LABORATORY DIAGNOSIS OF EPSTEIN BARR VIRUS IN DIFFUSE LARGE B CELL LYMPHOMA

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A dissertation submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in the branch of Anatomical Pathology.

21 July 2017

DECLARATION

I, Sharlene Naidoo declare that this Dissertation Report is my own work. It is being submitted for the Degree of Master of Science in the branch of Anatomical Pathology in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Signature of candidate

Student number: 1006371

Date: 21 July 2017

DEDICATION

In memory of late Professor Mario Altini

19 September 1949 to 10 February 2017

PRESENTATIONS/PUBLICATIONS ARISING FROM THIS STUDY

Conference Presentations

Sharlene Naidoo, Yvonne Perner, Mario Altini, Rindidzani Magobo, Michelle McCabe, Martin Hale. Identification of EBNA2 in a cohort of HIV associated diffuse large B cell lymphoma paraffin embedded tissue samples. Annual Pathology Congress 19-21 September 2014, Pretoria, South Africa.

ABSTRACT

Aims and objectives

The study design aimed to assess and validate various laboratory techniques in the detection of EBV in HIV positive patients with diffuse large B cell lymphoma. The sensitivity and specificity of each technique was determined, as was the presence of an asymptomatic (latent) or lytic phase infection and the viral strain. DLBL samples occurring in HIV seropositive patients were used as a vehicle for these laboratory procedures which included chromogenic in situ hybridisation (EBER), immunohistochemistry (EBNA 2, LMP 1), real time PCR, (EBNA 1, LMP 2 and BZLF 1) and nested PCR (EBNA 2).

Materials and Methods

46 cases of previously diagnosed DLBL from HIV positive individuals were identified and retrieved from the archives of the Department of Anatomical Pathology of the University of Witwatersrand and NHLS. All in-situ hybridisation, immunohistochemical and PCR laboratory procedures were carried out in accordance with the Standard Operating Procedures of the Anatomical Pathology Molecular Laboratory, using appropriate negative and positive controls throughout. Ethical clearance was obtained (M140273).

Results/Conclusion

A 20% frequency of EBV in HIV positive DLBL cases was established. All EBV infections were found to be in the lytic phase, with an almost equal distribution of latency patterns II and III and an equal distribution of EBV strains 1 and 2. EBER in situ hybridisation was confirmed to be the most sensitive and reliable method of viral detection, and the presence of the BZLF 1 gene determined by real time PCR was found to be a reliable indicator of a lytic infection.

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
BART	*BAMHI* A Rightward transcripts
BLAST	Basic local alignment search tool
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BL	Burkitt lymphoma
β globin	Beta globin
bp	base pairs
CC	Cell conditioning
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CMV	Cytomegalovirus
CNS	central nervous system
CTL	cytotoxic T lymphocyte
Ct.	cycle threshold
DAB	3,3' diaminobenzidine
DNA	Deoxyribonucleic acid

DPX	Distrene, Plasticiser, Xylene
DLBL	Diffuse large B cell lymphoma
DLBL NOS	Diffuse large B cell lymphoma, not otherwise specified
EBV	Epstein Barr virus
EBER	Epstein Barr virus early RNA
EBNA	Epstein Barr nuclear antigens
eV	electron volt
ELISA	Enzyme Linked Immunosorbent Assay
FRET	Fluorescent resonance energy transfer
Fig	Figure
FFPE	Formalin fixed paraffin embedded
HAART	Highly active antiretroviral therapy
HEV	High endothelial venule
H&E	Haematoxylin and Eosin
HHV	Human herpes virus
HSV	Herpes simplex virus
HPV	Human papilloma virus

HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HL	Hodgkin lymphoma
IL	interleukin
IE	immediate early
ISH	in situ hybridization
IHC	Immunohistochemistry
Ig	Immunoglobulin
JAK/STAT	Janus kinase/signal transducers and activators of transcription
LMP	Latent membrane protein
LP	Leader protein
LCS	Liquid coverslip medium
LCL	Lymphoblastoid cell lines
MAP	Mitogen-activated protein
mRNA	Messenger RNA
NHLS	National Health Laboratory Service
NBT	Nitro blue tetrazolium chloride

NF	Nuclear factor
NFκB	Nuclear Factor Kappa B
NKT	Natural killer T cell
NHL	Non Hodgkin lymphoma
NCBI	National centre for biotechnology information
P53	Phosphoprotein 53
PI3K / Akt	Phosphatidylinositol 3' kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PBGD	Porphobilinogen deaminase
PTCL	Peripheral T cell lymphoma
PTLD	Post transplant lymphoproliferative disorders
PBL	Plasmasblastic lymphoma
PEL	Primary effusion lymphoma
QPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
USP	Ubiquitin specific protease

VZV Varicella zoster virus

WHO World Health Organisation

CHAPTER 1

1.0 Introduction

Eight of the more than 100 known herpesviruses routinely infect humans. These are herpes simplex virus (HSV) types 1 and 2, varicella zoster virus (VZV), cytomegalovirus (CMV), Epstein Barr virus (EBV), human herpes virus (HHV) 6 and 7 and Kaposi sarcoma virus also known as human herpes virus 8 (HHV8) (Whitley 1996). A defining feature of herpes viruses is their ability to indefinitely maintain a latent infection within the host genome, being retained as an episome in the host cell nucleus without producing infectious virions.

In order to achieve a latent infection, the herpes virus, upon acute infection, fails to be cleared and remains dormant within the cell and persists in a non-infectious form with periodic reactivation. Latency is established within specific tissues which are characteristic for each virus. HSV1 and VZV infect epithelial cells and produce latent infection in neurons (Kinchington et, 2012). Cytomegalovirus and human herpes virus 6 latently infect monocytes and their bone progenitors (Minton et al, 1994) and Epstein Barr virus and HHV8 produce latent infection in lymphoid cells.

An understanding of the viral structure is required to facilitate an appreciation of the expression of viral genes and latency patterns.

Herpesviruses have a unique four-layered structure (Whitley, 1996):

- a core containing the large, double stranded DNA genome
- an icosapentahedral capsid which encloses the DNA genome and is composed of capsomers
- an amorphous protein coat, the tegument which surrounds the capsid
- a glycoprotein bearing lipid bilayer envelope encasing the entire structure

The process of transcription, genome replication and assembling of the virus capsid occurs within the host cell nucleus. The replicative lifecycle of a virus is divided into three phases which are the immediate-early, early and late phases. The viral genome is replicated as follows:

1. immediate-early (IE) genes – these gene products are transactivator proteins that trigger the expression of early genes
2. early genes which encode enzymes for replicating viral DNA
3. late genes which encode structural proteins

The viral membrane is formed as a result of the 'budding out' of the virions through the nuclear membrane or endoplasmic reticulum.

The Golgi complex facilitates the transportation of the virions to the cell membrane. The virions are released into the cell cytoplasm and the host cell may undergo lysis and be removed from the circulation by virus specific T lymphocytes, or the virions may be retained as nuclear episomes within the host cell nucleus (Kenney 2007), in a latent state. The latent state is maintained by a variety of genes which are peculiar to each virus type.

An appreciation of herpes virus latency patterns gains significance when considering that about 12% of all human cancers are caused by oncoviral infections and more than 80% of these occur in the developing world (Boyle & Levin, 2008; Bouvard et al, 2009; de Martel et al, 2012). Human viral oncogenesis has the following common characteristics (Bouvard et al, 2009; Zur Hausen, 2009).

a. Oncoviruses are necessary but not sufficient for cancer development. Therefore cancer incidence is much lower than the prevalence of the virus in the population.
b. Viral cancers appear in the context of persistent latent infections and may occur many years after acute infection.
c. The state of the immune system plays a role in the development of the cancer with some human virus associated cancers increasing in incidence with immunosuppression, while others appear in the context of chronic inflammation.
d. Most cancers occur in immunocompetent hosts and tumour cells must develop strategies that allow them to escape immune clearance.
e. In vivo, the growth of a tumour is mainly controlled by cell mediated immunity.
f. The ability of the immune system to keep the virus in check is one of the crucial barriers inhibiting the development of viral associated cancer.
g. An EBV infected cell carries double stranded viral DNA in a circularized episomal form which is then able to replicate and produce between 1 and 50 copies of viral genome. These clonal episomes are passed along to cellular progeny. If an infected cell undergoes malignant transformation, every neoplastic daughter cell inherits the same unique viral episomal structure,

making the viral genomic structure a marker for tumour clonality (Gulley & Tang, 2008).

Four DNA viruses have been identified in the pathogenesis of human cancers – human papilloma virus (HPV), EBV, hepatitis B virus (HBV) and HHV8 (Stricker & Kumar, 2010). Because of the increasing list of human cancers associated with EBV, the World Health Organisation classified EBV as a tumour virus in 1997 (Grywalska & Rolinski, 2015).

The Epstein Barr virus gains entry into the human body mainly by close contact with saliva, but also through sexual contact or vertically from mother to child. There is initial infection in the oropharyngeal lymphoid tissues, especially in the tonsils. EBV binds to B- lymphocytes through a direct interaction of the EBV glycoprotein gp350/gp220 with the complement receptor CD21 (Figure (Fig)1.1). In addition, the EBV glycoproteins gH-gL-gp42 interact with HLA class II molecules to allow endocytosis of the EBV virion into smooth endoplasmic reticulum of a B lymphocyte. The viral DNA is then carried to the nucleus of the cell. EBV infects epithelial cells through a CD21 independent mechanism, with viral glycoprotein gH mediating EBV attachment to CD21 negative epithelial cells, (Molesworth, 2000).

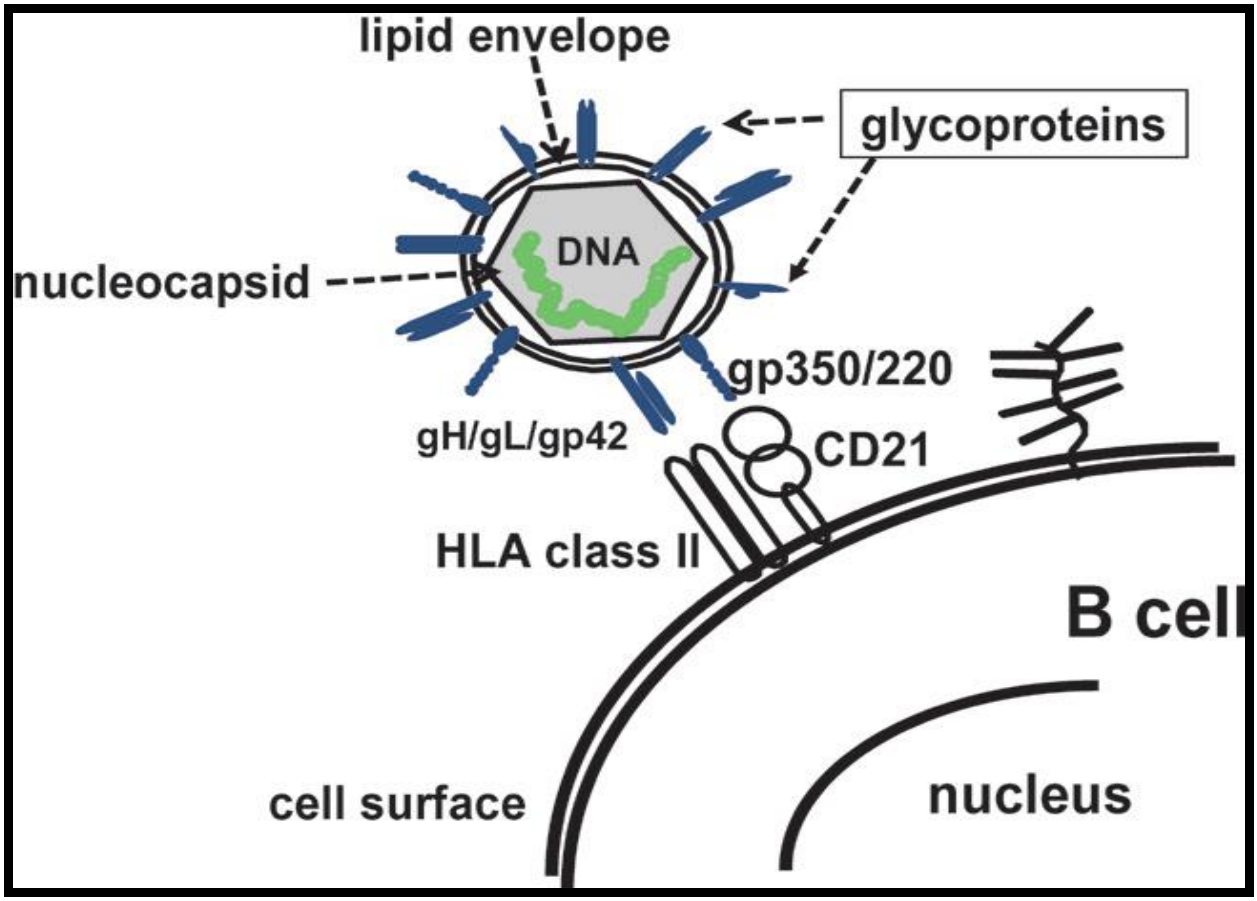


Figure.1.1 Attachment of EBV to B-cells through viral glycoproteins and cellular receptors (adapted from Kimura, 2013).

In the submucosa, infection of the B-cells occurs in one of two ways. In the minority of B cells, it is a productive infection with the virus causing cell lysis and release of virions which may in turn infect other B-cells, while in most of the B cells, EBV maintains a latent infection. A switch from lytic to latent infection or vice versa is always possible. A hypothetical model of EBV persistence has been explained by Thorley-Lawson, 2001 (Figure1.2) as follows:

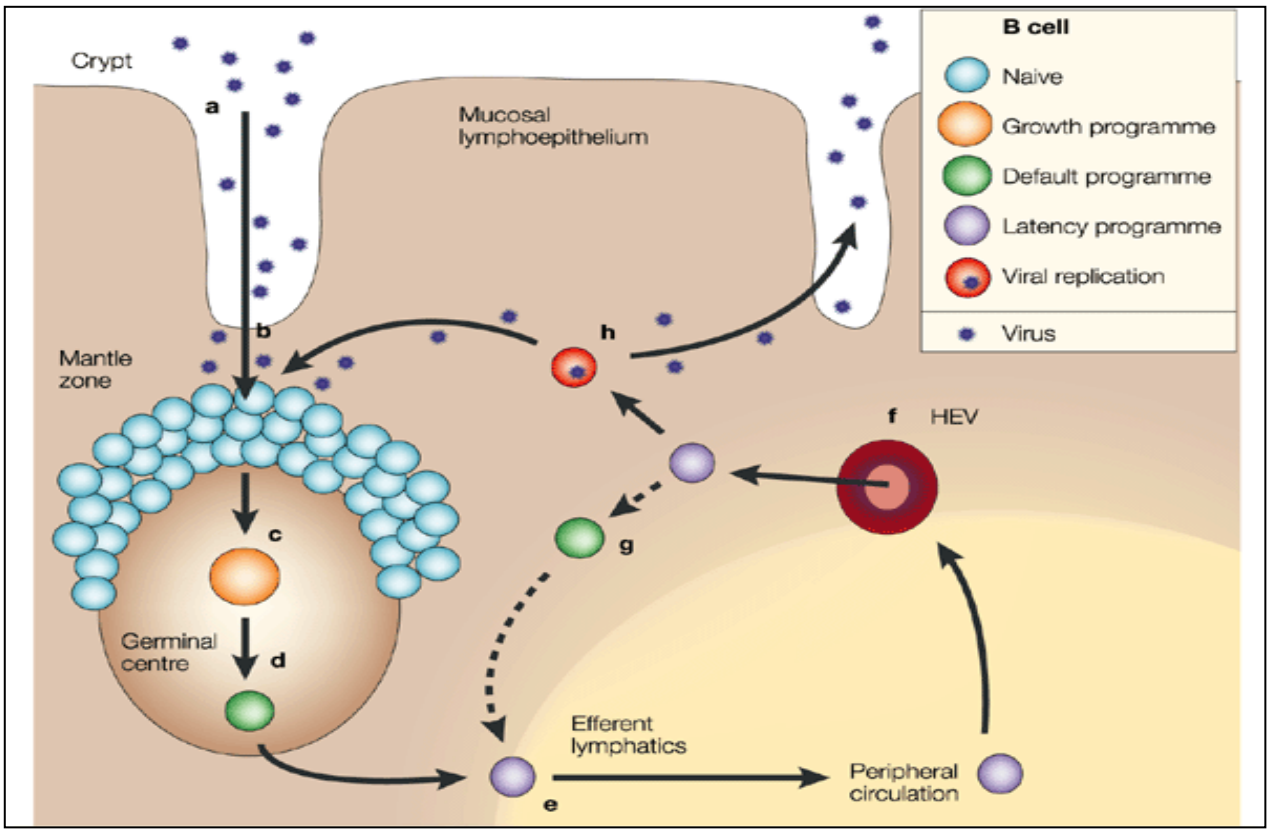


Figure 1.2 Hypothetical model of EBV persistence (adapted from Thorley-Lawson, 2001).

a. **Epstein Barr virus (EBV) gains entry into the body via saliva into the crypts of the lymphoepithelial structures such as the tonsils.**
b. **The virus crosses the epithelial barrier and infects the naïve B lymphocytes in the germinal centre.**
c. **The naïve B cells are driven to become blasts through the expression of the growth programme. This process is regulated by EBV nuclear antigen 2 (EBNA 2).**
d. **EBNA 2 is intensely immunogenic, and in order to bypass immune detection, it is down-regulated. In the absence of EBNA 2, the EBV infected blasts switch from the growth programme to the default programme. Expression of latent membrane protein 1 (LMP 1; a CD40 homologue) and LMP 2A (a B cell receptor homologue) in the absence of EBNA 2 provides the requisite survival signals for the default programme.**
e. **EBV latently infected resting memory cells leave the follicles and enter peripheral circulation through the efferent lymphatics. The EBV infected cells express little or no genetic information and are therefore non pathogenic, not recognised by the immune system and are hence able to evade the immune system. In this state, they lose the expression of LMP 1 and LMP 2A and are able to be maintained as normal memory cells.**
f. **Memory cells re-enter the tonsil from peripheral circulation through high endothelial venules (HEVs).**
g. **Survival of antigen specific memory cells requires an intact B cell receptor. EBV infected memory cells express the default programme which might provide the necessary signals for long term survival of the cells as memory cells. They then turn off the default programme and leave by the efferent lymphatics.**
h. **Occasionally a fraction of latently infected memory cells initiates replication of the virus to be shed into saliva. A failure to activate the default programme before the LMP 1 and LMP 2A proteins are lost might be a trigger to this action.**

Most individuals are infected by early adulthood. Although EBV preferentially infects B lymphocytes, the virus may also infect T cells, natural killer cells and possibly monocytes (Kimura et al, 2003). Children who are infected with EBV are usually asymptomatic, while adolescents and young adults usually become ill with infectious mononucleosis.

CHAPTER 2

2.0 Aims and objectives

The aim of the study was to characterize the frequency and heterogeneity in the pattern of expression of EBV latent genes in a series of DLBLs in HIV seropositive patients.

The objectives were:

1. To determine the frequency of EBV infection in DLBLs in HIV positive patients.
2. To determine the expression of the EBV *BZLF 1* lytic gene and hence show that the disease is in a lytic viral replication state.
3. To establish the pattern of expression of EBV latent genes
4. To compare IHC and PCR in determining the expression of the latent genes.
5. To determine whether the EBV viral isolates in DLBL are type 1 or type 2 in HIV positive patients.

CHAPTER 3

3.0 Literature Review

3.1. EBV infection

In order to maintain the integrity of the viral genome and evade the host immune system, EBV expresses several genes including genes for six EBV nuclear antigens (EBNA 1, 2, 3A, 3B, and 3C, and EBV nuclear antigen leader protein, EBNA LP); three EBV latent membrane proteins (LMP 1, 2A, and 2B); two short non-coding RNAs (EBV encoded small RNA, EBER 1 and 2) and *BamHI A* rightward transcripts (BART). The expressed latent proteins play important roles by regulating cell cycle and cell proliferation. In EBV associated cancers these genes contribute to oncogenesis. There are different patterns of expression of these latent genes which occur in different cancers and are discussed further in this chapter.

3.2. Latent Infection

According to Mendes, (2008), of the eighty EBV proteins encoded by the viral genome, only nine are expressed on establishment of a latent infection of naive B cells. Of the 9 proteins expressed 6 are localized in the nucleus (EBNAs) and three in the cell membrane (LMPs). These nuclear and membrane proteins are expressed in one of three distinct patterns, with switching occurring between patterns depending on the growth phase of the infected cell and the EBV infection cycle (Fig 1.2). Whilst there are only 3 patterns of protein expression, there are in fact 4 patterns of latent gene expression, designated: Latency 0, I, II and III (Thompson &

Kurzrock, 2004). The genes expressed in each pattern are as follows, (and are summarized in Table 3.1):

Latency pattern 0. This is characteristic of infected, resting, memory B cells in that only EBERs are expressed and the cell is immunologically silent.

Latency pattern I. This pattern` is also immunologically silent in that only EBNA 1 and EBERs are expressed. EBNA 1 allows the EBV episome to be segregated and retained in dividing cells.

Latency pattern II. With this pattern the B cells exhibit weak immunogenicity but are still susceptible to immune clearance. They express EBERs, EBNA 1, LMP 1, LMP 2A and LMP 2B.

Latency pattern III. This is the most immunogenic latency pattern, expressing EBER and all the nuclear and membrane proteins (EBNAs 1, 2, 3A, 3B, 3C and LP, LMP 1, LMP 2A and LMP 2B) rendering the cells most susceptible to immune clearance by cytotoxic T cells (El Bietar & Bollard 2011)

It has been shown, in vitro, that in the presence of removed or inhibited T lymphocyte immunity, EBV infected B lymphocytes give rise to immortalized cell lines, referred to as lymphoblastoid cell lines (LCLs). These exhibit a full complement of EBV encoded latent antigens comprising the six nuclear proteins, three latent membrane proteins and EBV early RNAs (EBERs) described above (Rickinson et al, 1984; Tsao, 2015; Kang & Kieff, 2015). This pattern of latent EBV gene expression in LCLs is an example of a latency III EBV infection and is present in most post transplant lymphoproliferative disorders (PTLD) in vivo.

When naïve B cells are first infected they exhibit a latency pattern III which drives the infected cells to proliferate, thus allowing episomal replication. Because of the high immunogenicity imparted by EBNA 2 and EBNA 3, a switch to latency pattern II soon ensues. With a latency pattern II the cells are driven to become memory B cells which then switch into one of the immune silent patterns i.e. 0 or I (Thorley-Lawson, 2001).

Table 3.1 Latent EBV gene expression

Latency Patterns	Genes expressed
Latency Pattern 0	*EBERs*
Latency Pattern 1	*EBERs, EBNA1*
Latency Pattern 11	*EBERs, EBNA1, LMP1,LMP2*
Latency Pattern 111	*EBERs, LMP1, LMP2, EBNAs 1,2,3A,3B,3C, EBNA LP*

In immunologically healthy people EBV driven polyclonal B cell proliferation is readily controlled and the individual develops a latent infection or transient episodes of infectious mononucleosis, characterised by the presence of large numbers of atypical mononuclear cells in the peripheral blood and tissues. These cells are predominantly EBV specific CD8+ HLA class I restricted cytotoxic T lymphocytes (CTLs) directed against latent and lytic cycle antigens of the Epstein Barr virus (Hopwood, 2000). Cell mediated mechanisms primarily control EBV infections. Cytotoxic T cells and natural killer T (NKT) cells constantly recognize and clear the

body of viral cells that express proliferation driving genes (Klein, 2010). The memory of the T cells is retained for life.

A humoral response also occurs. Antibodies to the early antigen complex and to the structural viral capsid/antigen complex including the gp350 complex occur early in infectious mononucleosis. By the time that symptoms present, there are high levels of immunoglobulin (Ig)M and IgG directed against the viral antigens.

A healthy EBV carrier state is established (latent infection) once the host recovers from the primary infection. IgG antibodies against the viral capsid antigen/membrane antigen complex gp 350, and against EBNA 1 are consistently detected and maintained. EBV infection is usually carried as a latency pattern 0 in healthy carriers. The host B cells are able to carry the virus in this state without manifesting symptoms.

In immunocompromised individuals, high levels of virally encoded cells are detected in the body. This occurs because the depressed immune system is not able to efficiently clear these cells. An accumulation of EBV infected B cells and increased viral replication occurs. The EBV infection may convert from a latent to a lytic pattern of expression.

3.3 EBV lytic infection and associated latent genes and proteins

The EBV lytic cycle is initiated by the transcription of the EBV immediate early *BZLF 1* and *BRF 1* genes (Ye et al, 2010). These two genes are expressed simultaneously and encode for the transactivator ZEBRA protein (*BZLF 1)* and Rta protein (*BRF 1*) which are essential in controlling the transition from viral latency to lytic replication. In this way the expression of *BZLF 1* in cells allows the distinction of EBV latent and

EBV lytic infections (Kraus et al, 2003). The *BZLF 1* and *BRF 1* are referred to as immediate early (IE) genes (Ye et al, 2010).

EBV encoded RNA (EBERs)

EBER 1 and EBER 2 commonly called EBERs are non-polyadenylated and non protein coding RNA transcripts. These RNA transcripts are well expressed in all EBV infected cells and serve as targets for the identification of EBV in histological material using in-situ hybridization (ISH). Although EBER ISH is now considered the 'gold standard' for the detection of EBV infection, it does not distinguish the EBV latency pattern.

The role of EBERs in transformation of B cells is contradictory, with both non essential and critical roles having been demonstrated. EBER gene expression auguments colony formation, confers resistance to apoptosis, stimulates growth of cells, induces cytokines and modulates innate immune responses (Kang & Kieff, 2015).

Latent membrane protein (LMP 1) and LMP 2A/2B

LMP 1 is important for EBV driven B cell transformation to immortalized lymphoblastoid cell lines. It appears to act by binding to tumour necrosis factor receptor (α & β) and activates signaling that mimics B cell activation by CD40, which is involved in normal B cell responses. It further activates nuclear factor (NF) kappa B, Janus kinase/signal transducers and activators of transcription (JAK/STAT), phosphatidylinositol 3' kinase (PI3K/Akt) and P38/mitogen activated protein (MAP) kinase signaling pathways which promotes B cell proliferation and survival. LMP 1 concurrently prevents apoptosis by activating BCL 2 (Kang & Kieff 2015, Ok et al, 2014).

LMP 2 can mediate the proliferation and survival of B cells. It may have a role in modifying normal B cell development, to facilitate the maintenance of EBV latency and prevent inappropriate activation of the lytic cycle. The consistent expression of LMP 2 in all EBV associated tumours with either latency pattern II or III suggests an important but not essential role in B cell transformation, although further work needs to be done to prove this (Tao et al, 2006).

EBV encoded nuclear antigen (EBNA 1, EBNA 2, EBNA 3A, 3B, 3C and EBNA LP)

EBNA 1 binds the EBV genome to the cell chromosomes, so that the virus is replicated in dividing cells as if it were part of the host genome, thus mediating episomal persistence and maintenance. It also blocks the interaction between herpesvirus-associated ubiquitin specific protease (USP7, also known as HAUSP) and phosphoprotein 53 (p53), facilitating p53 degradation according to the following mechanism (Grywalska & Rolinski, 2015). USP7 can bind to p53 and Mdm2 (an E3 ubiquitin ligase for p53) and cause these proteins to stabilize by removing the polyubiquitin chains that usually signal cell degradation. According to literature by Frappier, 2012 it is suggested by previous in vitro studies (Holowaty et al, 2003; Saridakis et al , 2005 & Sheng et al, 2006) that EBNA 1, p53 and Mdm2 compete for the same binding site in the N terminal TRAF domain of USP7. EBNA 1 has a higher affinity than p53 and Mdm2 and interferes with p53 and Mdm2 binding to USP7. P53 and Mdm2 are consequently destabilized and susceptible to degradation.

EBNA 2 drives B cell activation and proliferation by stimulating transcription of many host cell genes including those that drive viral entry into the cell.

In 2008, Mendes et al described EBNA 2 to be an important contributor to the EBV driven B cell growth transformation process.

In another study, the EBV cell line P3HR 1, carrying a deletion of the gene encoding *EBNA 2* and a deletion of the last two exons of *EBNA LP*, showed an inability of the EBV virus to cause B cell transformation (Kieff & Rickinson, 2007).

EBNA 2 activates viral and cellular target genes which initiates a cascade of events leading to viral cell entry and proliferation of infected B cells.

EBNA 2 activates *BFL 1*, a cellular anti-apoptotic gene which offers protection from apoptosis under conditions of depleted growth factor (Pegman et al, 2006).

The EBNA 3 family of proteins are transcriptional regulators with EBNA 3C able to upregulate both cellular antigens (CD21) and viral gene expression (LMP 1). Studies have demonstrated that EBNA 3A and EBNA 3C are essential for B cell transformation while EBNA 3B is dispensable (Tao et al, 2006). Cells expressing EBNA 3s are dominant CD8+ cytotoxic T lymphocyte (CTL) targets, hence cells expressing latency type III infection are usually eliminated by CTL. The function of the EBNA LP in B cell transformation is uncertain, however it is needed for the effective growth of LCLs in vitro (Allan et al, 1992).

3.4 EBV isolates Type 1 and 2

There are 2 major EBV strains (strains 1 and 2) as well as multiple minor strains. Strain variation is suspected to influence disease association, oncogenicity and drug resistance (Gulley and Tang, 2008).

The strain variation may have an impact on the aggressiveness of lymphomas and carcinomas with which they are associated (Gulley & Tang, 2008). Type 1 and 2 strains (which were previously known as type A and B strains) mainly occur due to polymorphisms in the viral gene encoding EBNA 2, although polymorphisms within the genes encoding EBNA 3A, EBNA 3B and EBNA 3C are also involved. Type 2

infections have a lower transforming efficiency and a slower initial proliferation (Mendes et al, 2008). EBV strains type 1 and 2 occur globally and show variation in geographical distribution. The Type 1 strain occurs predominantly in immunocompetent individuals. In certain T cell deficient hosts, especially those with acquired immunodeficiency syndrome (AIDS), there is an increased prevalence of type 2 infections (Mendes et al, 2008).

The genes and proteins expressed in each pattern and their functions are summarized in Table 3.2.

Table 3.2. Latency pattern of expression and functions of latent genes / proteins

LATENT GENE/PROTEINS	LATENCY PATTERN	FUNCTION
EBER	Latency pattern 0, I,II,III	EBER gene expression auguments colony formation, confers resistance to apoptosis, stimulates growth of cells, induces cytokines and modulates innate immune responses (Kang & Kieff, 2015).
BZLF 1 and *BRF 1* genes	Conversion from latent to lytic Infection	These two genes are expressed simultaneously and encode for the transactivator ZEBRA protein (*BZLF 1)* and Rta protein (*BRF 1*) which are essential in controlling the transition from viral latency to lytic replication.
LMP1	Latency I, II	LMP 1 is important for EBV driven B cell transformation to immortalized lymphoblastoid cell lines. It appears to act by binding to tumour necrosis factor receptor (α & β) and activates signaling that mimics B cell activation by CD40, which is involved in normal B cell responses. It further activates nuclear factor (NF) kappa B, Janus kinase/signal transducers and activators of transcription (JAK/ STAT), phosphatidylinositol 3' kinase (PI3K/Akt) and P38/mitogen activated protein (MAP) kinase signaling pathways which promotes B cell proliferation and survival. LMP 1 concurrently prevents apoptosis by activating BCL 2 (Kang & Kieff 2015, Ok et al, 2014).
LMP 2A / LMP 2B	Latency I, II	LMP 2 can mediate the proliferation and survival of B cells. It may have a role in modifying normal B cell development, to facilitate the maintenance of EBV latency and prevent inappropriate activation of the lytic cycle.
EBNA 1	Latency I	EBNA 1 binds the EBV genome to the cell chromosomes, so that the virus is replicated in dividing cells as if it were part of the host genome, thus mediating episomal persistence and maintenance.
EBNA 2	Latency III	EBNA 2 activates viral and cellular target genes which initiates a cascade of events leading to viral cell entry and proliferation of infected B cells. EBNA 2 activates *BFL 1*, a cellular anti-apoptotic gene which offers protection from apoptosis under conditions of depleted growth factor.
EBNA 3A/3B/3C	Latency III	The EBNA 3 family of proteins are transcriptional regulators with EBNA 3C able to upregulate both cellular antigens (CD21) and viral gene expression (LMP 1).
EBNA LP	Latency III	The function of the EBNA LP in B cell transformation is uncertain,however it is needed for the effective growth of LCLs in vitro (Allan et al, 1992).

3.5 EBV associated lymphomagenesis

There is a broad spectrum of EBV associated lymphomas that can be characterized by the pattern of viral latency and the occurrence in immunocompetent or immunocompromized hosts, as summarized in Table 3.3 (El -Bietar & Bollard, 2011; Ok et al, 2014).

Analysis of this table shows that lymphomas with a type III EBV latency pattern will develop only in immunodeficient hosts such as individuals with post transplant lymphoproliferative disorders (PTLD) and in lymphomas associated with HIV/AIDS. EBV associated lymphomas and cancers, in immunocompetent hosts, will show a latency type I or II pattern (table 2.2) (Kutok & Wang, 2006; Cesarman & Mesri, 2007).

Table: 3.3 Latency patterns for selected EBV associated lymphoid malignancies (adapted from El-Bietar & Bollard, 2011).

Disease	EBV Frequency	Latency
Immunocompetent patients		
Hodgkin lymphoma	40%	II
Burkitt lymphoma	20-95%	I
Diffuse large B cell lymphoma	10-35%	II
Angioimmunoblastic lymphoma	> 80%	II
Extranodal NK/T-cell lymphoma, nasal type	>95%	II
Immunosuppressed patients		
Post Hematopoeitic stem cell transplant lymphoproliferative disorder	>95%	III
Post solid organ transplant lymphoproliferative disorder	>95% (70%>1year post transplant	III (I or II if occurs late)
HIV associated NHL		
Primary CNS lymphoma	>95%	III
Primary effusion lymphoma	>90%	I
Diffuse large B-cell lymphoma	30-60%	II or III
Burkitt lymphoma	30-60%	I

The pathogenesis of Burkitt lymphoma provides some insight into the function of EBV in the development of lymphomas. Burkitt lymphoma can be classified into three types which differ in geographic distribution and Epstein Barr virus (EBV) association. The three types are endemic (eBL), sporadic (sBL) and HIV associated BL (Brady et al, 2007) (Table 3.4).

Table 3.4 Geographical distribution and EBV association of the three types of Burkitt lymphoma (adapted from Brady et al, 2007).

	Endemic	Sporadic	HIV associated
Distribution	Equatorial belt of Africa and Papua New Guinea	Worldwide	Worldwide
EBV association	98%	5−10%	30−40%
Co‑factors	EBV, malaria infection	–	HIV infection

Endemic Burkitt lymphoma is the most common paediatric tumour in central Africa and New Guinea and usually occurs in immunocompetent individuals. Most cases show a *MYC* translocation (Kimura et al, 2013). Although EBV is very closely involved in the development of Burkitt lymphoma and other lymphomas, additional factors must be involved causing dysregulation of the *MYC* oncogene characteristic of this tumour. A plausible scenario is one of sustained B cell proliferation, possibly due to a concomitant infection such as malaria. These cells persist indefinitely and acquire specific mutations, most notably the t(8;14) translocation which activates the *MYC* oncogene. Additional mutations then occur which ultimately release the cell from all growth restraints.

In other B cell lymphomas EBV has a more direct involvement. In contrast to Burkitt lymphoma, other EBV associated lymphomas uniformly express LMP 1 and EBNA 2 which drive entry into the cell cycle thus resulting in an initial polyclonal proliferation, with the accumulation of further mutations from which monoclonal neoplasms may emerge.

As regards targeted therapy in tumours with a Type I latency pattern, immunotherapy with specific CD8+ CTLs is likely to be effective (Ok et al, 2014).

Type II latency tumours targeted with therapy against LMP 2 may produce clinical improvement (Ok et al, 2014).

Type III latency tumours associated with a broad expression of latency proteins may prove to be susceptible to micro RNA targeted therapy, EBV vaccines and EBV signaling pathway inhibitors. A combination of EBV lytic phase induction and EBV drugs may prove valuable (Ok et al, 2014) in tumours with this latency pattern.

3.6 Lymphomas and HIV infection

The burden of HIV infected individuals is greatest in South Africa. According to the South African Statistical release P0302: Mid-year population estimates, 2016, 12.7% of the total South African population of 55,91 million are infected with the HIV virus. In the last decade, there has been a significant rise in the frequency of NHL, with HIV being a major contributor to this increase (Patel et al, 2015). Histologically, HIV-NHL typically manifests as a high grade B-cell lymphoma. There are several histological subtypes associated with HIV infection, including diffuse large B-cell lymphoma (DLBL); Burkitt lymphoma (BL); high-grade B-cell lymphoma, with *MYC* and *BCL2* and/or *BCL6* rearrangements, (formerly, B-cell lymphoma, unclassifiable, with features intermediate between DLBL and BL), primary effusion lymphoma, primary diffuse large B cell lymphoma of the central nervous system, KSHV-associated multicentric Castleman disease related large cell lymphoma and plasmablastic lymphoma (Swerdlow et al, 2016; Patel et al, 2015, Carbone et al, 2017). Many of the HIV associated DLBL cases have a high proliferation index and appear histologically aggressive.

Many studies have revealed an association between HIV and EBV infection (Tumwine et al, 2010). In the order of 50% of HIV associated B-cell lymphomas are associated with EBV (Gulley and Tang, 2008). It is suggested that immunosuppression leads to a decrease in T-cell immunity that gives rise to increased proliferation of EBV infected B-cells (Gulley and Tang, 2008). EBV infection persists for life in a human host and the ability to achieve life-long infection is related to the ability of EBV to hide from immune surveillance via a latent B lymphocyte infection in some cells and to actively replicate and shed from other B-cells in a lytic cycle, so providing infective virions to the systemic circulation which are then able to infect other B lymphocytes (Gulley, 2001). In this way, EBV infection contributes to aiding and abetting oncogenesis by facilitating unhindered B lymphocyte replication. The infected B-cells acquire additional mutations conferring malignant behaviour.

Before the onset of highly active antiretroviral therapy (HAART) the frequency of AIDS associated non-Hodgkin lymphoma (AIDS NHL) rose steadily and despite several improvements in treatment, the disease still has a high fatality rate and constitutes a major challenge for oncologists (Jacobson & Abramson, 2012). Since the introduction of HAART, the risk of NHL has substantially declined. Lymphomas that occur in HIV positive patients are mostly aggressive B cell lymphomas and some subtypes (EBV associated DLBL NHL and EBV associated central nervous system NHL) are considered AIDS associated conditions by the U.S. Centers for Disease Control and Prevention (Engels, 2009).

A multistep pathogenesis is suggested in the case of HIV related lymphomas including chronic antigenic stimulation, sustained B cell proliferation, high levels of interleukin (IL)6 and IL10, EBV latent infection and acquisition of some common

genetic abnormalities involving *MYC*, *BCL 6* and some tumour suppressor genes (Raphael et al, 2008).

EBV is identified in the tumour cells of 30-95 % of HIV related lymphomas, although this percentage varies with histological subtype and site, so that EBV occurs in nearly all primary central nervous system (CNS) and PEL lymphomas, in 30-60 % of DLBL and in 30-60% of Burkitt lymphoma (El-Bietar & Bollard, 2011). DLBL arises in the setting of longstanding AIDS with lower CD4+ cell counts, while Burkitt lymphoma occurs in less immunodeficient patients with higher CD4+ cell counts (Raphael et al, 2008).

3.7 Diffuse large B cell lymphoma

Diffuse large B cell lymphoma is defined by the WHO 2008 as a neoplasm of large B lymphoid cells. The nuclei of these cells exhibit a size exceeding the size of a macrophage nucleus and which has a diffuse growth pattern. A number of subgroups and distinct entities are recognized within the group of DLBL. Of this group, DLBL of the elderly, DLBL associated with chronic inflammation, plasmablastic lymphoma, primary effusion lymphoma and lymphomatoid granulomatosis are all associated with EBV infection. However a substantially high number of biologically heterogenous cases of DLBL occur and are classified as diffuse large B cell lymphoma, not otherwise specified (DLBL NOS) (Stein et al 2008). The group of HIV DLBL is also associated with EBV and it is this group of HIV DLBL which forms the cohort currently under investigation.

Collectively, DLBL account for 25-30% of adult NHL with a higher percentage in developing countries. The tumour is more common in the elderly reaching a peak in the 7th decade and showing a slight male predilection. The pathogenesis remains

unknown although underlying immunodeficiency is an important risk factor. EBV is present in only 10% of cases of DLBL occurring in the absence of immunodeficiency. In the presence of HIV, DLBL is more frequently EBV seropositive (Bietar & Bollard, 2011).

Immunophenotypically the tumour cells express one or more pan B cell markers such as CD19, CD20, CD22 and CD79a. Plasma cell markers such as CD38 and CD138 are rarely co-expressed in CD20+cells. CD30 may be expressed in the anaplastic variant of DLBL. CD5 is expressed in 10% of cases and CD10 in 30-60% of DLBL, indicating a germinal centre origin. BCL6 is expressed in 60-90% of cases and MUM 1 in 35-65 % of cases (Stein et al, 2008).

3.8 Therapeutic aspects for EBV associated lymphomas

The oncogenic effects of EBV latent proteins are well known. EBV is associated with a large range of lymphoproliferative and other disorders varying from benign disease to aggressive malignant neoplasms. The oncogenic role that EBV exerts varies in different clinical settings, lymphoma subtypes, ethnicity and in the nature of genomic aberrations (Ghosh et al, 2012).

In general the prognosis of EBV positive tumours is poor when compared to their EBV negative counterparts, especially in the context of concomitant HIV infection (Jacobsen & Abramson, 2012; Ghosh et al, 2012; Park et al, 2007). EBV driven neoplasia is refractory to usual treatment modalities and patient outcome is often poor.

The physical presence of EBV early in the genesis of many lymphoma subtypes represents a potential tumour specific target for various therapeutic approaches.

Such therapies can be divided into three groups viz; pharmacological, immunotherapy, and virus targeted therapy (Ghosh et al, 2012).

In order to continue developing new, effective treatment strategies, information on many aspects of the pathobiology of EBV is essential. These aspects can be summarized as follows (Castillo et al, 2011; Ghosh et al, 2012).

- The EBV viral DNA can be located within tumour cells. This implies that the virus can be used as a biomarker to determine the extent of tumour spread as well as monitor the disease burden in response to EBV targeted therapy
- The frequency of EBV expression in various lymphoma subtypes
- The effect of concomitant HIV infection on expression of EBV proteins
- Latency patterns associated with specific lymphomas
- The effects of pattern shifting in certain EBV associated tumours
- The potential predictive and prognostic value of the EBV viral load
- Switching back and forth from lytic to latent patterns of gene expression which is a major constraint to pharmacological intervention
- High risk individuals may benefit from EBV screening tests which may predict impending progression of EBV disease by the pattern of latency expression
- Detection of the viral gene products and their downstream mechanisms which facilitate cell proliferation, inhibit apoptosis and avoid immune clearance

Several studies have suggested that EBV infection of tumour cells is a prognostic factor that influences survival (Gulley et al, 2002). There is on-going progress in profiling the expression pattern of each EBV associated disease to allow for more precise diagnosis and in time, the development of EBV targeted treatments. Accurate, reliable and reproducible laboratory viral assays are continually improving and are widely used in the diagnosis, prediction of prognosis and prevention of EBV

associated malignant diseases (Gulley & Tang, 2008). Such assays may determine which patients will benefit from targeted treatment and may assist in monitoring the effects of such therapy.

This background led us to consider the extent to which EBV is involved in the development of DLBL in the high prevalence HIV setting of South Africa, and to consider the pattern of EBV latent gene expression in these lymphomas. The intention was to develop a framework for the reliable laboratory assessment of EBV in this group of tumours to improve our understanding of the pathobiology of DLBL and its relationship with EBV in the setting of HIV infection.

Our laboratory setting currently uses two tests in the identification of EBV related diseases. These are the EBER in situ hybridisation (ISH) and LMP 1 immunohistochemistry (IHC).

While the detection of EBER by in situ hybridisation is considered the ‘gold standard’ in determining the presence of EBV diseases in histological tissues, studies have revealed that EBV is present in some EBER negative tumours. This discrepancy has been explained by Ambinder, 2000 as the ‘hit and run’ hypothesis which postulates that segments of EBV DNA or EBV gene products are undetected in rearranged or in intergrated host chromosomal DNA – hence the need for other molecular and immunohistochemical assays to detect other latent gene products involved in the disease and it’s progression.

EBER ISH results can be morphologically interpreted so that the virus can be localised to malignant versus benign cells. The LMP1 immunohistochemistry (IHC) test has the similar advantage of identification of the virus within malignant cells but it is not uniformly expressed in all three EBV disease latency patterns. LMP 1 is

present in latency pattern II and III EBV diseases. Gulley et al (2002) recommended that LMP 1 protein expression should be interpreted with caution due to false positive results that may arise in poorly fixed tissue. The use of LMP 2 detection by polymerase chain reaction was incorporated into this study to offer a parallel test to characterize latency II and III pattern diseases.

To distinguish diseases that express a latency I pattern and a latency III pattern, the detection of EBNA 1 (latency I) and EBNA 2 (latency III) transcripts was developed for use in this study. EBNA 2 and EBNA 3 are consistently co-expressed in the Type III latency pattern. In view of this, it is considered unnecessary to independently establish the EBNA 3 status. The EBNA 2 PCR test used also offers the ability to distinguish between EBV Type 1 and Type 2 diseases identified by polymorphisms in the viral gene encoding EBNA 2.

Further, the EBV lytic cycle is initiated by the transcription of the EBV BZLF1 gene, whose product, the ZEBRA protein is an important factor in the switch from latency to lytic virus replication – therefore PCR detection of BZLF1 transcription was used to distinguish between latent and lytic infection.

CHAPTER 4

4.0 Materials and Methods

4.1 Research plan

In order to meet the objectives listed in Chapter 2, a research plan was devised (Fig 4.1) which is described as follows:

- A study cohort of DLBL which occurred in HIV seropositive patients, using both morphological and immunophenotypical characteristics as defined by the World Health Organisation (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues, Fourth Edition, Jaffe et al, 2008, was identified.
- *EBER* transcripts in these cases using chromogenic in situ hybridisation (CISH) were identified.
- Determination as to whether the EBV infection was in a lytic or latent phase was ascertained by identification of the *BZLF 1* transcripts using Real time polymerase chain reaction (PCR).
- The presence of LMP 1 and EBNA 2 antigens using immunohistochemistry (IHC) were detected.
- The expression of *LMP 2* and *EBNA 1* using Real time PCR were determined.
- Each case was characterised according to its latency pattern of expression, viz:
    - Latency Pattern 1 (*EBNA 1* positive)
    - Latency Pattern 2 (*EBNA 1, LMP 1, LMP 2* positive)
    - Latency Pattern 3 (*EBNA 1, LMP 1, LMP 2, EBNA 2* positive)

- In the latency pattern III cases, a differentiation of the viral isolates to type 1 and type 2 diseases was performed using EBNA 2 nested PCR to detect gene polymorphisms.

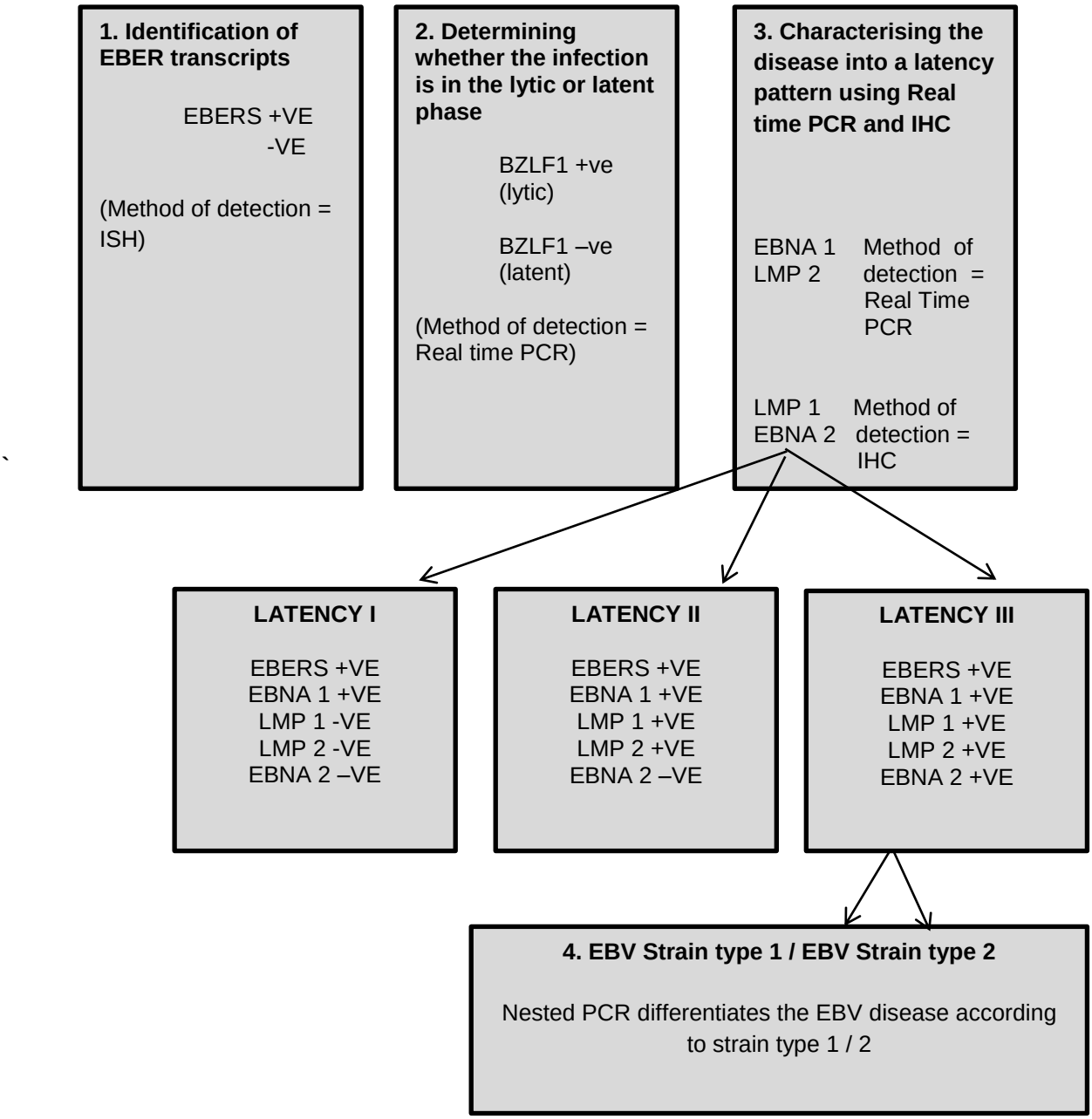


Figure. 4.1 Flowchart of research plan

4.2. Study design

The study design was retrospective, cross-sectional and descriptive.

4.3. Laboratory setting

The study took place in the Molecular Pathology Laboratory of the Division of Anatomical Pathology of the University of the Witwatersrand and the National Health Laboratory Service (NHLS). The patients were mainly from the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) but some had been referred from smaller peripheral hospitals or from neighbouring countries. The histopathology reports, formalin fixed paraffin embedded (FFPE) blocks and slides were subsequently reviewed and appropriate samples of DLBL were selected.

4.4. Study sample

Using the SNOMED classification, the DISA laboratory information system (LIS) and the Trakcare Information system of the NHLS, the Departmental archives were electronically searched and cases of DLBL accessioned during the period January 2010 to June 2016 were identified. The pathology reports were retrieved and the HIV status of the patients was established. Only those cases in which there was a definite history of HIV seropositivity and where the HIV status was recorded on the pathology report were included in the study. This formed the study sample cohort. The archived H&E and IHC slides were reviewed by the supervisor (Dr Y. Perner) in order to confirm the diagnosis. The minimum diagnostic criteria were a diffuse infiltrate of large round to ovoid cells which showed a centroblastic nuclear morphology and a positive CD20 immunophenotype.

4.5. Control sample

The SNOMED classification and the Trakcare Laboratory Information System of the NHLS were electronically searched and cases of HIV positive Burkitt lymphoma, plasmablastic lymphoma and Hodgkin lymphoma were retrieved from 2016 to serve as controls for this study.

4.6 Laboratory Methods

4.6.1 Preparation of the paraffin embedded tissue material for PCR, ISH and IHC

Tissue blocks were cut using new blades for each sample to avoid sample to sample cross contamination. The workbench area and tools used to perform all processes were cleaned with 3% Sodium Hypochlorite between each sample handled. In addition work areas were decontaminated with ultra violet light between each process. 2-4 Sections (10µm) were cut from each formalin fixed paraffin embedded sample for the PCR application. For ISH and IHC, a section from each paraffin embedded tissue block was cut at 4 µm and picked up onto commercially prepared adhesive coated slides (Superfrost®Plus, Fischer Scientific, UK).

To perform DNA extraction from paraffin embedded tissue sections, deparaffinisation was performed by exposing the samples to two washes in xylene for 5 minutes each at room temperature followed by two washes in 100% ethanol, also for five minutes each, to remove the xylene. Tissues were dehydrated on a hotblock at 37°C and an overnight digestion was performed at 56°C in 180 µl of Buffer ATL (tissue lysis buffer) (Qiagen GmbH, Germany) containing 20 mg/ml proteinase K (Qiagen GmbH, Germany). DNA Micro QIA amp kit (Qiagen GmbH, Germany) was used to perform DNA extraction in accordance to manufacturer's instructions.

4.6.2 Validation of laboratory assays

Positive, negative and housekeeping controls used in the various assays are summarised in Table 4.1(a).

Table 4.1(a) Controls used in laboratory assays

	Positive control	Negative control	Housekeeping gene	Blank wax control
ISH Detection of EBER transcripts	Previously diagnosed Hodgkin lymphoma.			
IHC LMP1 detection	Previously diagnosed EBV positive Hodgkin lymphoma.	Achieved by eliminating incubation with primary antibody.		
IHC EBNA 2 detection	Previously diagnosed HIV positive EBV positive plasmablastic lymphoma.	Achieved by eliminating incubation with primary antibody.		
Real time PCR Detection of: BZLF1, EBNA1,LMP1, LMP2	Previously diagnosed EBV positive Hodgkin and Burkitt lymphoma	A no template control in which nuclease-free water is substituted as a template.	The human β-globin gene to determine the integrity of the DNA extraction process.	The blank wax control is a paraffin embedded wax block which contains no tissue material.
Nested PCR EBNA2 detection	Previously diagnosed HIV positive EBV positive plasmablastic lymphoma.	A no template control in which nuclease-free water is substituted as a template.	The human β-globin gene to determine the integrity of the DNA extraction process.	The blank wax control is a paraffin embedded wax block which contains no tissue material.

The procedures used to validate the integrity of the DNA and RNA and the PCR assays (nested and Real time) are described in table 4.1 (b).

Table 4.1(b) Validation procedures for PCR based assays

Assay	Determining the integrity of DNA /RNA extracted using housekeeping genes	Limit of detection	Assay reproducibility and reliability	Confirmation of the PCR products by Sanger Sequencing
PCR	The human *β globin* locus is composed of five genes. Expression of all of these genes is controlled by a single locus control region (LCR), and the genes are differentially expressed throughout the development of a human thus serving as a suitable housekeeping gene to determine the integrity of the DNA yielded from PCR extraction. A Real time PCR assay using melt curve analysis and primers targeting the *β globin* housekeeping gene was employed to control the integrity of extraction and amplification of DNA from paraffin embedded tissue.	The limit of detection was determined by running the assays using 25, 50,100,300 ng/µl total genomic DNA. For Real time PCR reactions, the concentration of DNA template used is 50ng/µl of whole genomic DNA and for Nested PCR a concentration of 300 ng/µl of whole genomic DNA yielded a suitable reaction.	Assay reproducibility and reliability was evaluated on the same DNA sample amplified using five separate reactions. The inter sample reproducibility was evaluated on separate DNA extractions using the positively tested samples.	The PCR amplicons of the nested EBNA 2 reaction was confirmed by Sanger sequencing. Real time PCR products could not be sequenced due to the chemistry of the Taqman probes which interferes with the reagents(Big dye terminator kit) of Sanger Sequencing

4.6.3 Primers and Probes used in the Molecular Assays

The primers and probes that were used in the molecular assays are outlined in Tables 4.2; 4.3 and 4.4. To confirm the specificity of the primers used, a basic local alignment search tool (BLAST) search was done against the NCBI database to ensure that primers utilised only recognise the target of interest.

Table 4.2 Primers for Internal controls

Primer / Probe	Sequence	Amplicon size
β globin Forward primer: PCO4	5'-CAA CTT CAT CCA CGT TCA CC-3'	268 (base pairs) bp
Reverse primer: GH2O	5'-GAA GAG CCA AGG ACA GGT AC-3'	

Table 4.3 Primers for Nested PCR

EBNA2 Typing			
Primer/Probe	Sequence	Base pairs	*EBNA 2 Nucleotide location
EBNA-2F Forward primer (1st Round)	5'- TGG AAA CCC GTC ACT CTC-3'		48572-89
EBNA-2I Reverse primer (1st Round)	5'- TAA TGG CAT AGG TGG AAT G-'3		49355-73
EBNA-2C Forward Nested (2nd Round)	5'-AGG GAT GCC TGG ACA CAA GA-'3		48810-29
EBNA-2G Reverse Nested (2nd Round EBV Type 1)	5'-GCC TCG GTT GTG ACA GAG-3'	250bp	49048-65
EBNA-2B Reverse Nested (2nd Round EBV Type 2)	5'-TTG AAG AGT ATG TCC TAA GG-3'	300bp	2020-39

*Sequence locations for type-1 correspond to B95.8 coordinates (Accession number V01555) and for type-2 to AG876 isolate (Accession number K03332).

Table 4.4 Primers/Probes for Real time PCR

Primer/Probe	Sequence	Base pairs	Nucleotide location#
EBNA1			
Forward primer	5′-TAC AGG ACC TGG AAA TGG CC-3′	78 bp	107970 to 108048#
Reverse primer	5′-TCT TTG AGG TCC ACT GCC G-3′		
Probe	5′-(6FAM)AGG GAG ACA CAT CTG GAC CAG AAG GC(TAMRA)-3′		
LMP2			
Forward primer	5′-AGC TGT AAC TGT GGT TTC CAT GAC-3′	69bp	679 to 748#
Reverse primer	5′-GCC CCC TGG CGA AGA G-3′		
Probe	5′-(6FAM)CTG CTG CTA CTG GCT TTC GTC CTC TGG(TAMRA)-3′		
BZLF1			
Forward primer	5′-AAA TTT AAG AGA TCC TCG TGT AAA ACA TC-3′	91 bp	102214 to 102305#
Reverse primer	5′-CGC CTC CTG TTG AAG CAG AT-3′		
Probe	5′-(6FAM)ATA ATG GAG TCA ACA TCC AGG CTT GGG C(TAMRA)-3′		

Nucleotide # refers to location in the prototypic EBV B95.8 genome sequence (GenBank Accession No. V01555).

4.6.4 EBER chromogenic in situ hybridisation

In situ hybridization (ISH) is a method of localizing and detecting target messenger RNA (mRNA) sequences in morphologically preserved tissue sections or cell preparations. The process involves hybridizing a complementary strand of a nucleotide probe to the sequence of interest.

The EBER chromogenic in situ hybridisation method employed in this study uses a fluorescein labelled oligonucleotide probe which specifically hybridizes to EBER mRNA transcripts in sections of formalin fixed paraffin embedded tissue.

Sections from each paraffin embedded tissue block were prepared as described in section 4.5.1. Slides were incubated at 60ºC for 1 hour to aid adhesion of the section to the slide.

Chromogenic in situ hybridization for EBV encoded RNA (EBER) was performed using the INFORM® EBER Probe, (Ventana Medical Systems, Arizona) and stained on the Ventana BenchMark XT system (Roche, Germany) according to manufacturer's instructions. The INFORM® EBER probe targets the early RNA transcript of Epstein Barr virus (EBV) infection. Enzymatic digestion was accomplished using ISH Protease 2 (Ventana, Tucson, Arizona, United States). ISH Protease 2 is an endopeptidase (alkaline protease) of the serine protease family which is used in the in situ hybridisation process to permeabilise cell membranes and remove protein that surrounds the target DNA or RNA sequences of interest, (http://www.ventana.com/product/1366?type=1743). This protocol uses heat-induced epitope retrieval Cell Conditioning 1(CC1) solution, (Ventana Tucson, Arizona, United States) and the iView DAB detection kit (Ventana, Tucson, Arizona, United States) which is an indirect biotin streptavidin system for detecting fluorescein labelled probes. ISH iVIEW Blue Detection Kit utilises an alkaline

phosphatase enzyme and NBT/BCIP (Nitro blue tetrazolium chloride/ 5-bromo- 4-chloro- 3 idolyl-phosphate) substrate chromogen reaction, which yields an intense, blue, permanent colour deposition at the site of probe target interaction that is readily visualised by light microscopy. The Red Counterstain (Ventana, Tucson, Arizona, United States) provides a pale pink background staining. Liquid coverslip solution (LCS) (Ventana, Tucson, Arizona, United States) was used at each step of the staining reaction to act as a barrier between the aqueous reagents and the air and to prevent evaporation, thereby providing a stable aqueous environment for the in situ hybridisation reaction. After the ISH staining, slides were washed in a mild detergent to remove any residual LCS buffer, rinsed in running tap water, dehydrated in alcohol, cleared in xylene and mounted in Entellen (Merck® Darmstadt, Germany) mounting medium. Sections were reviewed with the supervisors under light microscopy.

4.6.5 Immunohistochemical detection of LMP 1 and EBNA 2 proteins.

Immunohistochemistry (IHC) specific antigen/antibody reactions are tagged with a visible label making it possible to visualize the distribution and localization of specific cellular antigens within a tumour using conventional light microscopy.

EBNA 2 immunohistochemistry staining was performed on the Ventana Benchmark XT autostainer (Roche, Germany). The anti EBNA 2 antibody (PE2) clone that was used for staining is directed at both type 1 and type 2 strains. The process of automated staining using the Benchmark XT can be summarised with the flow diagram represented in Figure 4.2.

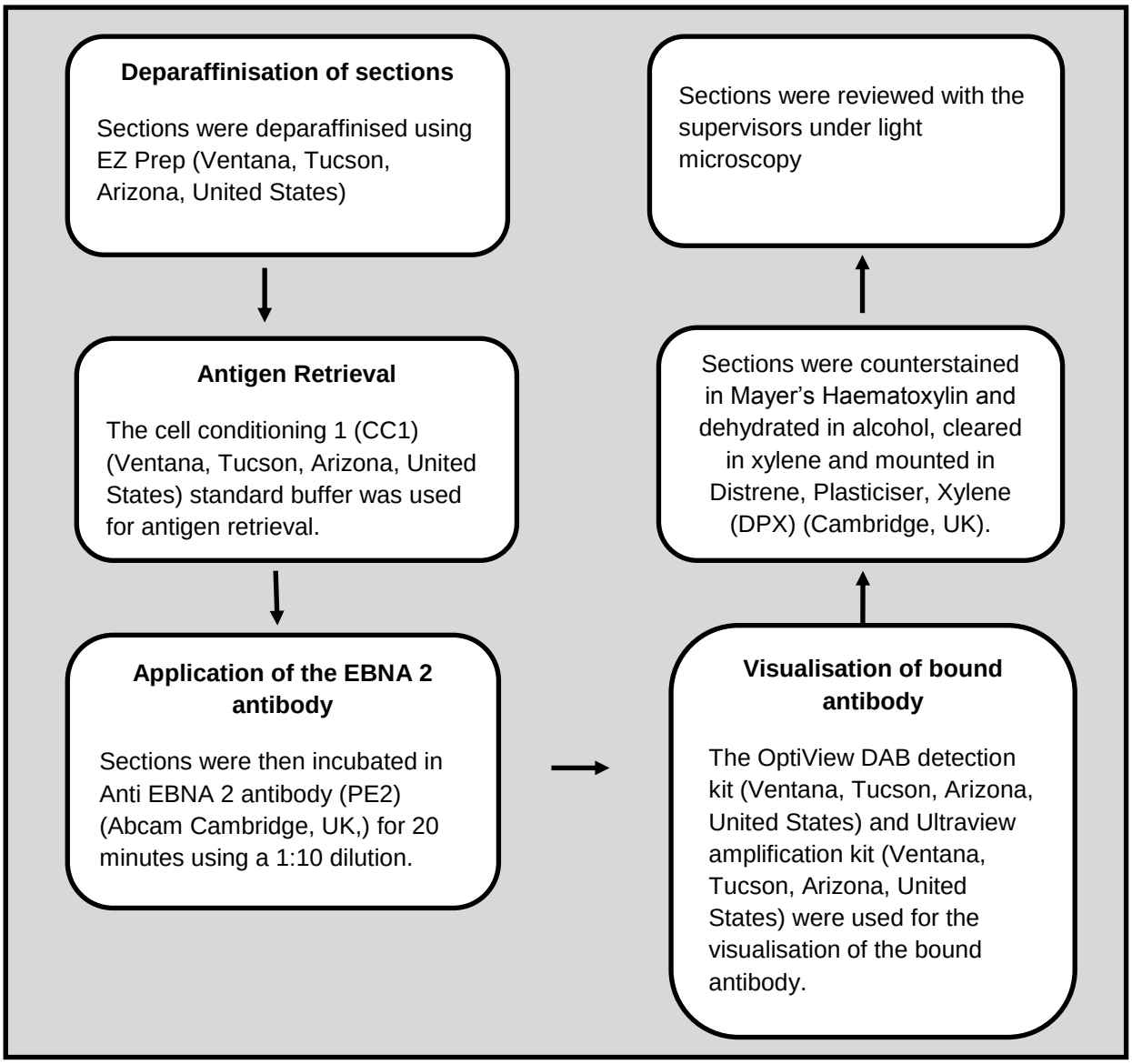


Figure 4.2 Flow diagram illustrating the staining of sections using the EBNA 2 antibody using the Ventana Benchmark XT autostainer

LMP 1 immunohistochemistry staining was performed on the DAKO autostainer Link (Dako, Carpinteria, CA). This staining procedure is illustrated in Figure 4.3.

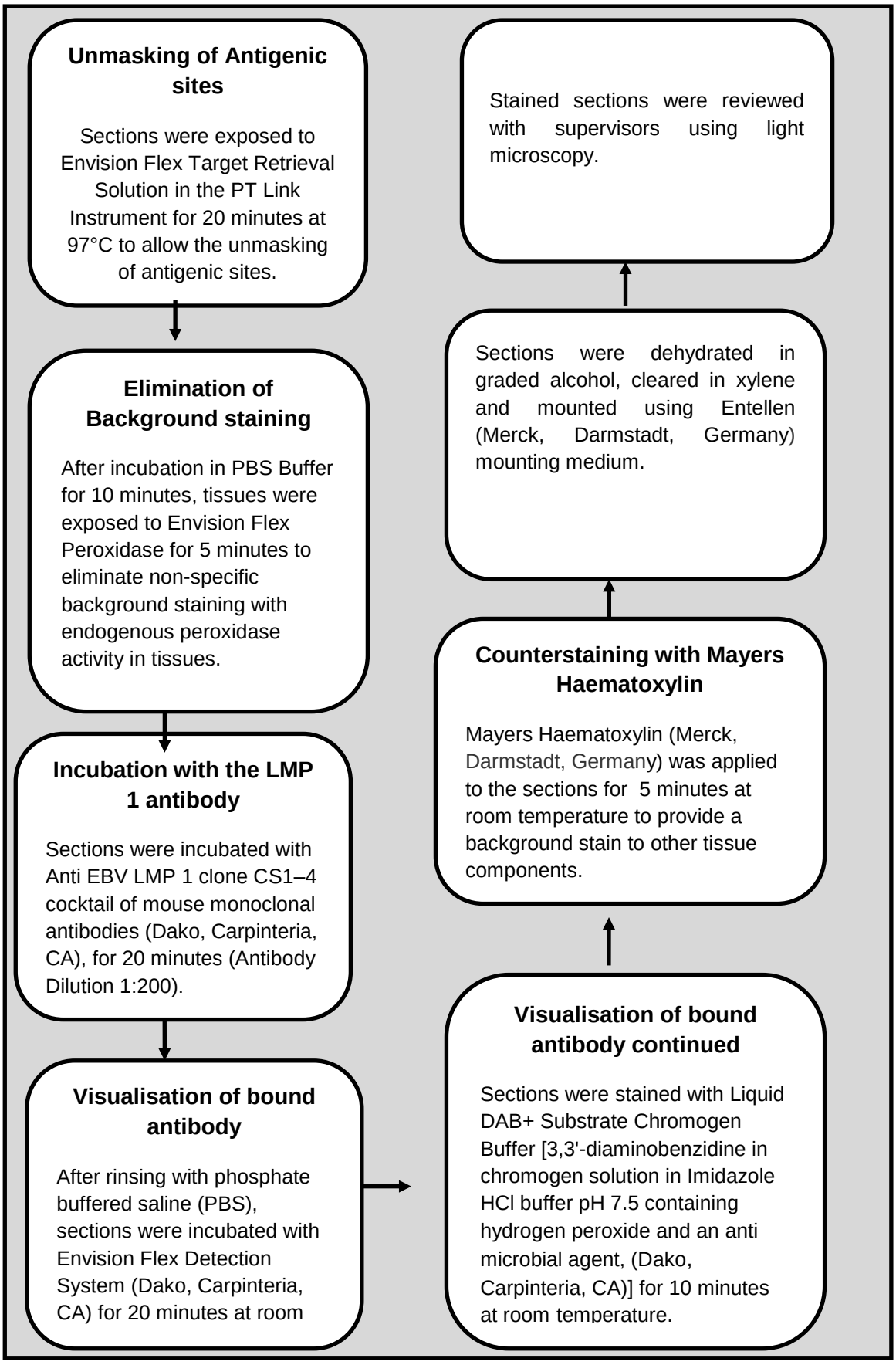


Figure 4.3 Schematic representation of the IHC staining procedure for LMP 1

4.6.6 Qualitative Real time PCR for the detection of *BZLF 1, LMP 1, LMP 2* and *EBNA 1* using Taqman Real time assay

For Real time PCR a Taqman assay was used. Taqman assays use the 5' nuclease PCR assay. The 5' nuclease assay has the ability to degrade DNA bound to a template, downstream of DNA synthesis. This assay uses a phenomenon called fluorescent resonance energy transfer (FRET) where the fluorescence emitted by one fluorescent dye can be strongly reduced by the presence of another dye (quencher dye) in close proximity to it (Basics of Real time PCR, lifetechnologies.com, 2012). To illustrate this phenomenon consider two dyes viz green and red (Fig 4.4). The green dye has a higher energy of emission compared to the red dye because green light has a shorter wavelength than red light (green light wavelength = 495–570 nm, photon energy = 2.17–2.50 electron volt (eV). Red light wavelength = 620–750 nm, photon energy = 1.65–2.00 eV). If the red light is in close proximity to the green light, energy will be transferred from a higher (green dye) to a lower level (red dye) when the green dye is excited (Fig 4.4) This causes the green signal to be suppressed (quenched). No transfer of energy will occur if the dyes are not in close proximity to each other (Real- time PCR Handbook, Lifetechnologies.com, 2012) (Fig 4.4 B).

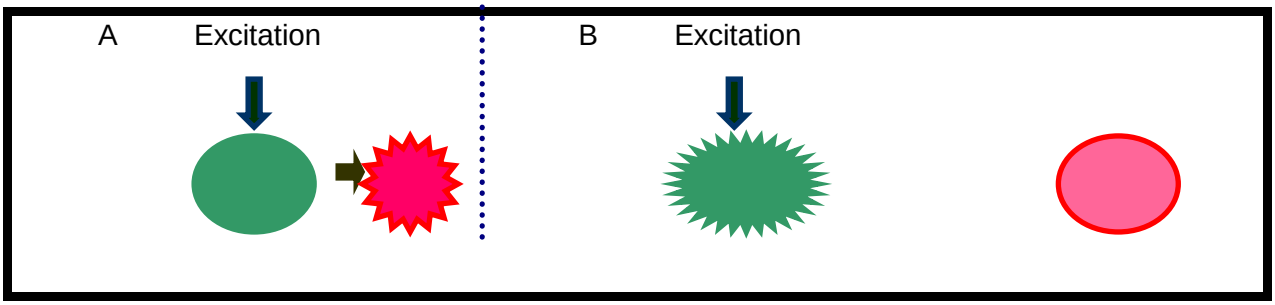


Figure 4.4. The FRET phenomenon. (A) FRET phenomenon occurs when a green light emitting fluorescent dye is in close proximity to a red light emitting fluorescent dye. (B) FRET does not occur when two fluorescent dyes are not in close proximity to each other.

Taqman assays consist of two primers and a fluorescent labelled taqman probe (consisting of a reporter signal and a quencher signal, designated R and Q in Fig 4.5 below). Before the PCR reaction occurs, the probe is intact. Both signals have an affinity for each other and lie in close proximity to each other which allows FRET to take place. During PCR the primers and probe anneal to the target sequence (Fig 4.5).

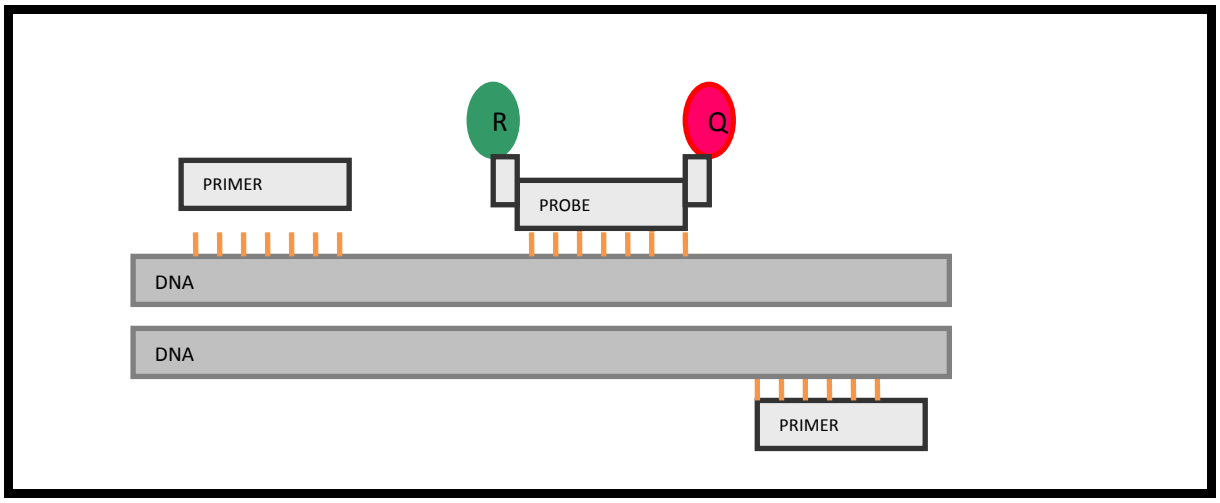


Figure 4.5 The taqman probe is designed to bind the target sequence between the two primers.

Using the FRET phenomenon, the quencher molecule (Q) quenches the fluorescence emitted by the reporter molecule (R) when the reporter is excited by

the thermocycler's light source. As long as the reporter and the quencher are in proximity, quenching inhibits any fluorescence signals and hence visualisation. During PCR, the primers and probe anneal to the target sequence (Fig 4.3) and the DNA polymerase (enzyme) extends the primer upstream (5'-3') of the probe. If the probe is bound to the correct sequence, the probe is cleaved to the target sequence and the fragment containing the reporter dye is released. Once this cleavage takes place the reporter and quencher dye are no longer attracted to each other, the released reporter fluorescence will no longer be quenched and will then emit fluorescence. As polymerisation takes place an increase in fluorescence will be seen and is visualised using the Real time platform software.

For this study a Real time assay using the Taqman Universal master Mix (Life Technologies, California) was performed according to manufacturer's instructions. The primers and probes (table 4.4) used in the reaction were designed for target genes viz*, LMP2, EBNA1,* and *BZLF1* (Integrated DNA Technologies, South Africa) as described by Ryan et al, 2004. PCR was performed and products were detected using a Rotorgene 6000 Real time PCR instrument and Rotorgene 6000 Detection System software. Thermocycling conditions are described in Table 4.5.

Table 4.5. Thermal profile of the Real time assay to detect *LMP2, EBNA1,* and *BZLF1* using the Rotorgene 6000.

	Temperature	Time
Hold 1	50°C	2 minutes
Hold 2	95°C	10 minutes
Amplification 40-50 cycles	95°C	15 seconds
	60°C	1 minute

The preparation of the Master Mix for this reaction is described in Table 4.6.

Table 4.6 Master Mix preparation

PCR reaction	1X
1X TaqMan Universal Master Mix	12.5 µl
Forward primer (15 ρmol)	2.78 µl
Reverse primer (15 ρmol)	2.78 µl
TaqMan probe (10 ρmol)	1.85 µl
Water	3.09 µl
Total	23.0 µl
Sample	2.0 µl

Each run contained at least two "no template" controls in which nuclease-free H_2O was substituted for template to ensure that the run was free of contamination. The PCR amplicons were further run on a 3% agarose gel using a 50bp molecular marker to confirm their base pair sizes as indicated in Table 4.4 (Ryan et al, 2004).

4.6.7 Typing the EBV disease according to strain differentiation (type 1/ type 2) using nested PCR

To further classify the disease as a type 1 or type 2 strain which involves the detection of the polymorphisms in the viral gene encoding the EBV nuclear antigen 2 *(EBNA 2*), a qualitative nested PCR test was incorporated. The nested PCR allows the distinction of the two types of EBV infection (Hassan et al, 2006).

Nested PCR is a modification of standard PCR that minimises non-specific primer binding due to the amplification of unintended primer binding sites (mispriming) and this improves the specificity of the reaction. In nested PCR two pairs of PCR primers are used to target a single locus. The first pair amplifies the locus as seen in any

PCR experiment. The second pair of primers (nested primers) bind within the first PCR product and produce a second PCR product that will be shorter than the first one. Nested PCR ensures that if the wrong locus is erroneously amplified, the probability is very low that it will be amplified a second time by a second pair of primers.

EBV genotyping was performed using primers (Integrated DNA Technologies, South Africa) as described by Hassan et al, 2006. The first PCR reaction amplified a common region of *EBNA 2* followed by two separate nested reactions amplifying distinctive regions (Table 4.3). The specificity of the PCR amplicons were confirmed using the Sanger sequencing technology and were aligned to reference sequences (ascension numbers V01555 and K03332.) obtained from NCBI (https://www.ncbi.nlm.nih.gov)

4.7 Data Analysis

The patient age, gender and site of the tumour were obtained from the Pathology report forms according to the clinical history as supplied by the clinicians submitting the biopsy. No patient hospital files were accessed. The histological diagnosis, morphology of the tumour and immunohistochemical profile were taken from the histology report as issued by the pathologist. The original sections were reviewed. Only those cases recording a definite positive HIV result and a morphology and immunophenotypic profile which was consistent with that seen in DLBL were included in the study. This data was recorded on an excel spreadsheet and analysis were conducted in R (R Core Team (2013) software: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

URL http://www.R-project.org/, the results of which are displayed in Table 5.2 on page 48 of Chapter 5. For statistical purposes, any equivocal results were interpreted as negative. All laboratory assay data were examined relative to the EBER ISH results since EBER ISH is the 'gold standard' for the demonstration of EBV diseases in paraffin embedded tissue sections. In other words, in the presence of adequate controls, the expectation would be that the EBER ISH positive cases might show positive assay results for the other viral proteins while the EBER ISH negative assays would not to be positive for any of the other viral proteins.

In situ hybridisation

The sections were examined by the student and an experienced pathologist using conventional microscopy. Cells positive for EBER mRNA transcripts showed a positive dark blue homogenous signal in the nuclei of the tumour cells. The positive signal may be present in few, a moderate number or in many cells. A tumour was defined as EBER positive if the nuclear signal was located in more than 5% of cells with a malignant morphology (Ziarkiewicz et al, 2016). If the tumour was only expressed in benign appearing cells, the sample was reported as EBER negative.

Immunohistochemistry

The sections were examined by the student and an experienced pathologist using conventional light microscopy. A positive reaction appeared as a granular, intense, golden brown staining of the cytoplasm and cytoplasmic membrane in the case of LMP 1 and of the nucleus with EBNA 2.

Real time PCR

Real time PCR results were assessed by the student in consultation with the supervisor. A positive result was demonstrated by a positive amplification curve with a cycle threshold (CT) value >15 and <36.

Nested PCR

Nested PCR results were assessed by the student in consultation with the supervisor. A positive result was demonstrated as an amplified band (within the correct molecular size range), assessed by gel electrophoresis.

4.8 Statistical Analysis

The study design aimed to determine the EBV latency pattern of gene expression in a sample of HIV positive patients with DLBL. For normally distributed data mean was used as a measure of centre and SD as a measure of variation. Differences in means were assessed using a two tailed t test with a p-value of < 0.05 indicating a significant difference. The validity of these tests was measured by the sensitivity and specificity tests using the EBER ISH test as the 'gold standard' test. Prevalence refers to the frequency of the disease in the total sample cohort. Prevalence was calculated as follows:

Prevalence: **(A+C)/N**

{where A = Total no of positive cases, C = false negatives and N= total sample population}

Sensitivity refers to the proportion of positive cases that can be detected by the test. Sensitivity is a measure of the reliability of the test when tested on positive individuals.

Specificity refers to the proportion of negative cases that can be reliably detected by the test. In other words, specificity measures the efficacy of the test when used on negative individuals.

Sensitivity and specificity were calculated as :

Sensitivity =A/(A+C) x 100

{where A = True Positive and C = False Negative }

Specificity = D/(D+B) x 100

{where D = True negative and B = False Positive }

Laboratory assay data and patient demographics were conducted in R (R Core Team (2013) software: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL, http://www.R-project.org/.

4.9 Ethics Clearance

The Department has blanket approval for retrospective studies on archived pathology reports, wax blocks and slides. Ethics clearance for this specific project was also received from the Human research ethics committee – Clearance certificate no. M140273.

CHAPTER 5

5.0 Results

The patient demographic details, site of the lesion and results of laboratory assays are presented in Table 5.1 and Table 5.2. The study sample consisted of 46 patients, all with a diagnosis of DLBL as ascertained from the patient histology report and confirmed by case review.

5.1 Morphology and immunophenotype

All DLBL tumours showed morphological features consistent with a diagnosis of DLBL. They consisted of diffuse sheets of infiltrating large round centroblasts with large nuclei and between 3–5 acidophilic nucleoli, often seen in apposition to the nuclear membrane. Mitoses ranged from 2-6 per single high power field. The centroblasts contained moderate amounts of amphophilic to pale eosinophilic cytoplasm. Apoptosis was generally inconspicuous (Fig 5.1).

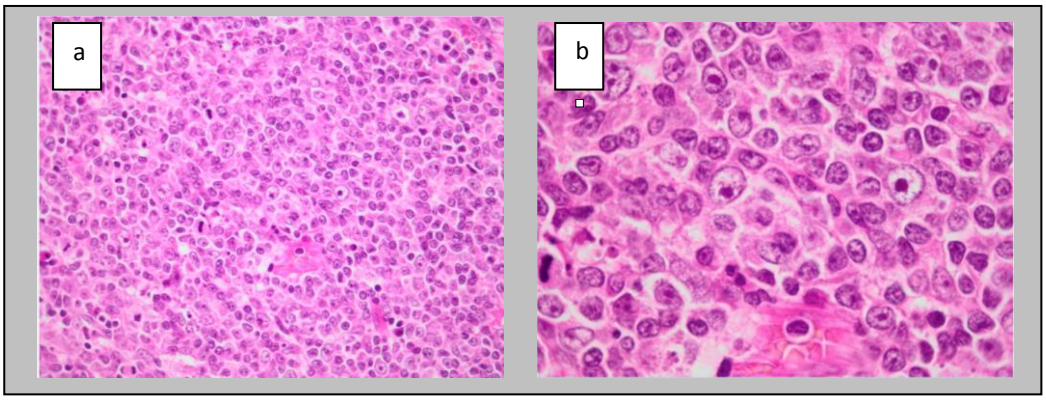


Figure 5.1 H&E stained section of a B-cell tumour showing morphological features consistent with that of DLBL. a. Magnification 40x. b. Magnification 100x

Immunohistochemistry showed consistent strong cytoplasmic membrane staining for LCA and CD20 (Figs 5.2a; 5.2b). There were variable numbers of interspersed reactive T lymphocytes (Fig 5.2c).

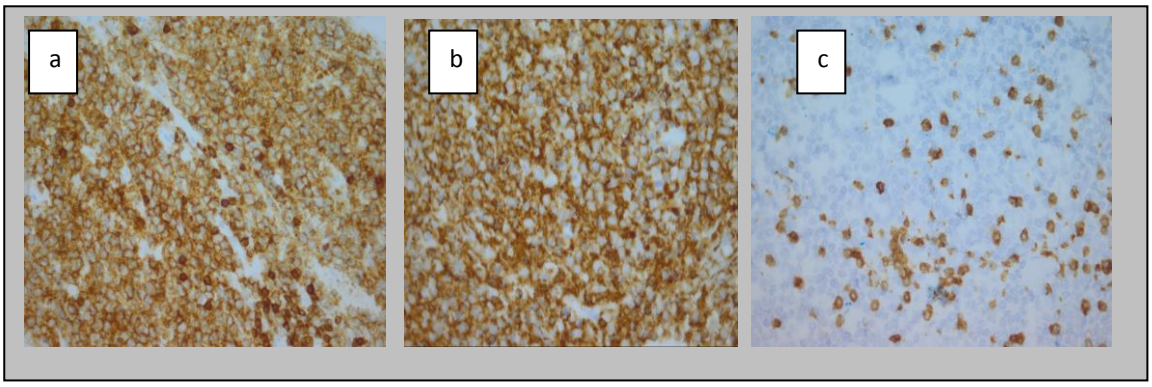


Figure 5.2 a. A strong positive membranous reaction for LCA (magnification 40x). b. CD20 confirming the B cell lineage of the tumour (magnification 40x). c. The tumour cells did not stain with CD3 but the background reactive cells showed a strong staining pattern (magnification 40x).

5.2 Age and Gender

The age distribution of the patients is shown graphically in Fig 5.3 with the majority of the patients being in the 4th and 5th decades. The age range was 17 to 71 years with a mean of 40, 6 (SD +/-10.7) years.

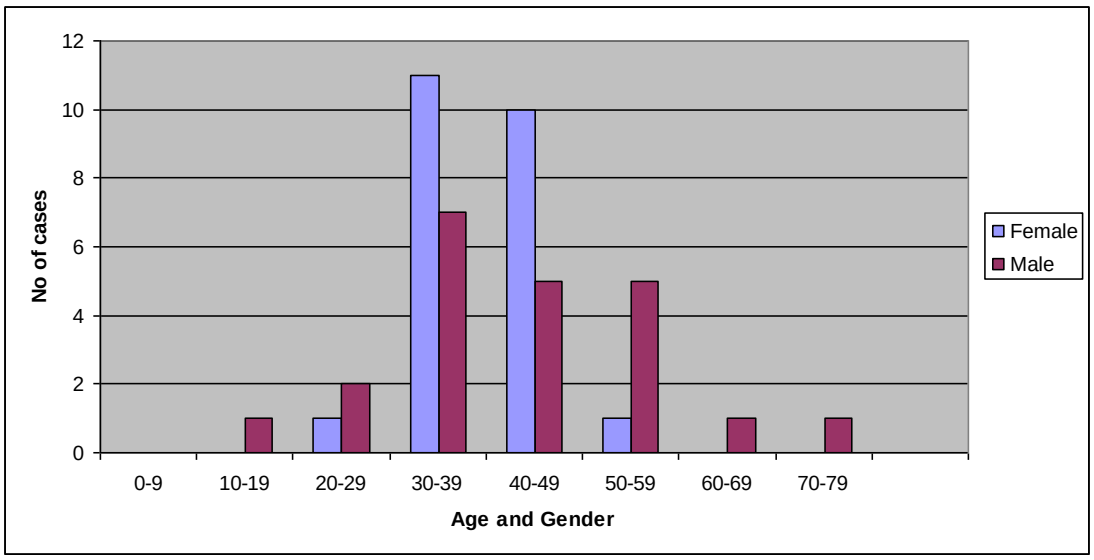


Fig 5.3 Age and Gender distribution of patients

There was no significant difference in the mean ages of the males (41.8 SD +/-14.0 years) and the females (39.5 SD +/- 6.3 years) (p= 0.48, two tailed t-test). In one patient the age was not known. There were equal numbers of males (23) and females (23) in the sample.

5.3 Site of biopsies

The biopsies were taken from many different anatomical sites including endometrium, skin and stomach. Most were found to have originated within lymph nodes (26/46; 57%) while the remainder arose in extranodal locations (18/46; 39%) (Table 5.1). In 2 patients (4%) the biopsy site was not known.

Table 5.1 Site distribution of the tumours

Site of biopsies	**No.**
Nodal	26
Extranodal: Skin	2
Soft tissue	9
Endometrium	1
Gastric	1
Distal femur	1
Tonsil	1
Oral cavity	2
Respiratory tract	1
Site unknown	2

5.4 HIV status

All patients tested positive for HIV using a standard ELISA (Enzyme-Linked Immunosorbent Assay) test, as ascertained from clinical information provided in the histology reports.

5.5 Laboratory assays

The raw data was analysed with the R Core Team (2013) software. A summary of the laboratory raw data is provided in Table 5.2.

Table 5.2 Results of laboratory assays and patients demographics

	Number of cases=46	Percentage of cases
Gender		
Male	23	50%
Female	23	50%
Age		
Range (yrs)	17-71	
Mean (+/- SD)	40.6 (10.7)	
Topography		
Nodal	26	57%
Extranodal	18	39%
Unknown	2	4%
EBER ISH		
Positive	9	20%
Negative	37	80 %
BZLF 1 PCR		
Positive	10	22%
Negative	36	78%
EBNA 1 PCR		
Positive	11	24%
Negative	35	76%
LMP 2 PCR		
Positive	8	17%
Negative	38	83%
LMP 1 IHC		
Positive	7	15%
Negative	39	85%
EBNA 2 IHC		
Positive	3	7%
Negative	43	93%
EBNA 2 PCR		
Positive	4	9%
Negative	42	91%

The consolidated laboratory assay data is summarised in Table 5.3.

Table 5.3 Consolidated data showing positive assay results with reference to EBER ISH positive and negative results

EBER ISH positive cases (9)							EBER ISH negative cases (37)						
BZLF1 PCR	*EBNA1* PCR	EBNA2 IHC	LMP1 IHC	*LMP2* PCR	*EBNA2* PCR TYPE1	*EBNA2* PCR TYPE2	*BZLF1* PCR	*EBNA1* PCR	EBNA2 IHC	LMP1 IHC	*LMP2* PCR	*EBNA2* PCR TYPE1	*EBNA2* PCR TYPE2
9	8	3	7	6	2	2	1	3	0	0	2	0	0

Such an approach identified a number of cases with aberrant or incongruent results which are shown in Table 5.4. These will be commented on further in the discussion.

Table 5.4 Cases which showed aberrant results

No.	Dx	HIV Status	Sex	Tissue	Age	EBER ISH	BZLF1 PCR	LMP2 PCR	LMP1 PCR	EBNA1 PCR	EBNA2 IHC	EBNA2 TYPE1	EBNA2 TYPE2	LATENCY PATTERN		
														1	2	3
11	DLBL	P	M	Extrano	30	N	N	P	N	N	N	N	N			
18	DLBL	P	F	Nodal	45	P	P	P	N*	P	N*	N	P			√
20	DLBL	P	M	Nodal	49	N	N	N	N	P	N	N	N			
26	DLBL	P	F	Nodal	45	P	P	N	P	P	N	N	N		√	
30	DLBL	P	M	Nodal	27	P	P	N	P	P	N	N	N		√	
31	DLBL	P	M	Nodal	35	N	N	P	N	P	N	N	N			
35	DLBL	P	M	Nodal	58	N	P	N	N	P	N	N	N			
53	DLBL	P	F	Nodal	46	P	P	N	P	N	N	N	N		√	
57	DLBL	P	M	Extrano	40	P	P	P	N	P	P	N	P			√

N=Negative P= Positive
***These were reported as equivocal but interpreted as negative for the purposes of statistics**

5.5.1. EBER ISH

A positive hybridisation reaction was defined as a dark purple/blue, circumscribed homogenous colouration of the nucleus which occurred in more than 5 % of the tumour cells. The percentage of EBER positive cells in our cases varied from 5-90%. The staining intensity varied from a weak nuclear signal, in which the nucleoli

were clearly visible to a dense staining pattern which obliterated the outline of the nucleoli completely (Fig 5.4).

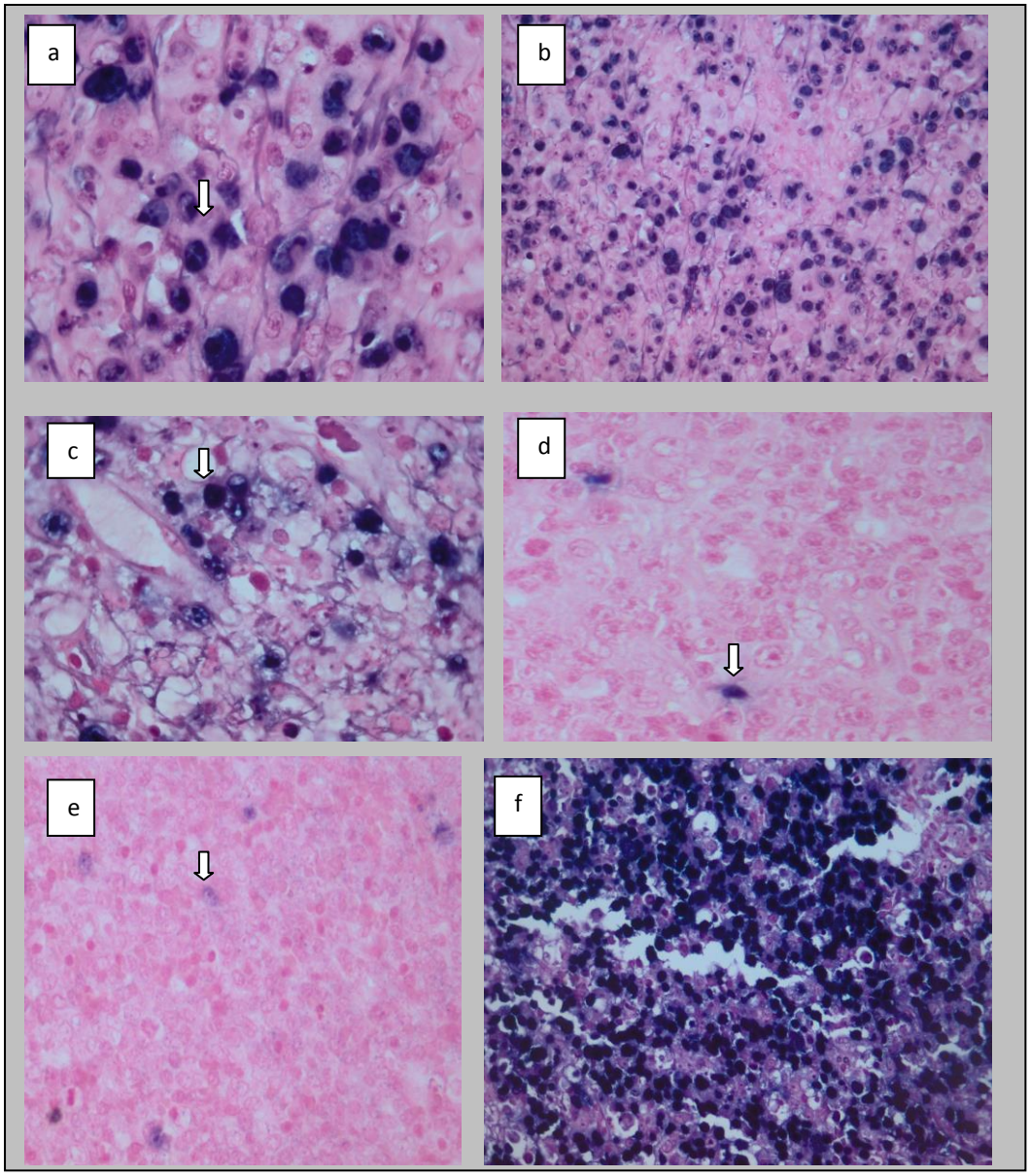


Figure 5.4. Cases demonstrating positive EBER ISH staining

a) Case 29 (magnification 100x) An EBER ISH positive tumour demonstrating cells with clear nuclear visibility
b) Case 29 (magnification, 40x) A diffuse EBER ISH staining pattern of tumour cells
c) Case 54 (magnification 100x) EBER ISH positivity, demonstrating some cells with a dense staining pattern obliterating the outline of the nuclei
d) Case 20 (magnification 40x) An EBER negative case showing isolated positive reactive cells
e) Case 26 (magnification 40x) EBER ISH staining in +/-5% tumour cells
f) Case 10 (magnification 100x) EBER ISH showing 90% staining within tumour cells

Nine cases (20%) were regarded as being EBER positive and 37 cases (80%) were EBER negative (Table 5.2). In the positive cases, tumour cells demonstrated a positive staining expression in up to 90% of cells (Figs 5.4 f). In the EBER negative cases, it was possible to discern isolated scattered positive cells but these were in reactive bystander cells, not in tumour cells (Fig 5.4 d).

5.5.2 BZLF 1 (PCR)

BZLF1 was consistently expressed in all 9 EBER positive cases. One EBER negative case (case 35) also showed BZLF1 expression (Table 5.2). Positive allelic amplification for BZLF1 occurred at 25-35 cycles (Fig 5.6). Gel electrophoresis confirmed the amplicon to consist of a 91 base pair (bp) product consistent with BZLF1 expression as described by Ryan, 2004 (Fig 5.5).

Table 5.5 The expression of BZLF 1 in the study cohort, relative to EBER expression

	BZLF1 POSITIVE	BZLF1 NEGATIVE	TOTAL
EBER POSITIVE	9	0	9
EBER NEGATIVE	1	36	37
TOTAL	10	36	46

Our study showed a 100% sensitivity and a 97% specificity with the BZLF1 expression (Table 5.5). The calculation for sensitivity and specificity is given in the Materials and Methods, page 48.

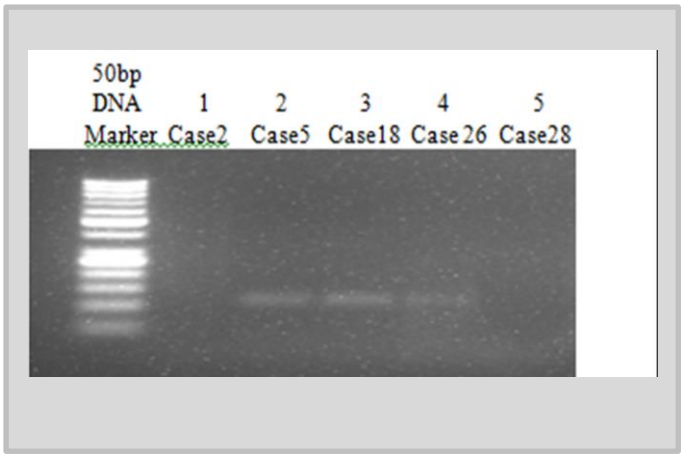
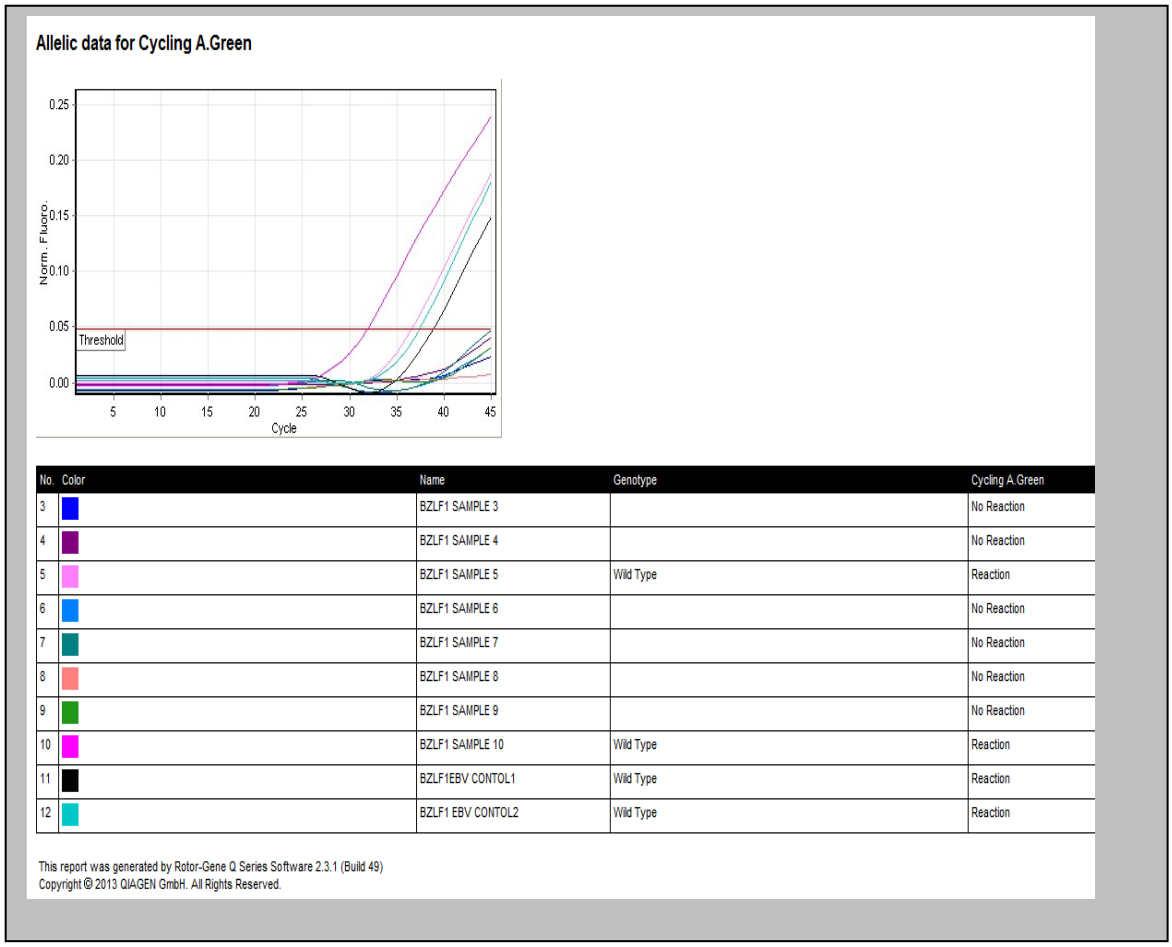


Figure 5.5 BZLF1 PCR amplicons



No.	Color	Name	Genotype	Cycling A.Green
3		BZLF1 SAMPLE 3		No Reaction
4		BZLF1 SAMPLE 4		No Reaction
5		BZLF1 SAMPLE 5	Wild Type	Reaction
6		BZLF1 SAMPLE 6		No Reaction
7		BZLF1 SAMPLE 7		No Reaction
8		BZLF1 SAMPLE 8		No Reaction
9		BZLF1 SAMPLE 9		No Reaction
10		BZLF1 SAMPLE 10	Wild Type	Reaction
11		BZLF1EBV CONTOL1	Wild Type	Reaction
12		BZLF1 EBV CONTOL2	Wild Type	Reaction

This report was generated by Rotor-Gene Q Series Software 2.3.1 (Build 49)

Figure. 5.6 Allelic discrimination curves of PCR reactions for samples 3-10 produced by the Rotorgene 6000 software for the detection of *BZLF1*

5.5.3 EBNA 1 (PCR)

Eight of the 9 EBER positive cases also showed positive expression for EBNA 1, while EBNA 1 expression also occurred in 3 of the EBER negative cases.

Table 5.6 The expression of EBNA 1 in the study cohort, relative to EBER expression

	EBNA 1 POSITIVE	EBNA 1 NEGATIVE	TOTAL
EBER POSITIVE	8	1	9
EBER NEGATIVE	3	34	37
TOTAL	11	35	46

In this study the EBNA 1 test showed a sensitivity of 88.89% and a specificity of 92.5%. Amplification was seen to occur at a threshold cycle (Ct) value of 24-35 cycles (Fig 5.7).

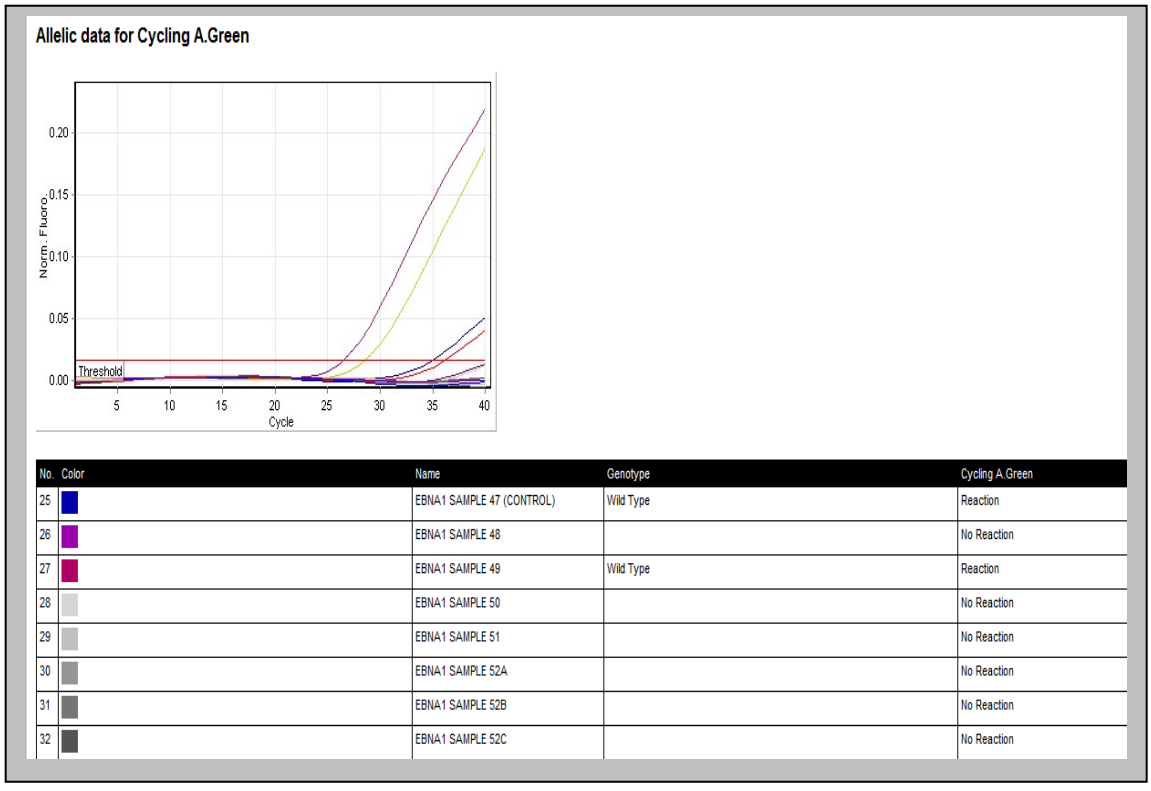


Figure 5.7 Allelic discrimination curves for samples 47-52 produced by the Rotorgene 6000 software for the detection of EBNA1.

Gel electrophoresis demonstrated the EBNA 1 PCR amplicon to consist of a 78 bp product (Fig 5.8).

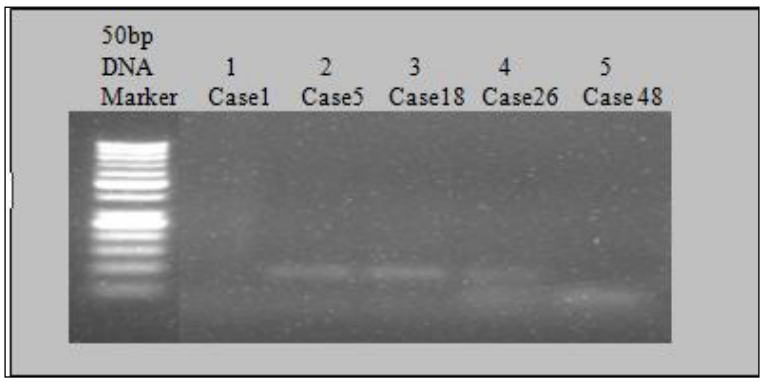


Figure. 5.8 EBNA 1 PCR amplicons

5.5.4 LMP *2* (PCR)

Six of the 9 EBER positive cases also showed positive LMP 2 expression and two EBER negative cases expressed a positive LMP 2 amplification. The Ct value ranged from 20-35 cycles (Fig 5.10). To validate this test, positive amplicons were electrophoresed on a gel, (Fig 5.9) where a 69 bp product was confirmed.

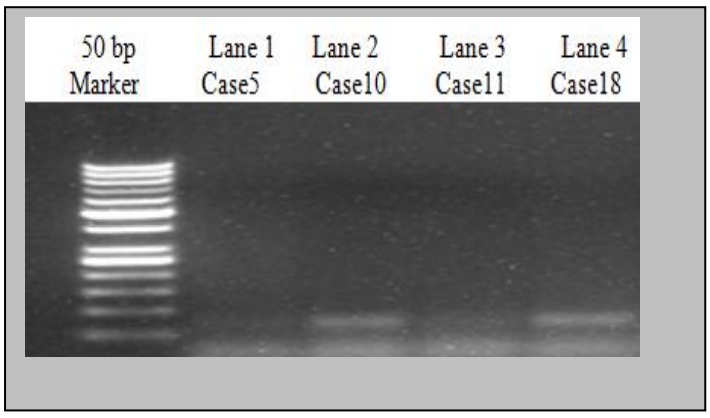


Figure. 5.9. LMP 2 positive amplicons (cases 5, 10, 11, 18), 69bp

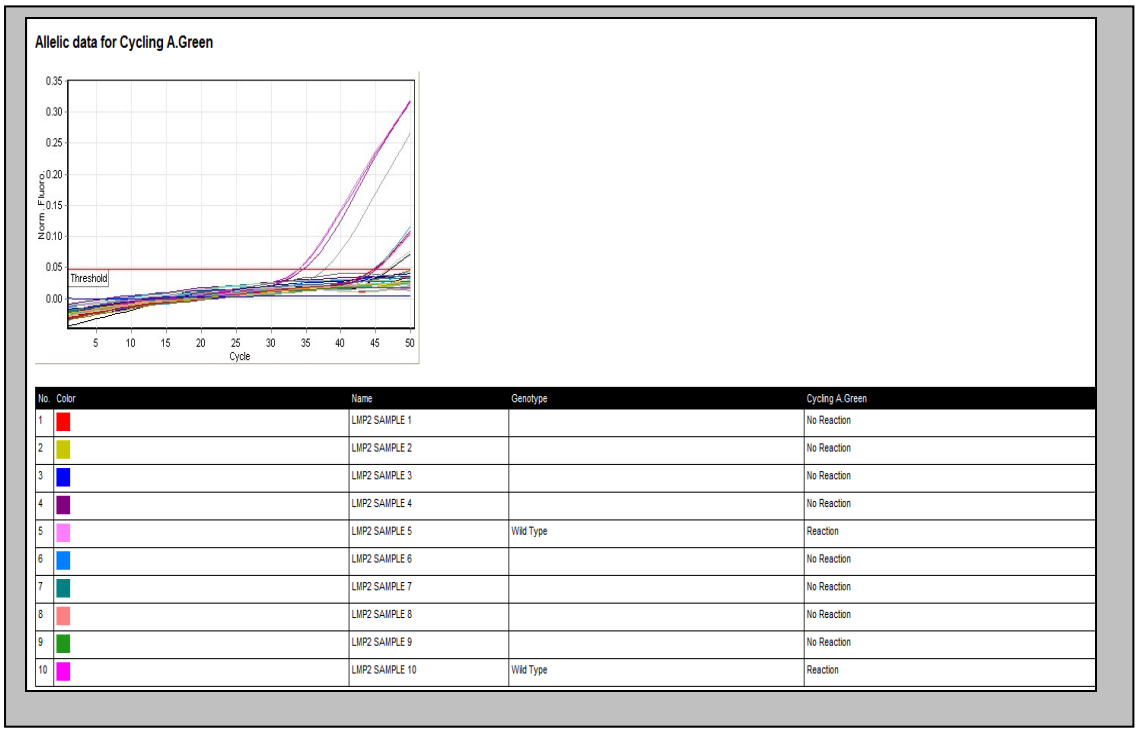


Figure. 5.10 Allelic discrimination curves for samples 1-10 produced by the Rotorgene 6000 software for the detection of LMP 2

Table 5.7 The expression of LMP 2 in the study cohort, relative to EBER expression

	LMP 2 POSITIVE	LMP 2 NEGATIVE	TOTAL
EBER POSITIVE	6	3	9
EBER NEGATIVE	2	35	37
TOTAL	8	38	46

Overall the LMP 2 test demonstrated a 66.67% sensitivity and a specificity of 94.74% (Table 5.7).

5.5.5 LMP 1 (IHC)

Of the 9 EBER positive cases, 7 demonstrated positive LMP 1 IHC staining (Fig 5.11).

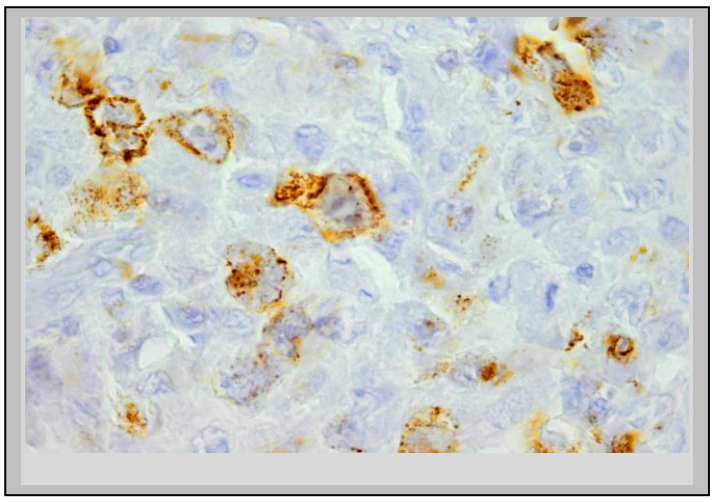


Figure 5.11 Case 54 (magnification 100x). LMP 1 IHC staining

One case, LMP1 was negative and another (case 18) showed a very faint signal which was interpreted as an equivocal signal.

In this study, the LMP 1 showed a 94.74% sensitivity and a 100% specificity.

5.5.6 EBNA 2 (IHC)

Only 3 of the 9 EBER positive cases showed positive EBNA 2 IHC staining. A conclusive identification of the EBNA 2 signal could not be identified on case 18 and the EBNA 2 IHC results were reported as equivocal.

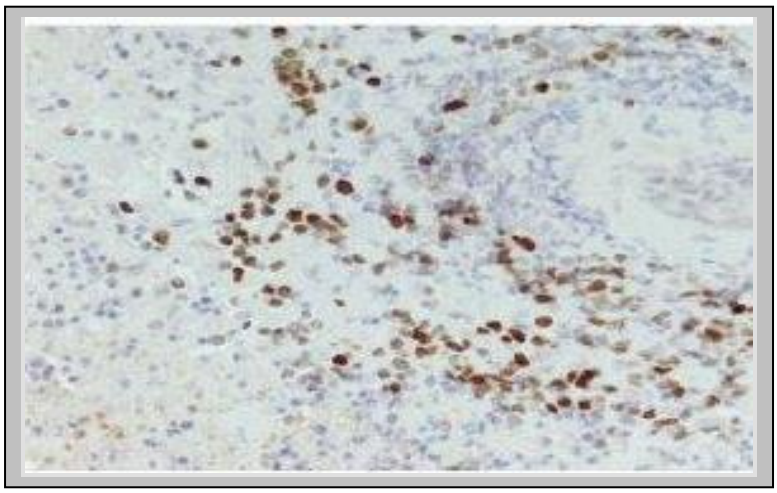


Figure 5.12 Case 5 (magnification 20x). EBNA 2 IHC staining

The EBNA 2 signal is a brown deposit located within the nucleus of tumour cells (Fig 5.12). The EBNA 2 IHC showed a 33% sensitivity and a 100% specificity (Table 5.8).

Table 5.8. The expression of EBNA 2 IHC in the study cohort, relative to EBER expression

	EBNA2 IHC POSITIVE	EBNA2 IHC NEGATIVE	TOTAL
EBER POSITIVE	3	6	9
EBER NEGATIVE	0	37	37
TOTAL	3	43	46

5.5.7 EBNA 2 NESTED PCR

Five of the 9 EBER positive cases were found to have a type 2 latency pattern. The remaining four cases showed a type 3 latency pattern.

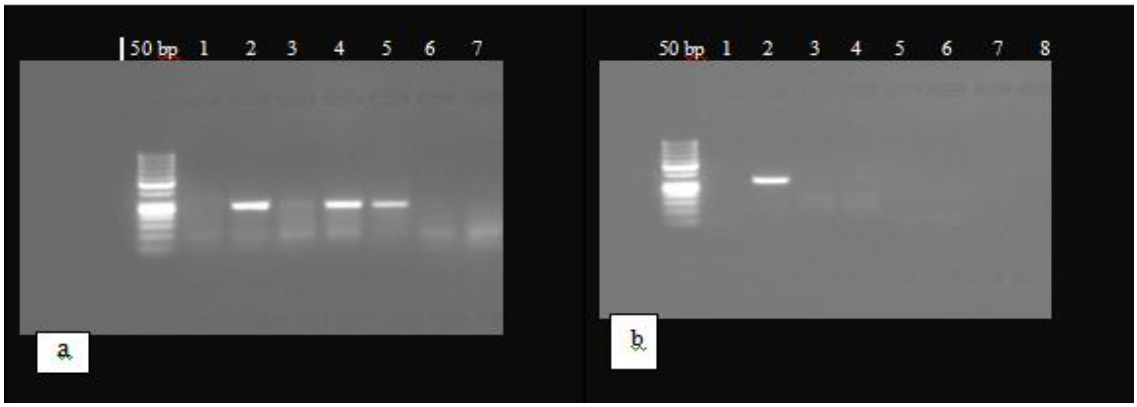


Figure 5.13 Gel electrophohoresis of *EBNA 2* nested PCR using a 50bp DNA ladder and a 3% agarose gel. a. Products of *EBNA 2* type 1. b. Products of *EBNA 2* type 2

In 2 of 9 cases, EBNA 2 polymorphism was identified as Type 1 and in another 2 cases as Type 2 (Table 5.2). The remaining 5 cases did not give a reaction for EBNA 2 (PCR) because they were latency pattern 2. Gel electrophoresis showed the Type 1 amplicon to consist of a product of 250 bp while the Type 2 amplicon consisted of a product of 300bp (Fig 5.13). All 4 cases demonstrated EBER ISH positivity.

Table 5.9 The expression of EBNA 2 PCR in the study cohort, relative to EBER expression

	EBNA 2 PCR POSITIVE	EBNA 2 PCR NEGATIVE	TOTAL
EBER POSITIVE	4	5	9
EBER NEGATIVE	0	37	37
TOTAL	4	42	46

The EBNA 2 PCR showed a 44% sensitivity and a 100% specificity (Table 5.9).

5.6 Latency patterns

The latency patterns in the 9 EBER positive cases are shown in Table 5.10.

Of the 9 EBER positive cases, 5 demonstrated a latency 2 pattern of expression and 4 demonstrated a latency 3 pattern of expression.

Table 5.10 Latency Patterns in the EBER positive cases

Latency Pattern	Number of cases	Minimum criteria
1	0	EBER positive, BZLF1 positive, LMP1 negative, LMP2 negative, EBNA1 positive, EBNA2 negative
2	5	EBER positive, BZLF1 positive, LMP1 positive, LMP2 positive, EBNA1 positive, EBNA2 negative
3	4	EBER positive, BZLF1 positive, LMP1 positive, LMP2 positive, EBNA1 positive, EBNA2 positive
Total	9	

Of the 4 EBER+ cases that occurred in males, 3 showed a Type 2 EBV latency pattern and 1 showed a Type 3 EBV latency pattern. The 5 EBER positive cases that occurred in females showed 2 with a Type 2 EBV latency pattern and 3 with a Type 3 EBV latency pattern. Overall, 6 of the 9 EBER positive cases occurred as nodal disease and the remaining 3 arose in extranodal sites, as summarised in Table 5.11.

Table 5.11 Distribution of latency pattern 1 and 2 diseases relative to gender and biopsy site

	Male	Female	Extranodal	Nodal
Latency Pattern 2 Expression	3	2	1	4
Latency Pattern 3 Expression	1	3	2	2

CHAPTER 6

6.0 Discussion

Approximately 12.7 % of the South African population is infected with HIV (South African Statistical release P0302: Mid-year population estimates, 2016). The last decade has seen a significant rise in the frequency of NHL, with HIV being a major contributor to this increase (Patel et al, 2015). Histologically, HIV-NHL typically manifests as a high grade B-cell lymphoma. There are several histological subtypes associated with HIV infection, including diffuse large B-cell lymphoma (DLBL); Burkitt lymphoma (BL); high-grade B-cell lymphoma, with *MYC* and *BCL2* and/or *BCL6* rearrangements, (formerly, B-cell lymphoma, unclassifiable, with features intermediate between DLBL and BL), primary effusion lymphoma, primary diffuse large B cell lymphoma of the central nervous system, KSHV-associated multicentric Castleman disease related large cell lymphoma and plasmablastic lymphoma (Swerdlow et al, 2016; Patel et al, 2015, Carbone et al, 2017). Many of the HIV associated DLBL cases have a high proliferation index and appear histologically aggressive.

EBV infection is more common in DLBLs occurring in HIV infected individuals and is present in 30% of these cases and in HIV negative individuals EBV is present in less than 5% of the DLBLs with EBV infection in this entity being more prevalent in the older individuals acquiring immunosuppression (Cesarman E, 2016). In addition some of the EBV positive DLBLs in HIV positive patients have an immunoblastic morphology such as those presenting as primary in the central nervous system. The immunoblastic pnenotype is rare in HIV negative tumours. Immunoblastic lymphomas associated with EBV, most commonly have a type III latency pattern of

expression. The widespread implementation of combined antiretroviral therapy (CART) to control HIV has led to a decrease in frequency of immunoblastic DLBL. DLBL cases with a centroblastic morphology occurs more frequently in cases with or without AIDS and most commonly have a type II latency pattern

In the order of 50% of HIV associated B-cell lymphomas are associated with EBV (Gulley and Tang, 2008). It is suggested that immunosuppression leads to a decrease in T-cell immunity that predisposes to massive proliferation of EBV infected B-cells (Gulley and Tang, 2008). EBV infection persists for life in a human host and the ability to achieve life-long infection is related to the ability of EBV to hide from immune surveillance via a latent B lymphocyte infection in some cells and to actively replicate and shed from other B-cells in a lytic cycle, so providing infective virions to the systemic circulation which are then able to infect other B lymphocytes (Gulley, 2001). In this way, EBV infection contributes to aiding and abetting oncogenesis by facilitating unhindered B lymphocyte replication. The infected B-cells acquire additional mutations conferring malignant behaviour.

Several studies have suggested that EBV infection of tumour cells is a prognostic factor that influences survival (Gulley et al, 2002). There is on-going progress in profiling the expression pattern of each EBV associated disease to allow for more precise diagnosis and in time, the development of EBV targeted treatments.

This background led us to consider the extent to which EBV is implicated in the pathogenesis of DLBL in the high prevalence HIV setting of South Africa, and to consider the pattern of EBV latent gene expression in these lymphomas. The

intention was to develop a framework for the reliable laboratory assessment of EBV in this group of tumours.

Our study demonstrated approximately 20% EBV association with DLBL, with either a Type II or a Type III latency pattern in approximately equal numbers, all of which displayed a lytic pattern of infection as demonstrated by the expression of the *BZLF1* gene. A lytic pattern indicates the virus is in a replicative state and is therefore immunogenic. A latent infection indicates a non-replicative viral state whereby infected cells acquire protection from host immunity by the expression of various EBV latent genes.

EBER expression by in situ hybridisation was used as the gold standard against which the immunohistochemistry and PCR investigations for establishing latency pattern were assessed. A latency pattern could be ascribed to all EBER positive cases. In addition, there were a few cases that were EBER negative that never the less displayed expression of some of the EBV latent genes.

The EBV viral strain differentiation was investigated in all cases and 4 cases with a Type III pattern, identified with EBNA 2 PCR demonstrated both Type 1 and Type 2 EBV strains in equal numbers.

There was variable correlation between the immunohistochemistry and PCR results in the detection of these latency patterns, and each of these latent components and the laboratory methods used for their detection will be addressed.

EBER ISH

EBER transcripts are abundant in latently infected cells with levels exceeding 1 million copies per cell making the EBER ISH test the best natural biomarker for the detection of the virus. RNA-based tests like *EBER* ISH are prone to false negative

results. To overcome this, a control stain for the ubiquitous U6 cellular transcript is recommended. U6 transcripts are similar in size, abundance and intra-nuclear location as EBER transcripts. The positive demonstration of the U6 cellular transcript indicates appropriate preservation of RNA within the tissue. EBER ISH can only be regarded as truly negative if the RNA is shown to be preserved and available for hybridisation. This was not undertaken in our study and is a potential limitation in the assessment of the 4 EBER negative cases that showed expression of some of the latent genes.

The interpretation of a positive EBER ISH signal is based on demonstration of the signal in the nucleus of target cells and not bystander cells. The literature provides variable guidelines in assessing a positive signal and the number of lesional cells required to interpret a result as positive. Some authors believe that a single positive lesional cell should be interpreted as indicative of EBV association (Gulley and Tang, 2008). Other authors have set a lower limit of 5% positivity of lesional cells (Ziarkiewicz, 2016). Leticia Quintanilla-Fendt refers to 20% as being the lowest level at which EBV should be accepted as positive. Positivity within greater numbers of lesional cells than background bystander cells has also been accepted as a positive signal (Quintanilla-Fendt, personal communication).

We chose to interpret any positivity within lesional cells as indicative of a positive result, and the lowest level of positivity recorded in our cases was consistently above 5% of tumour cells (1 case), with the remaining 8 DLBLs expressing EBER positivity in 50-100% of tumour cells.

BZLF 1

The EBV lytic cycle is initiated by the transcription of the EBV immediate early *BZLF1* and *BRF 1* genes (Ye et al, 2010). *BZLF 1* is expressed early in the lytic

infection of a B lymphocyte. Wen et al (2006), demonstrated that the Epstein Barr virus *BZLF1* gene, is expressed as early as 1.5h after EBV infection in Burkitt lymphoma derived, EBV negative Akata and Daudi cells and primary B lymphocytes. These cell lines and lymphocytes were originally EBV positive and subcloned to become EBV negative. They were re-infected with Akata virus derived recombinant EBV carrying the enhanced green fluorescence protein (EGFP) and were examined at designated time intervals for the expression of BZLF 1 by RT PCR.

Whereas all 9 EBER positive cases displayed a lytic pattern of EBV infection with expression of BZLF 1, an additional single EBER negative case was also shown to have a lytic infection, but failed to demonstrate the presence of other latency genes. This may possibly be accounted for by the hit-and-run hypothesis proposed by Ambinder (2000). It also suggests that viral infection only plays a triggering or accessory role in most cancers, with host oncogenes being the main drivers of neoplasia (Stevenson et al, 2010). The same authors provided evidence using a mouse recombinant model, showing that emerging cancers demonstrate the expected genetic changes but lack viral genomes at the time of presentation. They also showed that vaccination with a non-persistent muroid herpes-virus confers complete protection from tumourigenesis. They concluded that vaccines could potentially prevent not only viral genome positive cancers but also cancers where viral origin is unsuspected, on account of the loss of viral genomic material (Stevenson et al, 2010).

Antibodies for immunohistochemical detection of the ZEBRA protein, a product of the *BZLF-1* gene, are commercially available and may offer a useful parallel test to the *BZLF1* PCR test. The consistent expression of BZLF1 in all 9 EBER ISH positive cases and in only 1 of 37 EBER ISH negative cases, providing a sensitivity of 100%

and specificity of 97% indicates that both diagnostic platforms offer reliable results and could be used interchangeably in future studies.

EBNA1

Multiple anti EBNA 1 reagents are available, some of which have potential false reactivity, such as the 2B4 clone of EBNA 1 antibody which cross-reacts with human MAGEA 4, (melanoma-associated antigen 4), causing false positive signals (Gulley & Tang, 2008).

This prompted the decision in our study to use PCR detection of EBNA 1 transcripts in preference to that of immunohistochemical detection, where 8 of the 9 EBER positive cases expressed EBNA 1.

Three of the EBNA1 positive cases were EBER negative (cases 20, 31 & 35). A possible explanation might be the presence of EBNA 1 expression in bystander lymphocytes, rather than lesional tumour cells, which would signal as a “falsely” positive PCR product. This raises the principle of quantitative as opposed to qualitative PCR. In 2008, Gulley suggested that quantitative PCR (QPCR), expressed as a percentage, may constitute a more accurate reflection of latent gene expression, whereby a low positive percentage reflects bystander infection and a high positive percentage indicates EBV associated disease.

In case 53, which was EBER positive, the negative expression of EBNA 1 may be accounted for by the ‘hit and run’ hypothesis, which refers to cumulative host mutations that allow viral genomes to be lost, such that the viral origin of a tumour may be undetected (Stevenson et al, 2010). Case 53 also demonstrated a negative expression of LMP 2 and this may also be accounted for by the ‘hit and run’ hypothesis.

LMP 2

Our study suggests that LMP 2 PCR is the least sensitive method (66.67% sensitivity) for the detection of EBV. Only 4 of the 7 cases that co-expressed EBER and LMP 1, also expressed LMP 2. Whilst technical considerations such as fixation and tissue processing may play a role, additional biological considerations could account for diminished sensitivity of LMP 2 PCR. Ryan et al (2004) proposed that a global defect in the EBV LMP 2 gene region such as a gene deletion or chromosomal integration may result in diminished detection of

LMP 2.

LMP 1

LMP1 IHC is a further method used to detect EBV transcripts and it is only rarely expressed in latently infected background lymphocytes. According to Gulley et al (2002), LMP 1 protein expression is preserved in cases with poor RNA preservation and an equivocal EBER result. Furthermore, LMP1 is useful in the diagnosis of Hodgkin lymphoma, where a positive immunohistochemical signal is considered as informative as *EBER in situ* hybridization in cases that are EBV-related (Gulley, 2002). The demonstration of LMP1 and other EBV encoded proteins is less reliable in other tumours such as Burkitt lymphoma, PTLD, and NPC, which may have diffuse, focal, or completely undetectable expression of viral proteins by immunostains. As with LMP 2, the slightly diminished sensitivity of 94.74% may also be attributed to technical considerations.

Although the detection of LMP1 is less consistent than that of EBER (Gulley, 2002), it carries the advantage of indicating a type II or III latency pattern, whereas EBER is ubiquitously expressed in all three latency patterns.

LMP 1 immunohistochemistry must be interpreted with caution, as false positive staining is reported in poorly fixed tissue, in glial cells of the central nervous system (CNS), in some uninfected haematopoietic elements and in decalcified tissues. LMP 1 can be down-regulated, as occurs in Burkitt lymphoma and this could be a function of the degree of tumour cell differentiation or the immune system's ability to recognize and destroy cells expressing LMP 1 (Gulley et al, 2002). This may account for the 2 EBER positive cases which failed to identify LMP 1 transcripts, as reflected in table 5.3 and 5.4.

EBNA 2

Types 1 and 2 EBV strains vary according to polymorphisms in the sequence of the *EBNA 2* gene (Mendes, 2008; Tzellos and Farrell, 2012) and genes encoding for EBNA 3A, EBNA 3B and EBNA 3C (Baarle et al, 1999). EBNA 2 is a direct transcriptional activator of viral and cellular genes necessary for B cell proliferation. Type 1 strains are found worldwide and transform B lymphocytes into lymphoblastoid cell lines much more efficiently than type 2 strains (Lucchesi et al, 2008).

Both EBV strains occur equally in Africa, and in several other regions of the world. The type 1 strain is however much more frequent in Europe and the United States (Lucchesi et al, 2008). Type 1 strains were detected in higher rates in HIV positive individuals in a Brazilian study population (Santos et al, 2014). Our study shows an equal prevalence of type 1 and type 2 strains. Furthermore, approximately 9% of the study cohort demonstrated positive EBNA 2 expression and hence a type 3 latency pattern.

According to studies performed by Cancian et al (2011), the greater transforming activity of type 1 EBV was found to correlate with a stronger and more rapid induction of the viral oncogene LMP1 and the cell gene chemokine, CXC motif and receptor (CXCR7) which are both required for proliferation of EBV LCLs during primary infection of B cells. In immunocompetent patients, in vitro studies have shown that although EBNA 2 is important in the initial stages of the transformation of EBV infected B lymphocytes, its expression may be less important during tumour progression (Grywalska and Rolinski, 2015).

Our study showed that the EBNA 2 PCR has an increased sensitivity in comparison to the EBNA 2 IHC assay. The EBNA 2 IHC may nevertheless offer usefulness in instances where one is able to identify the chromogen within tumour cells, to rule out the possibility of false positive results of the EBNA 2 PCR due to detection of the EBV infection in positive memory cells and non-tumour, bystander lymphocytes.

Latency patterns

DLBL has been shown to have a Type II or III latency pattern in several other studies (El-Bietar & Bollard, 2011) and our study shows a similar prevalence and pattern of EBV latency, despite the high HIV prevalence and expectation of a greater association with EBV.

The pattern of EBV latent gene expression points to different aspects of virus cell interactions and indicates the role of EBV proteins in cell growth and survival. EBER is present all 3 latency patterns and is implicated in cell survival by upregulation of *BCL2* which confers resistance to apoptosis. This was shown in latency pattern type I, in Burkitt lymphoma, by Komano et al in 1999.

In latency pattern II associated Hodgkin lymphoma and nasopharyngeal carcinoma, LMP 1 and LMP 2 expression contribute to cell survival by the activation of nuclear

factor κB (NFκB), MAPK/ERK, JAK/STAT and phosphatidylinositol 3' kinase (PI3K) pathways (Kang and Kieff, 2015; Korkolopoulou et al, 2016).

In PTLD with a type III latency pattern, EBNA 2, EBNA LP, EBNA 3A and EBNA 3C co-ordinately upregulate *cMYC* expression and cell proliferation, with EBV LMP 1 enhancing cell survival by the same mechanism of NFκB activation.

In addition, EBV-coded viral miRNAs may target p53 function, B-cell receptor (BCR) signalling, oxidative stress response or apoptosis, so facilitating immune evasion and maintaining viral latency (Ok et al, 2015).

By understanding the specific functions of EBV latent proteins that are responsible for the maintenance of EBV infection and the control of various signalling and transcriptional pathways that facilitate the proliferation and survival of infected cells, targets for novel treatment can be identified (Young et al, 2004). This has been suggested by Korkolopoulou et al, (2016) where targeted inhibition of JAK/STAT, PI3K/AKT or NFκB pathways was described in the context of DLBL. Cohen (2009) proposed target directed treatment against EBNA 1 in Burkitt lymphoma and against LMP 2, with LMP 2 specific CD8+ T-cells in Hodgkin lymphoma.

PCR is more cost effective than ISH. Its applicability to specimens with poor quality RNA, and its ability to detect (pure lytic) infection lacking *EBER* transcripts is considered an advantage over ISH techniques. Where EBER ISH is not run in complementarity with PCR investigations to detect the expression of other latent gene products, such as that done for BZLF 1, EBNA 1 and LMP 2 presented in this study, it is important that quantitative PCR analysis be done instead of the qualitative PCR approach. Quantitative PCR (Q-PCR) differentiates low level infection of healthy carriers from the high levels characteristic of EBV related disease. In addition, these assays must be run parallel with Q-PCR for an endogenous human

gene to normalize for the number of nucleated cells represented in the reaction. A threshold value of around 100 EBV copies per 100 000 cells distinguishes EBER positive from EBER negative cancers (Ryan et al, 2004). In a study by Hsieh et al in 2007, it was shown that EBV viral load in NK/T lymphoma tissue can be used as a prognostic indicator. Patients that had a low EBV viral load (< 1 copy per cell) frequently survived for more than two years, as compared to patients with a high EBV viral load. Likewise, a biopsy or fine needle aspirate that is suspicious for NPC contains high levels of EBV if the cancer is indeed present and is EBV-related (Gulley et al, 2008). Low levels or undetectable levels of EBV by Q-PCR indicate the absence of EBV related neoplastic cells and rather, the presence of EBV carrier B lymphocytes. This points to the need to interpret EBV Q-PCR results in the context of histopathological findings.

Commercial quantitative EBV viral load tests that amplify specific viral targets are available and as these are already commercially validated, require only verification for laboratory implementation. Exploration of the usefulness of these commercial assays provides an opportunity for further studies. Further studies using a larger cohort may also improve the statistical significance of this study.

CHAPTER 7

7.0 Conclusion

1. This study demonstrates a twenty percent EBV association with HIV positive DLBL.
2. All cases of EBV positive DLBL in this HIV positive cohort show either a latency type 2 or a latency type 3 pattern of EBV infection.
3. There are no latency type 1 tumours.
4. In the determination of EBNA 2 expression, PCR has a greater sensitivity than IHC.
5. In the detection of latency pattern in this cohort of HIV positive DLBLs, the use of EBER ISH to detect prevalence of EBV disease and the detection of EBNA 2 by PCR or IHC is all that is required to determine a latency pattern, i.e. a sample that is EBNA 2 negative is identified as having a latency pattern 2 and one that is EBNA 2 positive, a latency pattern 3.
6. In a resource constrained environment we recommend that only two tests are performed viz. EBER ISH and EBNA 2 PCR. However, given the small amount of EBER ISH positive cases in the study cohort, these results require validation with greater case numbers before being considered generally applicable to assessing for the presence of EBV in DLBL in HIV positive individuals.
7. The high correlation we observed between EBER ISH and PCR suggests that PCR offers a potentially useful diagnostic platform to confirm EBV infection (with BZLF 1 PCR), the latency pattern of expression and EBV strain subtype (by EBNA-1, LMP2 and EBNA 2 PCR).

8. Nested PCR has the ability to differentiate between viral strain types 1 and 2. The clinical relevance of strain type differentiation in therapeutic management of the disease is still to be determined by clinical studies.

9. EBER RNA in situ hybridization is the ‘gold’ standard for EBV diagnosis in tumour cells. All EBV PCR tests must be interpreted in conjunction with EBER ISH studies as the viral DNA in positive bystander cells can be incorrectly reported as positive by PCR.

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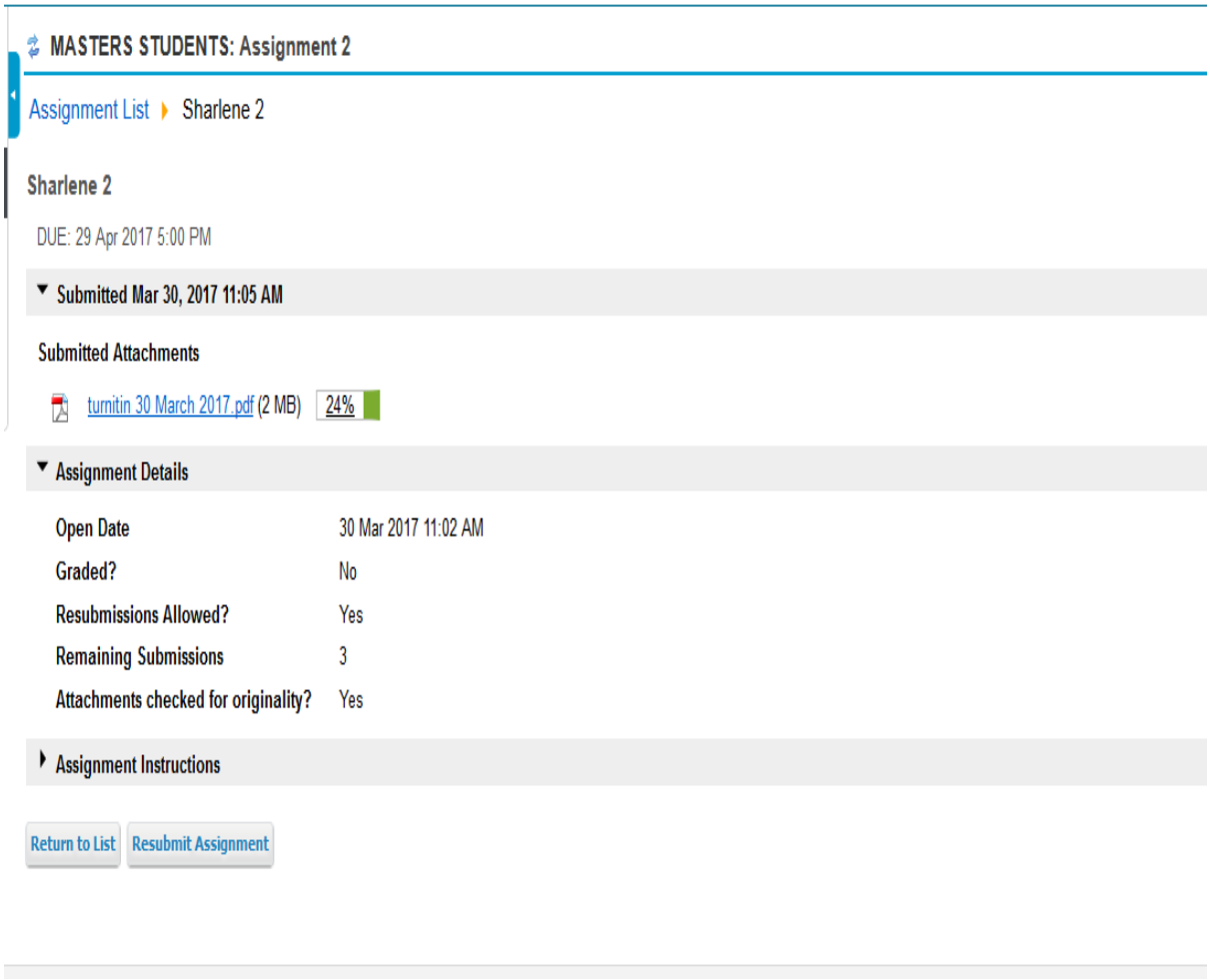
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Dr. Yvonne Perner Signed: 31/03/2017

Supervisor)

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CLEARANCE CERTIFICATE NO. M140273

NAME: (Principal Investigator)	Ms Sharlene Naidoo
DEPARTMENT:	School of Pathology National Health Laboratory Services
PROJECT TITLE:	Laboratory Diagnosis of Epstein Barr Virus in Diffuse Large B-cell Lymphoma
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Yvonne Perner

APPROVED BY: Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 24/03/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a**

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