System 2700 PCR thermocycler (Applied Biosystems, Foster City, CA, USA) with denaturation at 94°C for 30 sec, annealing at 64°C for 1 min, extension at 72°C for 1 min. Amplified sequences were visualized on a 1.5% agarose gel using standard conditions. A 50bp DNA marker was used as a molecular size marker.

2.6.5 DNA Sequencing of EBNA-2 PCR Products

Prior to sequencing, the PCR products were first purified with the QIAquick Gel Extraction Kit (Thermo Scientific, Lithuania) following the manufacturer's instructions. Forward or reverse linear amplification were performed in 10µl using 2µl of the purified PCR product (about 20 to 200ng), 2µl BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA**)**, 1µl BigDye Sequencing Buffer (Applied Biosystems, Foster City, CA, USA**)** and 1µM of primer. Linear amplification consisted of 25 cycles of denaturation at 96^{o}C for 10s, annealing at 60^{o}C for 30s and elongation at 72^{o}C for 60s using the iCycler Thermocycler (Bio-Rad, Hercules, CA, USA). Fluorescence-labelled DNA was purified using the ethanol precipitation method.

Briefly, the entire extension products were then transferred into 80µl of freshly prepared precipitation solution (3µl of 3M sodium acetate [pH 4.6], 62.5µl of non-denatured 95% ethanol and 14.5µl deionised water), incubated for at least 1hr at room temperature and centrifuged at 14000rpm for 20min. After carefully removing the supernatant, 250µl of 70% ethanol was added to the pellet, vortexed and the contents re-centrifuged at 14000rpm for 8min. The ethanol was then carefully aspirated and the pellet air-dried for 15min at room temperature. The samples

were then analysed on an ABI PRISM 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA.

The genetic relatedness within each EBV subtype was determined by phylogenetic analysis based on the maximum likelihood method (ML) by using MEGA 6 Software. (Centre for Evolutionary Medicine and Informatics, S. McAllister Ave, USA). Reference sequences for the two subtypes of EBV (EBV subtype 1 and subtype 2) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The reference DNA sequences used for EBV subtype 1 and EBV subtype 2 were sLCL-IS1.10 and sLCL-2.15, respectively.

2.7 Data Analysis

Data were analysed with GraphPad Prism Software Version 6.0 for Windows (GraphPad Software, San Diego, California, USA). Chi square or fisher's exact test was used to evaluate the association between EBV status, type of lymphoma and gender. The level of statistical significance was set at $p \leq 0.05$. DNA sequences were edited with Ridom Trace Edit Software (Ridom GmbH, Münster, Germany) and analysed by BioEdit Software Version 7.0.9.1 (Ibis Biosciences, Faraday Avenue Carlsbad, California, USA). Identification of the sequences was performed by using the BLAST algorithm (basic local Alignment Search Tool; http://blast.ncbi.nlm.gov/Blast.cgi). EBNA2 gene sequences were used to construct phylogenetic tree based on the maximum likelihood method (ML) by using MEGA 6 (Centre for Evolutionary Medicine and Informatics, Tempe, AZ, USA). The reference strain sequences were obtained from the public database, GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

2.8 Ethics Approval

This was a laboratory-based study, with no direct contact with patients. Ethics approval for the study was obtained from the University of Zambia Biomedical Research and Ethics Committee. The Ethics Clearance Certificate reference number was 017-03-14 (Appendix 1).

Chapter 3

Results

3.1 Determination of the Commonly Diagnosed Lymphomas

A total of 150 lymphoma blocks, collected from January 2011 to December 2014 at the UTH, were analysed in this study. All cases were confirmed as either HL or NHL on the basis of different histopathological changes which were observed under light microscopy (Figure 3.1 below).

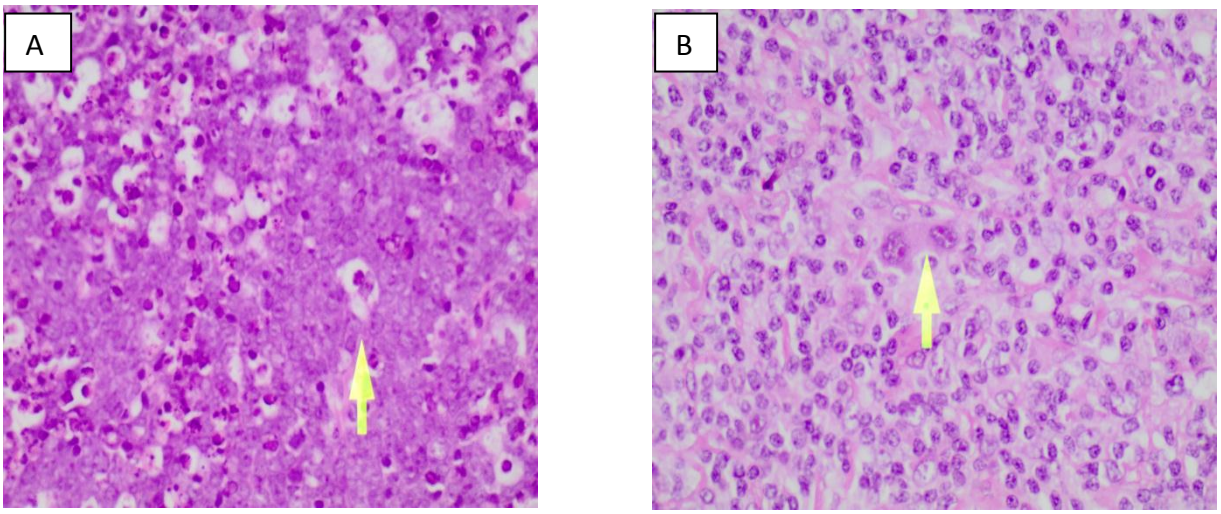

Figure 3.1 Histopathological features of lymphomas after H and E staining.

Some examples of features seen in Burkitt's lymphoma, a NHL, were presence of histiocytes with phagocytosis of nuclear debris (arrow), accumulated cellular debris due to increased apoptosis, starry-sky pattern (Figure 3.1A). Some features seen in lymphocyte predominance a HL, included inflammatory cell infiltrate composed of abundant eosinophils, plasma cells, and Reed-Sternberg cell (arrow) (Figure 3.1B).

Of all the tissues examined, 73.3% (110/150) were confirmed as lymphoma cases, while 26.7% (40/150) were not lymphomas (Figure 3.2A). Of the 110 lymphomas, 80% (88/110) were identified as NHL, while 20% (22/110) were identified as HL (Figure 3.2B).

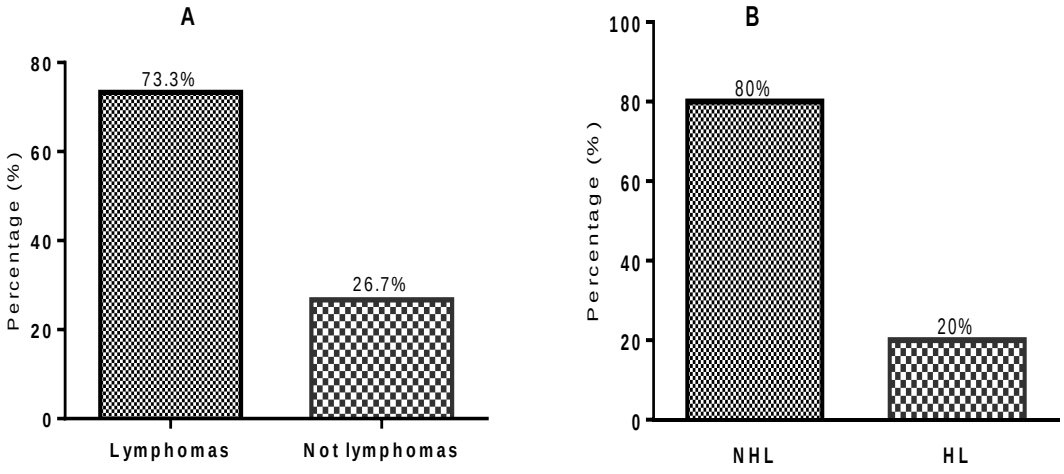

Figure 3.2: Histological diagnosis of lymphomas. A). Total detection of Lymphomas. B) Distribution of lymphomas identified in this study

NHL affected 55.7% (49/88) males and 44.3% (39/88) females, while HL affected 72.7% (16/22) males and 27.3% (6/22) females (Figure 3.3).

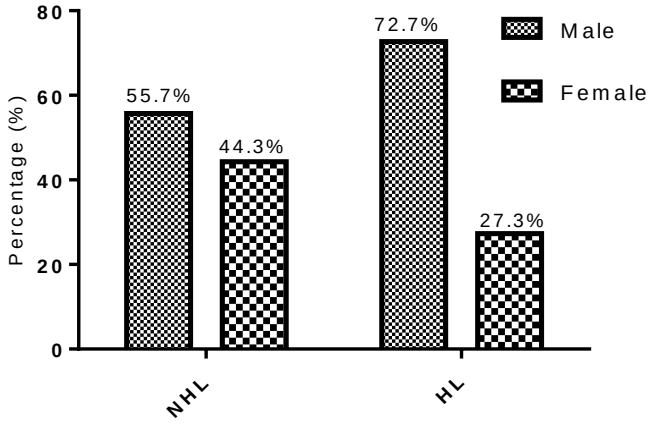

Figure 3.3: Gender Distribution in Lymphomas

In NHL the most affected age group were those aged 31-40 years and 41-50 years age groups (Figure 3.4 A), while most of the HL cases were mostly in the between the ages 0-10 years, followed by the age groups 11-20, 21-30 and 31-40 year age groups (Figure 3.4 B).

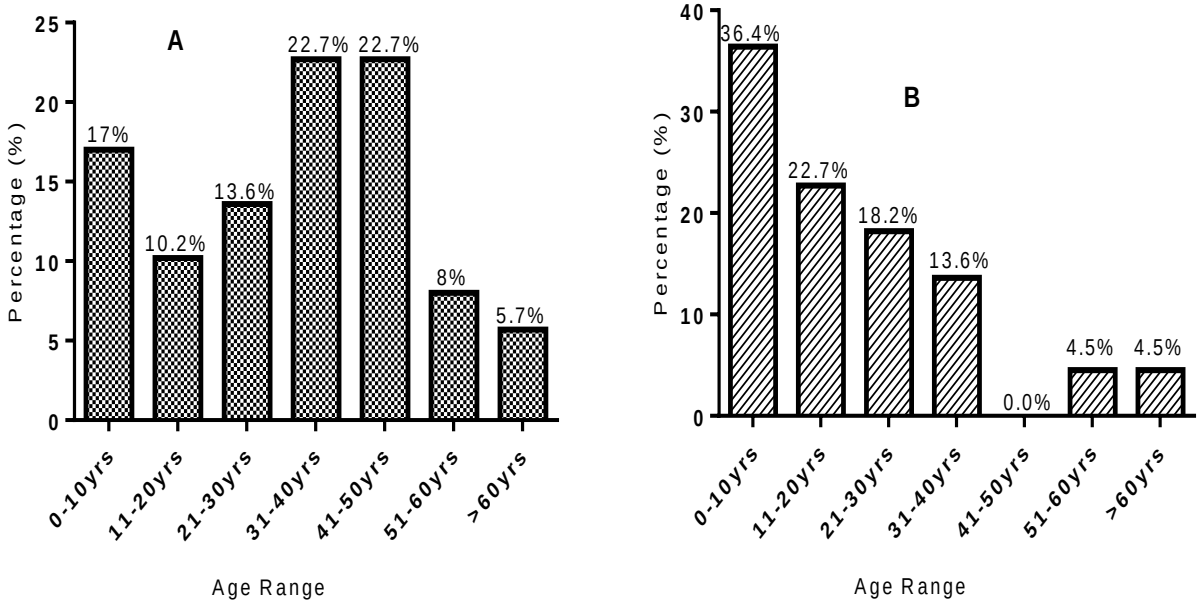

Figure 3.4: Age distribution in lymphomas. A) NHL cases B) HL cases

3.2 Detection of EBV DNA and Subtypes

Of the lymphomas analysed, EBV DNA was detected in 51.8% (57/110) of the cases (Figure 3.5A). Both EBV subtype 1 and EBV subtype 2 were detectable in these cases (Figure 3.5A). The quality of DNA used was assessed by PCR amplification of a 200bp fragment of the human β-actin gene which acted as an internal control for the DNA extraction process (Figure 3.5 B).

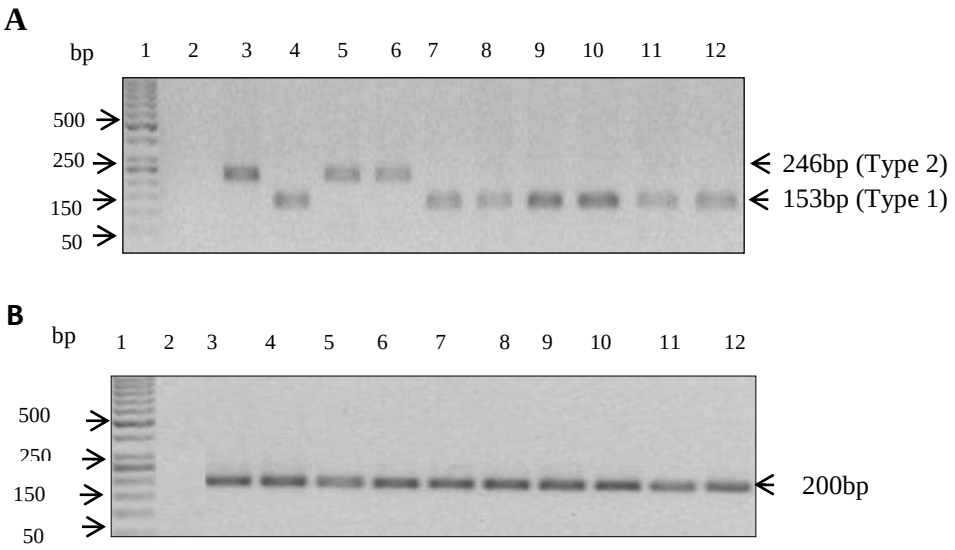

Figure 3.5: (**A)** EBV detection and subtyping targeting the EBNA3C region. Lane 1, 50bp DNA marker; lane 2, negative control, lane 3 and 4, positive controls for type 2 and type 1, respectively; lanes 5-12 EBV-positive representative samples. (**B)** β-actin internal control on selected samples. Lane 1, 50bp DNA marker; lane 2, Negative control; lane 3 and 4, positive controls for type 2 and type 1, respectively; lanes 5-12 representative samples.

In one case of HL both EBV type 1 and 2 were detected as shown in Figure 3.6, lane 8

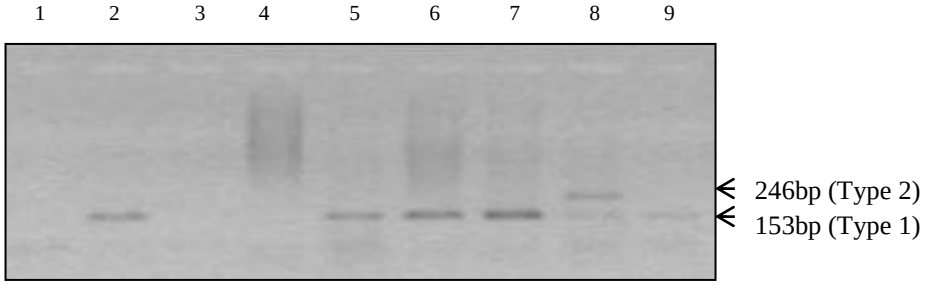

Figure 3.6: Detection of Mixed infection in an HL case. Lane 1, Negative control; lane 2, positive control; lane 3, DK007; lane 4, DK006, lane 5, DK016; lane 6, DK039, lane 7, DK035; lane 8, DK015; lane 9, DK044.

Of the 110 lymphomas, EBV was detected in 51.8% (57/110) and 48.2% (53/110) were negative for EBV (Figure 3.7A). Of the 88 NHL cases, 54.5% (48/88) were positive for EBV in contrast to 40.9% (22/88) of the HL cases, ρ=0.252 (Figure 3.7B).

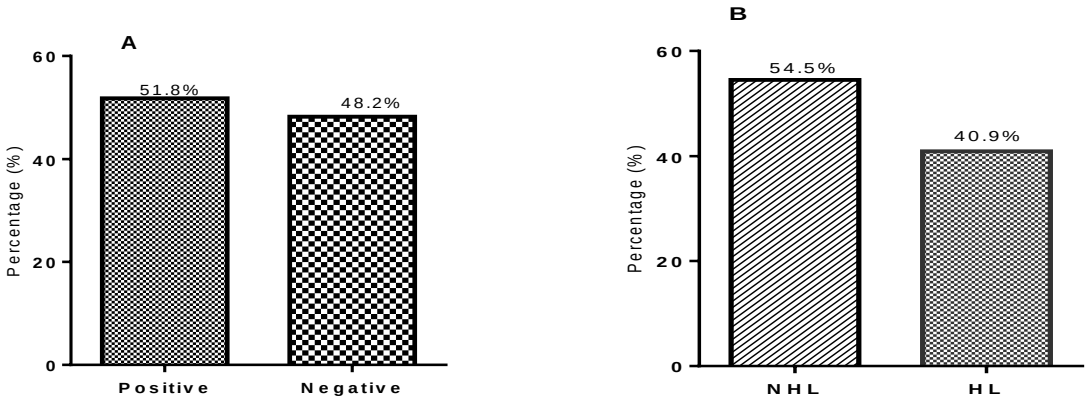

Figure 3.7: EBV DNA detection in lymphomas. A) In all lymphomas B) In each subtype of lymphoma

In the positive NHL cases, 81.3% (39/48) were of EBV subtype 1, while 18.8% (9/48) were of EBV subtype 2 (Figure 3.8 A). For the HL EBV-positive cases, 55.6% (5/9) were of the EBV subtype 1, while 33.3% (3/9) were of the EBV subtype 2. Mixed infection was noted in one case of HL (Figure 3.8 B).

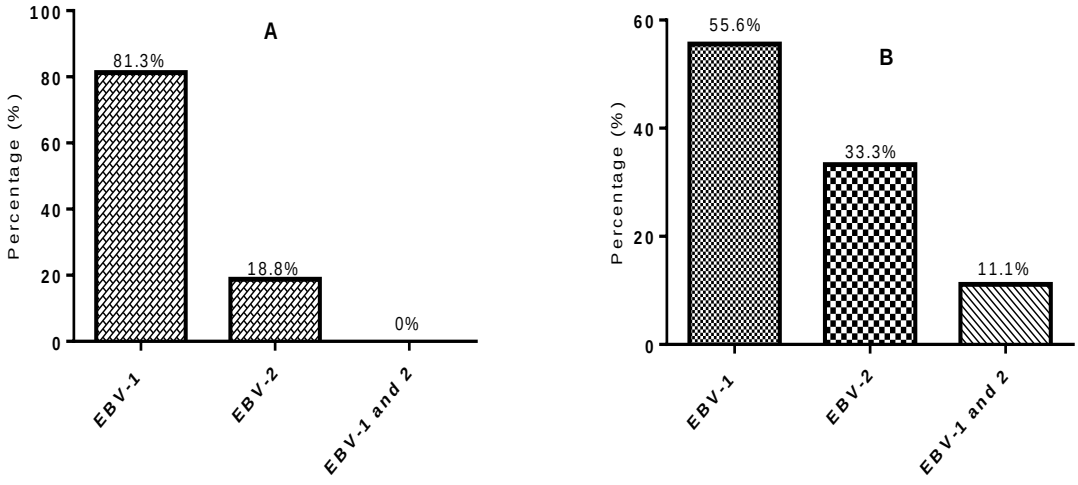

Figure 3.8 Distribution of EBV subtypes in lymphomas. A) NHL cases B) HL cases

Among the 48 EBV-positive NHL cases, there were 59.2% male patients and 48.7% female patients. The difference in the EBV positivity rate between male and female NHL was not statistically significant (ρ=0.327) (Figure 3.9 A). In the 9 EBV-positive HL cases, there were 43.8% of male patients and 33.3% female patients. The difference in the EBV positivity rate between male and female NHL was also not significant (ρ=1.00) (Figure 3.9 B).

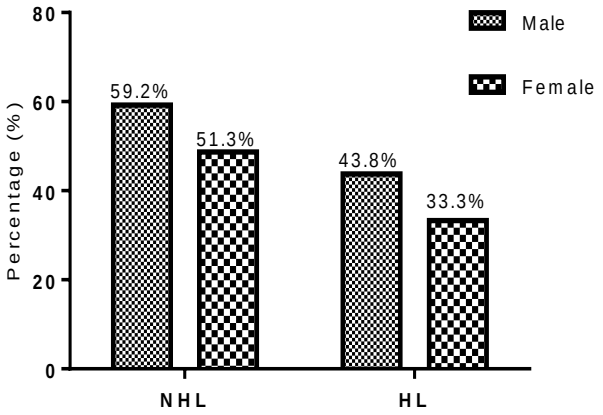

Figure 3.9: Distribution of lymphoma cases by gender and EBV-positivity results. A) NHL cases B) HL cases

Those identified as EBV-1 and EBV-2 were later subjected to further PCR typing (Figure 3.10) and DNA sequencing by amplifying a portion of the EBNA-2 gene in order to determine the genetic relatedness within each subtype.

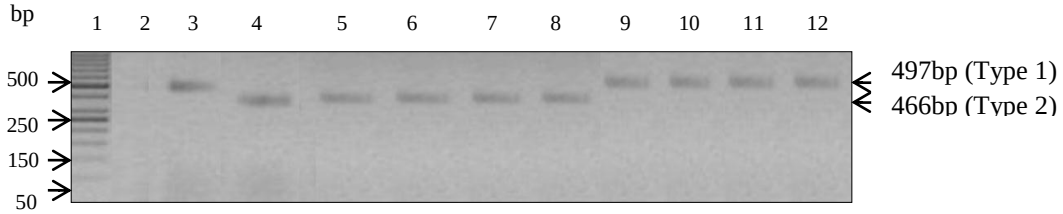

Figure 3.10: Demonstration of PCR products of Epstein-Barr virus (EBV) type 1 and 2 EBV nuclear antigen 2. Lane 1, 50bp DNA marker; lane 2, negative control, lane 3 and 4, positive controls for type 2 and type 1, respectively; lanes 5-12 EBV-positive representative samples. bp, base pair.

Phylogenetic analysis revealed that the EBV subtypes were segregated into four groups. The first three groups were for EBV subtype 1, whilst the fourth was for EBV subtype 2 (Figure 3.11). The strains within groups 1 and 2 were indistinguishable, but showed genetic diversity when the two groups were compared (Appendix IIIA). Group 3 only had one strain, and this strain, when compared to the other two groups, revealed that it was distantly related to the other strains in groups and 1 and 2 (Appendix III A).

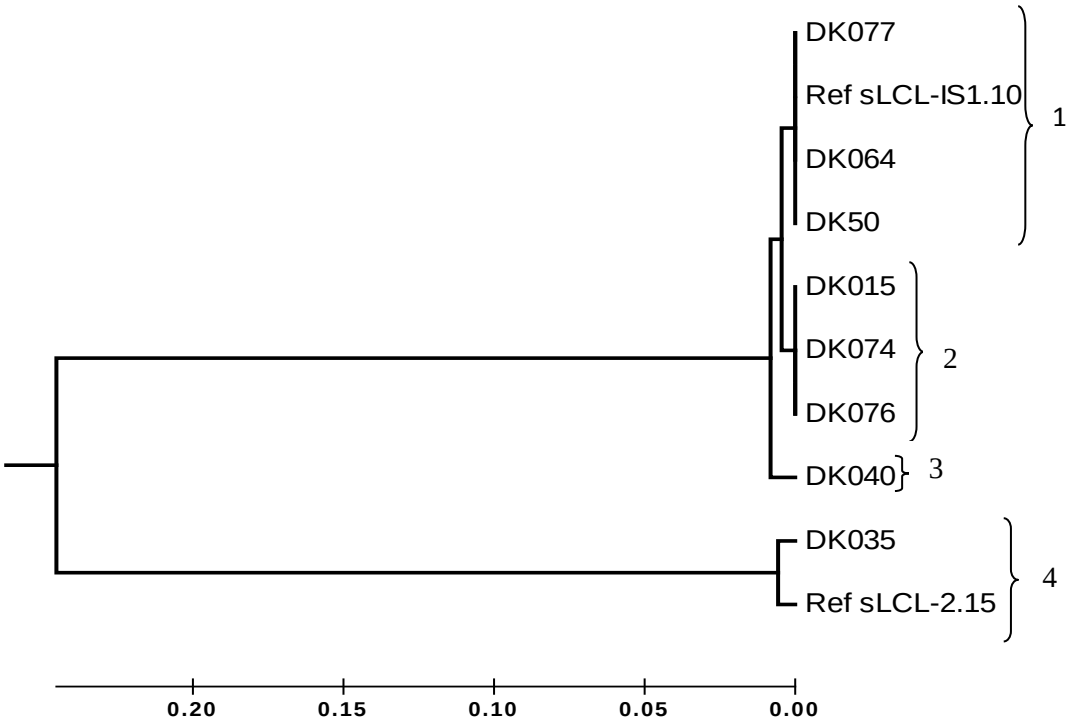

Figure 3.11: Phylogenetic tree of EBV based on the maximum likelihood method (ML). Ref sLCL-IS1.10 is the reference sequence for EBV type 1, while Ref SLCL-2.15 is the reference sequence for EBV subtype 2.

There was only one sequence for the subtype 2 strain available for the comparison as the other sequences failed to amplify. For this subtype, minor base differences were mainly observed at positions 36890 (A→T), 36891 (C→T), 36893 (A→C) and 37000 (C→T) and 37002(C→ G) with regard to the subtype 2 reference strain, Ref sLL-2.15 (Appendix IIIB).

Chapter 4

Discussion

4.1 Discussion

The detection of EBV strains in tissue has been useful in studies involving its transmission and persistence in tissue (Keegan *et al*, 2005; Dojcinov *et al*, 2011). EBV has been closely linked to the pathogenesis of the majority of lymphomas arising in patients with iatrogenic or congenital immunosuppression (Shimoyama *et al*, 2006; Cohen *et al*, 2015). Previous studies have shown that detection of EBV in lymphomas may aid in making correct diagnosis and treatment for EBV-associated lymphomas (Sugita *et al*, 2010; Qi *et al*, 2012).

Several studies have examined the histopathological features of lymphomas, with the majority of NHL cases showing a diffuse pattern, while the presence of Reed-Sternberg cells in a cellular background were associated with HL (Hingorjo and Syed, 2008; Al-Maghrabi and Sayadi *et al*, 2012). This was consistent with the morphological features found in the majority of lymphomas in this study. Identification of HL was mainly based on the presence of Reed-Sternberg cells in a cellular background of eosinophils, plasma cells, non-neoplastic small lymphocytes and neutrophils. The majority of NHL cases showed a diffuse pattern with large cells, prominent nucleoli, abundant cytoplasm and many mitoses.

Data generated from this study showed that both HL and NHL were identified, NHL being the most commonly diagnosed lymphoma. This was consistent with studies conducted in Nigeria, Japan, Iraq, and India which reported cases ranging from 60% to 84% of NHL (Aoki *et al*, 2008; Audouin *et al*, 2010; Yaqo *et al*, 2011; Mondal *et al*, 2013). NHL is not a single disease, but a

complex and diverse group which encompasses more than 65 different subtypes and this may explain its predominance when compared to HL (Huh, 2012). Other reasons may be attributed to the prevailing HIV pandemic and an aging world population (Epeldegui *et al*, 2010; Huh, 2012; Corti *et al*, 2014). The difference in the distribution of lymphomas in different countries reflects that both genetic and environmental factors may play a role in the development of lymphomas (Sun *et al*, 2012).

Worldwide, the occurrence of NHL and HL has been reported to be higher in males than in females (Yeole, 2008), and this mirrored findings in this study. This male preponderance has also been reported in other countries such as Pakistan, Uganda, Nigeria and India (Mushtaq *et al*, 2008; Tumwine *et al*, 2010; Olu-Edo *et al*, 2011 and Sharma *et al*, 2014). Gender-based differences in immune response are known to contribute to differences in lymphoma development. Males lack factors that have a protective role against lymphoma development in comparison to females. The reduced rates among females can be attributed to the direct effects of oestrogen on lymphoma cell proliferation or by its effect on anti-tumour immune response. In addition, pregnancy and the use of oral contraceptives may also contribute to this protective role in females (Villano *et al*, 2011; Horesh and Horowitz, 2014).

It was observed that a high incidence of NHL in both sexes was mostly in the 31 to 40 and 41 to 50 year age groups, the latter being similar to findings of an Indian study in which the most affected age group was from 40 to 50 years (Padhi *et al*, 2012). However, this was in contrast to findings of a German study which reported NHL to mainly affect individuals who were aged 55 years and above (Muller *et al*, 2005). In our study, NHL seemed to have afflicted a much younger age group as compared to the German study. This may be attributed to the poor

socioeconomic conditions and the predominance of immunodeficiency states such as HIV infection prevailing in developing countries such as Zambia (Horesh and Horowitz, 2014). However, it was not possible to link these NHL or HL cases to HIV infection as clinical data for the patients was difficult to obtain due to poor patient record keeping at the University Teaching Hospital.

It was, however, observed that the age group mostly afflicted by HL was below 30, with highest peak in those aged between 0-20 years. This finding was consistent with studies carried out in Iraqi, Kuwait, Jordan and Egypt (Makar *et al*, 2003; Almasri and Khalidi, 2004; Al-Safi, 2007; Audouin *et al*, 2010). In the developed world, the disease seems to peak in individuals over 60 years of age (Cartwright and Watkins, 2004). This finding seems to suggest that HL occurs at an earlier age in developing countries than in developed countries (Al-Mudallal & Al-Sinjery, 2012). This may be attributed to environmental, genetic and racial factors (Olu-Eddo *et al*, 2011). Increased risk of HL in the young adult population has also been linked to poor childhood socioeconomic status, which is a major problem among the adolescent population in developing countries (Maggioncalda *et al*, 2011).

The rate of EBV detection in this study was found to be high, and was similar to a Nigerian study which reported 54.5% EBV-positivity in all lymphomas (Iliyasu *et al*, 2014). This can be attributed to a similarity in the types of lymphomas and subtypes evaluated, as well as the extent of immune suppression of the study population (Ashraf *et al*, 2012). The EBV positivity rate in NHL was slightly higher than that in HL, although the difference was not statistically significant. This corroborated findings in a Chinese study in which the rate of EBV detection in NHL was also higher (42.6%) than that of HL (26.3%), (p=0.213) (Zhang *et al*, 2010). Despite

the fact that many studies confirm a strong association between EBV and lymphomas, the detection of EBV has not produced coherent results due to biological heterogeneity of the virus and diversity of lymphoma subtypes (Hassan *et al*, 2006; Gonin *et al*, 2011; Ishtiaq *et al* 2012).

The detection rate of EBV in HL tissues was observed to be low. This finding was consistent with North American and European findings where EBV expression in HL ranged from 20% to 50% (Herling *et al*, 2003; Chang *et al*, 2004; Diepstra *et al*, 2009). However, our findings were different from observations in other developing countries such as India, Iran, Nigeria, Egypt and Iraq which reported rates ranging from 60 to 90% (Karnik *et al*, 2003; Katebi *et al*, 2008; Adelusola *et al*, 2009; Audouin *et al*, 2010; Al-Mudallal and Al-Sinjery, 2012). This difference may be attributed to the fact that the association between EBV infection and HL depends on the geographical location, age, subtype of HL, and the extent of immunosuppression of the study population (Gulley *et al*, 2002; Adelusola *et al*, 2009).

A single case of mixed EBV infection (i.e. subtype 1 and 2) was detected, and this was similar to findings in a Spanish study which reported two cases of HL to have mixed infection (Garcia-Cosio *et al*, 2008). Dual infection could reflect immune dysfunction, with HIV being a cofactor in the process (Garcia-Cosio *et al*, 2008). However, in this study, we could not confirm whether or not this case of co-infection was attributed to HIV infection due to lack of patient data.

The rate of EBV positivity for NHL cases in this study was similar to those reported in Brazil, Uganda and Nigeria, which reported rates from 54.5% to 79.8% (Hassan *et al*, 2006; Tumwine *et al*, 2010; Iliyasu *et al*, 2014). This similarity can be ascribed to the anatomical site or types of lymphomas evaluated (Ashraf *et al*, 2012). The rate of EBV in lymphomas is also dependent on

the overall rate of EBV infection in a specific geographical region. For instance, in an Egyptian study, 90% of the EBV-positive cases in lymphomas of head and neck were ascribed to the high frequency of EBV infection in the general Egyptian population (Bahnassy *et al,* 2006). However, the findings in this study are in contrast to those reported in an Iranian study which reported a low rate of NHL (10%) (Ashraf *et al,* 2012). This can be attributed to the low prevalence of EBV infection in this population.

There were no significant differences in terms of gender and EBV association. This corroborated studies conducted in China and Uganda (Zhang *et al,* 2010; Tumwine *et al*, 2010). This is not surprising since it is known that EBV is usually acquired horizontally by intimate contact, and more than 90% of the world's population carry EBV as a lifelong, latent infection of B lymphocytes (Irshaid *et al*, 2010).

The predominant subtype of EBV detected in both NHL and HL was subtype 1. This finding was consistent with findings reported Brazilian and Chinese studies (Zhou *et al,* 2001; Peh *et al*, 2003; Hassan *et al*, 2006; Queiroga *et al,* 2008; Ai *et al*, 2012). However, studies conducted in Turkey and Mexico showed that EBV-2 was the predominant subtype (Tinguely *et al,* 2000; Palma *et al,* 2013). EBV subtype-2 infection has been described to be more prevalent in immunocompromised patients in Western populations, particularly in immunoblastic lymphomas arising in HIV-positive homosexual patients (Fassone *et al*, 2002; Santos *et al*, 2014). This difference in the prevalence of the two EBV subtypes can be attributed to the differences in their oncogenicity and the extent of immune suppression of the study population (Mendes *et al,* 2008; Ibrahim *et al,* 2013). In our study, this association was not possible due to lack of access to clinical information.

Phylogenetic analysis revealed that there were four groups of the EBV strains. The first three groups belonged to the EBV subtype 1 category, whilst the fourth one was for EBV subtype 2. The strains within each of group 1 and 2 were indistinguishable from each other. However, there were some major genetic differences between the strains in the two groups. The single strain in group 3, when compared with the other subtype 1 strains, showed it was not closely related to those in the other groups. These findings corroborate those in a Kenyan study (Simbiri *et al*, 2015). This shows that inter and intra-genetic variations in EBV genomes exist, and that within a geographical region different EBV genetic strains can coexist (Palser *et al*, 2015; Simbiri *et al*, 2015). However, a study conducted in the United Kingdom, which examined EBV strains from different geographical locations, showed that strains from the same region clustered together as opposed to those from different regions (Palser *et al*, 2015). This is attributed to genetic differences in the strains circulating in different geographical locations (Renzette *et al*, 2014).

The single strain in group 4 was the only one amplifiable from the subtype 2 category. The other sequences failed to amplify, and thus it was only on available for comparison. When compared to the reference strain, three-base pair differences were observed. This genetic similarity was interesting in that the reference, obtained from GenBank, from originally isolated strain from the United Kingdom. This may suggest that the two strains may have a common ancestry. A similar study conducted in Japan also detected only one case of EBV subtype 2 strain with no mutations when compared to the reference strain (Higa *et al*, 2002).

4.2 Conclusion

Data in this study demonstrate that the commonly diagnosed lymphomas are of the NHL type, accounting for 80% of all lymphomas. Males were more affected than females in both NHL and HL types of lymphomas, and mostly affect the young age group, a common pattern seen in most developing countries. The study also demonstrated that EBV was detectable in the majority of the lymphomas analysed, the rate of detection being higher in NHL than in HL. The predominant EBV subtype detected was subtype 1. There was no significant difference in the EBV detection rate with regards to gender in both types of lymphomas. Phylogenetic analysis of EBV strains revealed genetic diversity amongst EBV type 1, and that the EBV type 2 strain detected showed close genetic relatedness to the reference strain. It may be of interest to target EBV during lymphoma diagnosis as this may lead to improved patient outcomes during treatment. To our knowledge this is the first study of its kind in Zambia to detect and subtype EBV in lymphoma tissues.

4.3 Limitations of Study

A major limitation of this study was the non-availability of clinical information on the HIV status of patients due to lack of access to patient records. It would have been of interest to associate the findings with immune status of the patients to know if rate of EBV detection and subtypes in this study were influenced by patient's immune status. Another limitation was failure to amplify DNA sequences from the EBNA2 genomic region of EBV, and this was probably due to degradation of DNA in formalin fixed paraffin embedded tissues. Therefore, only a few strains were sequenced.

4.4 Future Directions

It would be of interest to collect clinical information on patients' immune status by carrying out a prospective study in order to associate EBV infections with HIV infection. Further studies on the characterisation of lymphomas by using immunohistochemistry should also be done in order to associate EBV infection with histological subtypes of lymphomas. Despite the UTH being a national reference hospital that processes most of the pathological specimens in Zambia, the findings in this study cannot be extrapolated to representative data from the whole country. Therefore, further work is warranted to determine the distribution of EBV strains in other parts of Zambia in order to have an accurate picture of EBV strains circulating in the country. This can be achieved by conducting national surveys involving lymphoma cases from other hospitals which would allow more systematic collection of epidemiological data.

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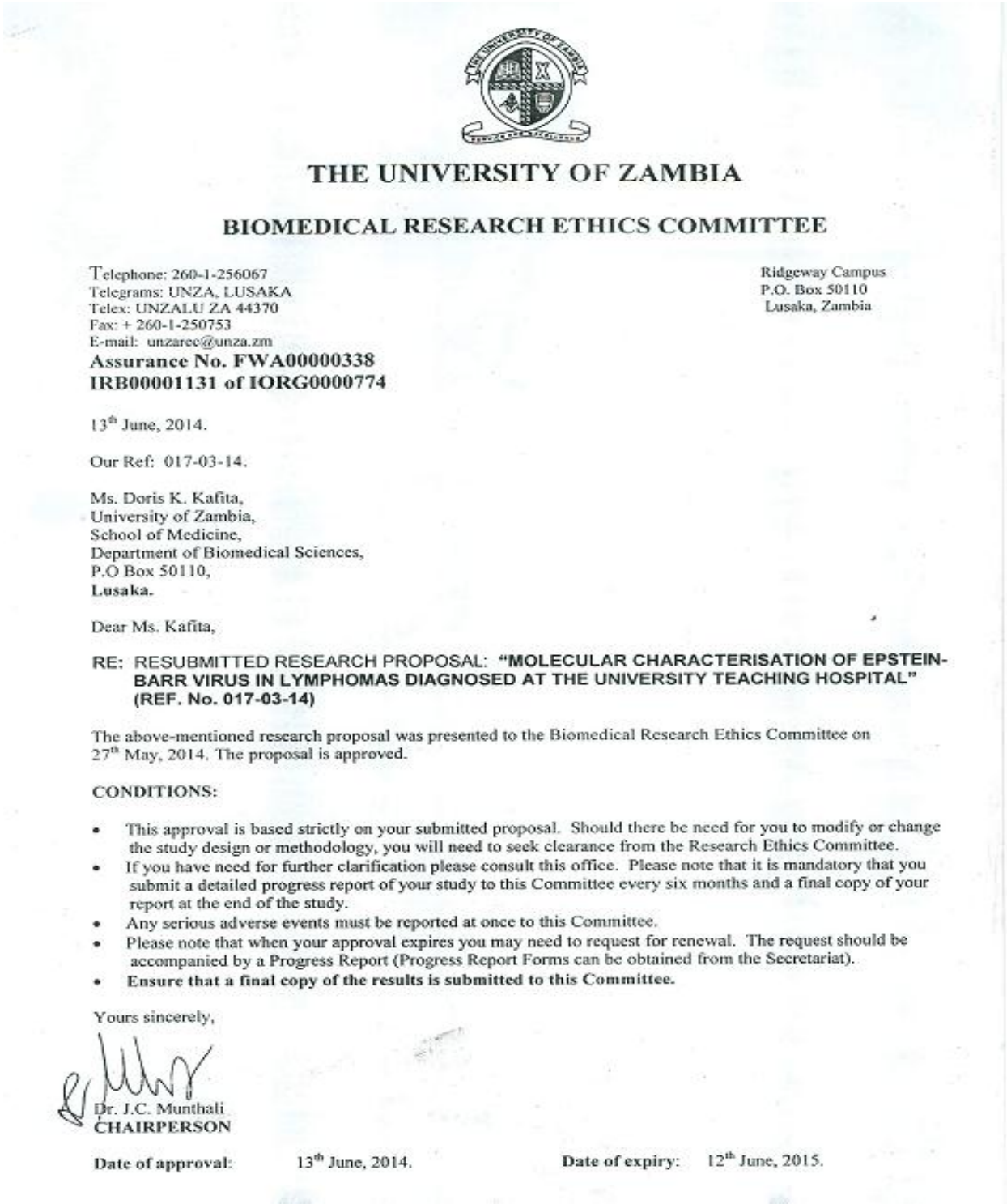
Appendices

Appendix I: Ethics Approval Letter



THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

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Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

Assurance No. FWA00000338
IRB00001131 of IORG0000774

13th June, 2014.

Our Ref: 017-03-14.

Ms. Doris K. Kafita,
University of Zambia,
School of Medicine,
Department of Biomedical Sciences,
P.O Box 50110,
Lusaka.

Dear Ms. Kafita,

RE: RESUBMITTED RESEARCH PROPOSAL: **"MOLECULAR CHARACTERISATION OF EPSTEIN-BARR VIRUS IN LYMPHOMAS DIAGNOSED AT THE UNIVERSITY TEACHING HOSPITAL" (REF. No. 017-03-14)**

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 27th May, 2014. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. J.C. Munthali
CHAIRPERSON

Date of approval: 13th June, 2014. **Date of expiry:** 12th June, 2015.

Appendix II: Representative DNA Sequences of EBV Strains Detected in this Study

A) Sample I.D Number: DK015F (EBV-1)

Human herpesvirus 4 strain GC1, complete genome
Sequence ID: gi|810056545|gb|KP735248.1|Length: 170390Number of Matches: 3
Related Information
Range 1: 36538 to 36973GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
833 bits(433)	0.0	435/436(99%)	0/436(0%)	Plus/Plus

```
Query  5      ATGCTTTGGATGGCTAATTATATTGTAAGACAATCACGGGGTGACCGGGGCCTTATTTTG  64
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36538  ATGCTTTGGATGGCTAATTATATTGTAAGACAATCACGGGGTGACCGGGGCCTTATTTTG  36597

Query  65     CCACAAGGCCCACAAACAGCCCCTCAGGCCATGTTGGTACAGCCACATGTCCCCCCTCTA  124
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36598  CCACAAGGCCCACAAACAGCCCCTCAGGCCATGTTGGTACAGCCACATGTCCCCCCTCTA  36657

Query  125    CGCCCGACAGCACCCACCATTTTGTCACCTCTGTCACAACCGAGGCTTACCCCTCCACGA  184
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  36658  CGCCCGACAGCACCCACCATTTTGTCACCTCTGTCACAACCGAGGCTTACCCCTCCACAA  36717

Query  185    CCACTCATGATGCCACCAAGGCCTACCCCTCCTACCCCTCTGCCACCTGCAACACTAACG  244
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36718  CCACTCATGATGCCACCAAGGCCTACCCCTCCTACCCCTCTGCCACCTGCAACACTAACG  36777

Query  245    GTGCCACCAAGGCCTACCCGTCCTACCACTCTGCCACCCACACCACTACTCACGGTACTA  304
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36778  GTGCCACCAAGGCCTACCCGTCCTACCACTCTGCCACCCACACCACTACTCACGGTACTA  36837

Query  305    CAAAGGCCTACCGAACTTCAACCCACACCATCACCACCACGCATGCATCTCCCTGTCTTG  364
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36838  CAAAGGCCTACCGAACTTCAACCCACACCATCACCACCACGCATGCATCTCCCTGTCTTG  36897

Query  365    CATGTGCCAGACCAATCAATGCACCCTCTTACTCATCAAAGCACCCCAAATGATCCAGAT  424
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36898  CATGTGCCAGACCAATCAATGCACCCTCTTACTCATCAAAGCACCCCAAATGATCCAGAT  36957

Query  425    AGTCCAGAACCACGGT  440
              ||||||||||||||||
Sbjct  36958  AGTCCAGAACCACGGT  36973
```

B) Sample I.D Number: DK015R (EBV-1)

Human herpesvirus 4 strain GC1, complete genome
Sequence ID: gi|810056545|gb|KP735248.1|Length: 170390Number of Matches: 3
Related Information
Range 1: 36477 to 36916GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
840 bits(437)	0.0	439/440(99%)	0/440(0%)	Plus/Minus

```
Query  2      TTGATTGGTCTGGCACATGCAAGACAGGGAGATGCATGCGTGGTGGTGATGGTGTGGGTT  61
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36916  TTGATTGGTCTGGCACATGCAAGACAGGGAGATGCATGCGTGGTGGTGATGGTGTGGGTT  36857

Query  62     GAAGTTCGGTAGGCCTTTGTAGTACCGTGAGTAGTGGTGTGGGTGGCAGAGTGGTAGGAC  121
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36856  GAAGTTCGGTAGGCCTTTGTAGTACCGTGAGTAGTGGTGTGGGTGGCAGAGTGGTAGGAC  36797

Query  122    GGGTAGGCCTTGGTGGCACCGTTAGTGTTGCAGGTGGCAGAGGGGTAGGAGGGGTAGGCC  181
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36796  GGGTAGGCCTTGGTGGCACCGTTAGTGTTGCAGGTGGCAGAGGGGTAGGAGGGGTAGGCC  36737

Query  182    TTGGTGGCATCATGAGTGGTCGTGGAGGGGTAAGCCTCGGTTGTGACAGAGGTGACAAAA  241
              |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  36736  TTGGTGGCATCATGAGTGGTTGTGGAGGGGTAAGCCTCGGTTGTGACAGAGGTGACAAAA  36677

Query  242    TGGTGGGTGCTGTCGGGCGTAGAGGGGGGACATGTGGCTGTACCAACATGGCCTGAGGGG  301
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36676  TGGTGGGTGCTGTCGGGCGTAGAGGGGGGACATGTGGCTGTACCAACATGGCCTGAGGGG  36617

Query  302    CTGTTTGTGGGCCTTGTGGCAAAATAAGGCCCCGGTCACCCCGTGATTGTCTTACAATAT  361
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36616  CTGTTTGTGGGCCTTGTGGCAAAATAAGGCCCCGGTCACCCCGTGATTGTCTTACAATAT  36557

Query  362    AATTAGCCATCCAAAGCATTCGCATAGCAGATGCTAGAGGTCCATGCCCGACGTCATATC  421
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36556  AATTAGCCATCCAAAGCATTCGCATAGCAGATGCTAGAGGTCCATGCCCGACGTCATATC  36497

Query  422    CTAGCGGATCCCTATCAAGA  441
              ||||||||||||||||||||
Sbjct  36496  CTAGCGGATCCCTATCAAGA  36477
```

C) Sample I.D Number: DK035F

Human herpesvirus 4 genome assembly sLCL-2.15, chromosome : I
Sequence ID: gi|764012596|emb|LN827591.1|Length: 172137Number of Matches: 1
Related Information
Range 1: 36594 to 36996GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
775 bits(403)	0.0	403/403(100%)	0/403(0%)	Plus/Plus

```
Query  1      TGCTTTGCATGGCTCAATACCTTCTTAGAAACGCACGGGGCCAACAAGGCCTTCTTAGGC  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36594  TGCTTTGCATGGCTCAATACCTTCTTAGAAACGCACGGGGCCAACAAGGCCTTCTTAGGC  36653

Query  61     CACTAGGACCACAAACACGCTCTCAGGTCACCTTGGAACGTCAACCTGTCCACAACCCTC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36654  CACTAGGACCACAAACACGCTCTCAGGTCACCTTGGAACGTCAACCTGTCCACAACCCTC  36713

Query  121    GCCAGGAGGCACCCATCATTTTGTTACAGAGTCCCGCGCCCCCCCGATTCACACCAGTGC  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36714  GCCAGGAGGCACCCATCATTTTGTTACAGAGTCCCGCGCCCCCCCGATTCACACCAGTGC  36773

Query  181    CCATGGTAGCCTTAGGACATACTCTTCAACCCACACCACCACCAAGGCTTACTCTTCCTC  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36774  CCATGGTAGCCTTAGGACATACTCTTCAACCCACACCACCACCAAGGCTTACTCTTCCTC  36833

Query  241    AACCCAGAATACCACTGATAATACCACCAAGGCATACTAATCAACCAGCCACAACACCAC  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36834  AACCCAGAATACCACTGATAATACCACCAAGGCATACTAATCAACCAGCCACAACACCAC  36893

Query  301    CCACGGCGCCACAAAGGCTCACACTAGGGCATCAACTAAGTCTACCACCGCATCCTCCCC  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36894  CCACGGCGCCACAAAGGCTCACACTAGGGCATCAACTAAGTCTACCACCGCATCCTCCCC  36953

Query  361    CGCATCAGAGCACCCCACATTGTAGTTCTGATAGTACAGGACT  403
              |||||||||||||||||||||||||||||||||||||||||||
Sbjct  36954  CGCATCAGAGCACCCCACATTGTAGTTCTGATAGTACAGGACT  36996
```

D) Sample I.D Number: DK035R (EBV-2)

Human herpesvirus 4 genome assembly sLCL-2.15, chromosome : I
Sequence ID: gi|764012596|emb|LN827591.1|Length: 172137Number of Matches: 1
Related Information
Range 1: 36535 to 36932GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
748 bits(389)	0.0	395/398(99%)	0/398(0%)	Plus/Minus

```
Query  1      TTAGTTGATGCCCTAGTGTGAGCCTTTGTGGCGCCGTGGGGGAAGTTGTGGCTGGTTGAT  60
              |||||||||||||||||||||||||||||||||||||||| |  ||||||||||||||||
Sbjct  36932  TTAGTTGATGCCCTAGTGTGAGCCTTTGTGGCGCCGTGGGTGGTGTTGTGGCTGGTTGAT  36873

Query  61     TAGTATGCCTTGGTGGTATTATCAGTGGTATTCTGGGTTGAGGAAGAGTAAGCCTTGGTG  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36872  TAGTATGCCTTGGTGGTATTATCAGTGGTATTCTGGGTTGAGGAAGAGTAAGCCTTGGTG  36813

Query  121    GTGGTGTGGGTTGAAGAGTATGTCCTAAGGCTACCATGGGCACTGGTGTGAATCGGGGGG  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36812  GTGGTGTGGGTTGAAGAGTATGTCCTAAGGCTACCATGGGCACTGGTGTGAATCGGGGGG  36753

Query  181    GCGCGGGACTCTGTAACAAAATGATGGGTGCCTCCTGGCGAGGGTTGTGGACAGGTTGAC  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36752  GCGCGGGACTCTGTAACAAAATGATGGGTGCCTCCTGGCGAGGGTTGTGGACAGGTTGAC  36693

Query  241    GTTCCAAGGTGACCTGAGAGCGTGTTTGTGGTCCTAGTGGCCTAAGAAGGCCTTGTTGGC  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36692  GTTCCAAGGTGACCTGAGAGCGTGTTTGTGGTCCTAGTGGCCTAAGAAGGCCTTGTTGGC  36633

Query  301    CCCGTGCGTTTCTAAGAAGGTATTGAGCCATGCAAAGCATTCTTATGGAAGATGCCAGAG  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36632  CCCGTGCGTTTCTAAGAAGGTATTGAGCCATGCAAAGCATTCTTATGGAAGATGCCAGAG  36573

Query  361    GGCCTTGACTGGCGTCACTGCCCAGGGGATTCATATCC  398
              |||||||||||||||||||||||||||||||||||||
Sbjct  36572  GGCCTTGACTGGCGTCACTGCCCAGGGGATTCATATCC  36535
```

Appendix III: EBNA2 DNA Sequences Analysed in this Study

A) EBV Type 1

```
                          10         20         30         40         50
                 ....|....| ....|....| ....|....| ....|....| ....|....|
DK015            GGGATCCGCT AGGATATGAC GTCGGGCATG GACCTCTAGC ATCTGCTATG
DK040            GGTTTTGGAT AGGATATGAC GTCGGGCATG GACCTCTAGC ATCTGCTATG
DK074            GGGATCCGCT AGGATATGAC GTCGGGCATG GACCTCTAGC ATCTGCTATG
DK076            GGGATCCGCT AGGATATGAC GTCGGGCATG GACCTCTAGC ATCTGCTATG
DK077            GGGATCCGCT AGGATATGAC GTCGGGCATG GACCTCTAGC ATCTGCTATG
DK064            GGGATCCGCT AGGATATGAC GTCGGGCATG GACCTCTAGC ATCTGCTATG
DK050            GGGATCCGCT AGGATATGAC GTCGGGCATG GACCTCTAGC ATCTGCTATG
Ref sLCL-IS1.10  GGGATCCGCT AGGATATGAC GTCGGGCATG GACCTCTAGC ATCTGCTATG

                          60         70         80         90        100
                 ....|....| ....|....| ....|....| ....|....| ....|....|
DK015            CGAATGCTTT GGATGGCTAA TTATATTGTA AGACAATCAC GGGGTGACCG
DK040            CGAATGCTTT GGATGGCTAA TTATATTGTA AGACAATCAC GGGGTGACCG
DK074            CGAATGCTTT GGATGGCTAA TTATATTGTA AGACAATCAC GGGGTGACCG
DK076            CGAATGCTTT GGATGGCTAA TTATATTGTA AGACAATCAC GGGGTGACCG
DK077            CGAATGCTTT GGATGGCTAA TTATATTGTA AGACAATCAC GGGGTGACCG
DK064            CGAATGCTTT GGATGGCTAA TTATATTGTA AGACAATCAC GGGGTGACCG
DK050            CGAATGCTTT GGATGGCTAA TTATATTGTA AGACAATCAC GGGGTGACCG
Ref sLCL-IS1.10  CGAATGCTTT GGATGGCTAA TTATATTGTA AGACAATCAC GGGGTGACCG

                         110        120        130        140        150
                 ....|....| ....|....| ....|....| ....|....| ....|....|
DK015            GGGCCTTATT TTGCCACAAG GCCCACAAAC AGCCCCTCAG GCCATGTTGG
DK040            GGGCCTTATT TTGCCACAAG GCCCACAAAC AGCCCCTCAG GCCATGTTGG
DK074            GGGCCTTATT TTGCCACAAG GCCCACAAAC AGCCCCTCAG GCCATGTTGG
DK076            GGGCCTTATT TTGCCACAAG GCCCACAAAC AGCCCCTCAG GCCATGTTGG
DK077            GGGCCTTATT TTGCCACAAG GCCCACAAAC AGCCCCTCAG GCCGTGTTGG
DK064            GGGCCTTATT TTGCCACAAG GCCCACAAAC AGCCCCTCAG GCCGTGTTGG
DK050            GGGCCTTATT TTGCCACAAG GCCCACAAAC AGCCCCTCAG GCCGTGTTGG
Ref sLCL-IS1.10  GGGCCTTATT TTGCCACAAG GCCCACAAAC AGCCCCTCAG GCCGTGTTGG

                         160        170        180        190        200
                 ....|....| ....|....| ....|....| ....|....| ....|....|
DK015            TACAGCCACA TGTCCCCCCT CTACGCCCGA CAGCACCCAC CATTTTGTCA
DK040            TACAGCCACA TGTCCCCCCT CTACGCCCGA CAGCACCCAC CATTTTGTCA
DK074            TACAGCCACA TGTCCCCCCT CTACGCCCGA CAGCACCCAC CATTTTGTCA
DK076            TACAGCCACA TGTCCCCCCT CTACGCCCGA CAGCACCCAC CATTTTGTCA
DK077            TACAGCCACA TGTCCCCCCT CTACGCCCGA CAGCACCCAC CATTTTGTCA
DK064            TACAGCCACA TGTCCCCCCT CTACGCCCGA CAGCACCCAC CATTTTGTCA
DK050            TACAGCCACA TGTCCCCCCT CTACGCCCGA CAGCACCCAC CATTTTGTCA
Ref sLCL-IS1.10  TACAGCCACA TGTCCCCCCT CTACGCCCGA CAGCACCCAC CATTTTGTCA

                         210        220        230        240        250
                 ....|....| ....|....| ....|....| ....|....| ....|....|
DK015            CCTCTGTCAC AACCGAGGCT TACCCCTCCA CGACCACTCA TGATGCCACC
DK040            CCTCTGTCAC AACCGAGGCT TACCCCTCCA CGACCACTCA TGATGCCACC
DK074            CCTCTGTCAC AACCGAGGCT TACCCCTCCA CGACCACTCA TGATGCCACC
DK076            CCTCTGTCAC AACCGAGGCT TACCCCTCCA CGACCACTCA TGATGCCACC
DK077            CCTCTGTCAC GACCGAGGCT TACCCCTCCA CAACCACTCA TGATTCCACC
DK064            CCTCTGTCAC GACCGAGGCT TACCCCTCCA CAACCACTCA TGATTCCACC
DK050            CCTCTGTCAC GACCGAGGCT TACCCCTCCA CAACCACTCA TGATTCCACC
Ref sLCL-IS1.10  CCTCTGTCAC GACCGAGGCT TACCCCTCCA CAACCACTCA TGATTCCACC
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                        260         270         280         290         300
                    ....|....| ....|....| ....|....| ....|....| ....|....|
DK015               AAGGCCTACC CCTCCTACCC CTCTGCCACC TGCAACACTA ACGGTGCCAC
DK040               AAGGCCTACC CCTCCTACCC CTCTGCCACC TGCAACACTA ACGGTGCCAC
DK074               AAGGCCTACC CCTCCTACCC CTCTGCCACC TGCAACACTA ACGGTGCCAC
DK076               AAGGCCTACC CCTCCTACCC CTCTGCCACC TGCAACACTA ACGGTGCCAC
DK077               AAGGCCTACC CCTCCTTCCC CTCTGCCACC TGCAACACTA CTCACGGTGC
DK064               AAGGCCTACC CCTCCTTCCC CTCTGCCACC TGCAACACTA CTCACGGTGC
DK050               AAGGCCTACC CCTCCTTCCC CTCTGCCACC TGCAACACTA CTCACGGTGC
Ref sLCL-IS1.10     AAGGCCTACC CCTCCTTCCC CTCTGCCACC TGCAACACTA CTCACGGTGC

                        310         320         330         340         350
                    ....|....| ....|....| ....|....| ....|....| ....|....|
DK015               CAAGGCCTAC CCGTCCTACC ACTCTGCCAC CCACACCACT ACTCACGGTA
DK040               CAAGGCCTAC CCGTCCTACC ACTCTGCCAC CCACACCACT ACTCACGGTA
DK074               CAAGGCCTAC CCGTCCTACC ACTCTGCCAC CCACACCACT ACTCACGGTA
DK076               CAAGGCCTAC CCGTCCTACC ACTCTGCCAC CCACACCACT ACTCACGGTA
DK077               CACCAAGGCC TACCCGTCCT ACCACTTTGC CACCCACACC ACTACTCACG
DK064               CACCAAGGCC TACCCGTCCT ACCACTTTGC CACCCACACC ACTACTCACG
DK050               CACCAAGGCC TACCCGTCCT ACCACTTTGC CACCCACACC ACTACTCACG
Ref sLCL-IS1.10     CACCAAGGCC TACCCGTCCT ACCACTTTGC CACCCACACC ACTACTCACG

                        360         370         380         390         400
                    ....|....| ....|....| ....|....| ....|....| ....|....|
DK015               CTACAAAGGC CTACCGAACT TCAACCCACA CCATCACCAC CACGCATGCA
DK040               CTACAAAGGC CTACCGAACT TCAACCCACA CCATCACCAC CACGCATGCA
DK074               CTACAAAGGC CTACCGAACT TCAACCCACA CCATCACCAC CACGCATGCA
DK076               CTACAAAGGC CTACCGAACT TCAACCCACA CCATCACCAC CACGCATGCA
DK077               GTACTACAAA GGCCTACCGA ACTTCAACCC ACACCATCAC CACCACGCAT
DK064               GTACTACAAA GGCCTACCGA ACTTCAACCC ACACCATCAC CACCACGCAT
DK050               GTACTACAAA GGCCTACCGA ACTTCAACCC ACACCATCAC CACCACGCAT
Ref sLCL-IS1.10     GTACTACAAA GGCCTACCGA ACTTCAACCC ACACCATCAC CACCACGCAT

                        410         420         430         440         450
                    ....|....| ....|....| ....|....| ....|....| ....|....|
DK015               TCTCCCTGTC TTGCATGTGC CAGACCAATC AATGCACCCT CTTACTCATC
DK040               TCTCCCTGTC TTGCATGTGC CAGACCAATC AATGCACCCT CTTACTCATC
DK074               TCTCCCTGTC TTGCATGTGC CAGACCAATC AATGCACCCT CTTACTCATC
DK076               TCTCCCTGTC TTGCATGTGC CAGACCAATC AATGCACCCT CTTACTCATC
DK077               GCATCTCCCT GTCTTGCATG TGCCAGACCA ATCAATGCAC CCTCTTACTC
DK064               GCATCTCCCT GTCTTGCATG TGCCAGACCA ATCAATGCAC CCTCTTACTC
DK050               GCATCTCCCT GTCTTGCATG TGCCAGACCA ATCAATGCAC CCTCTTACTC
Ref sLCL-IS1.10     GCATCTCCCT GTCTTGCATG TGCCAGACCA ATCAATGCAC CCTCTTACTC

                        460         470         480         490
                    ....|....| ....|....| ....|....| ....|....| .
DK015               AAAGCACCCC AAATGATCCA GATAGTCCAG AACCACGGTA A
DK040               AAAGCACCCC AAATGATCCA GATAGTCCAG AACCACGGTA A
DK074               AAAGCACCCC AAATGATCCA GATAGTCCAG AACCACGGTA T
DK076               AAAGCACCCC AAATGATCCA GATAGTCCAG AACCACGGTA T
DK077               ATCAAAGCAC CCCAAATGAT CCAGATAGTC CAGAACCACG G
DK064               ATCAAAGCAC CCCAAATGAT CCAGATAGTC CAGAACCACG G
DK050               ATCAAAGCAC CCCAAATGAT CCAGATAGTC CAGAACCACG G
Ref sLCL-IS1.10     ATCAAAGCAC CCCAAATGAT CCAGATAGTC CAGAACCACG G
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B) EBV Type 2

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                    10         20         30         40         50
                ....|....| ....|....| ....|....| ....|....| ....|....|
DK035           TGAATCCCCT GGGCAGTGAC GCCAGTCAAG GCCCTCTGGC ATCTTCCATA
Ref sLCL-2.15   TGAATCCCCT GGGCAGTGAC GCCAGTCAAG GCCCTCTGGC ATCTTCCATA

                    60         70         80         90        100
                ....|....| ....|....| ....|....| ....|....| ....|....|
DK035           AGAATGCTTT GCATGGCTCA ATACCTTCTT AGAAACGCAC GGGGCCAACA
Ref sLCL-2.15   AGAATGCTTT GCATGGCTCA ATACCTTCTT AGAAACGCAC GGGGCCAACA

                   110        120        130        140        150
                ....|....| ....|....| ....|....| ....|....| ....|....|
DK035           AGGCCTTCTT AGGCCACTAG GACCACAAAC ACGCTCTCAG GTCACCTTGG
Ref sLCL-2.15   AGGCCTTCTT AGGCCACTAG GACCACAAAC ACGCTCTCAG GTCACCTTGG

                   160        170        180        190        200
                ....|....| ....|....| ....|....| ....|....| ....|....|
DK035           AACGTCAACC TGTCCACAAC CCTCGCCAGG AGGCACCCAT CATTTTGTTA
Ref sLCL-2.15   AACGTCAACC TGTCCACAAC CCTCGCCAGG AGGCACCCAT CATTTTGTTA

                   210        220        230        240        250
                ....|....| ....|....| ....|....| ....|....| ....|....|
DK035           CAGAGTCCCG CGCCCCCCCG ATTCACACCA GTGCCCATGG TAGCCTTAGG
Ref sLCL-2.15   CAGAGTCCCG CGCCCCCCCG ATTCACACCA GTGCCCATGG TAGCCTTAGG

                   260        270        280        290        300
                ....|....| ....|....| ....|....| ....|....| ....|....|
DK035           ACATACTCTT CAACCCACAC CACCACCAAG GCTTACTCTT CCTCAACCCA
Ref sLCL-2.15   ACATACTCTT CAACCCACAC CACCACCAAG GCTTACTCTT CCTCAACCCA

                   310        320        330        340        350
                ....|....| ....|....| ....|....| ....|....| ....|....|
DK035           GAATACCACT GATAATACCA CCAAGGCATA CTAATCAACC AGCCACAACT
Ref sLCL-2.15   GAATACCACT GATAATACCA CCAAGGCATA CTAATCAACC AGCCACAACA

                   360        370        380        390        400
                ....|....| ....|....| ....|....| ....|....| ....|....|
DK035           TCCCCCACGG CGCCACAAAG GCTCACACTA GGGCATCAAC TAAGTCTACC
Ref sLCL-2.15   CCACCCACGG CGCCACAAAG GCTCACACTA GGGCATCAAC TAAGTCTACC

                   410        420        430        440        450
                ....|....| ....|....| ....|....| ....|....| ....|....|
DK035           ACCGCATCCT CCCCCGCATC AGAGCACCCC ACATTGTAGT TCTGATAGTA
Ref sLCL-2.15   ACCGCATCCT CCCCCGCATC AGAGCACCCC ACATTGTAGT TCTGATAGTA

                   460        470        480        490
                ....|....| ....|....| ....|....| ....|....| .
DK035           CAGGACTACT TGCACCTCCC ACATCTTACA GCATTCCTTC T
Ref sLCL-2.15   CAGGACTCCC TCCACCTCCC ACATCTTACA GCATTCCTTC T
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Appendix IV: Reagents

10X TBE Buffer

108g Tris

55g Boric acid

9.3g EDTA

Up to a 1000ml of distilled water.

TBE (x1)

1 volume of 10x TBE buffer

9 volumes distilled water

Ethidium bromide solution

10mg/ml in distilled water.

Stored in a dark bottle at 4°C

Agarose Gel (1.5%)

1.5% Agarose gel

Up to 100ml 1X TBE buffer