Detection of Human Herpes Virus-8 in Kaposi's Sarcoma Tissues at the University Teaching Hospital, Lusaka

By

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A Dissertation submitted to the University of Zambia in Partial fulfilment of the requirements for the Degree of Master of Science in Pathology (Clinical Pathology)

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Declaration

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

Rabecca Tembo 2nd November, 2015.

Certificate of Approval

Dissertation Title: Detection of Human Herpes Virus 8 in Kaposi's Sarcoma Tissues at the University Teaching Hospital, Lusaka.

This dissertation of Rabecca Tembo has been approved in partial fulfilment of the requirements for the degree of Master of Science in Pathology (Clinical Pathology) at the University of Zambia.

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Abstract

Background: Human herpes virus-8, a γ2-herpesvirus, is the aetiological agent of Kaposi's sarcoma. Recently, there has been an increase in kaposi's sarcoma cases in Zambia with the seroprevalence reported to range from 20-48%. In a recent study, the incidence of paediatric KS at the University Teaching Hospital in Lusaka was shown to have increased from 3.2% to 19% in just one decade, demonstrating the enormity of the problem of KS. Despite this increase, the diagnosis of kaposi's sarcoma is based on morphological appearance of the affected tissues using histological techniques and the association with its causative agent, Human herpes virus 8 is not sought. This means poor prognosis for affected patients since the causative agent is not targeted during diagnosis and KS lesions may be mistaken for other reactive and neoplatic vascular proliferations when only histological techniques are used.

Objectives: This study was therefore aimed at detecting Human Herpes virus 8 in Kaposi's sarcoma tissues at the University Teaching Hospital in Lusaka.

Methods and Results: One hundred and twenty suspected Kaposi's sarcoma archival formalin-fixed paraffin-wax embedded tissues stored from January 2013 to December 2014 in the Histopathology Laboratory were analysed using histology and Polymerase Chain Reaction targeting the *ORF26* gene of Human Herpes virus 8. The following histological types of Kaposi's sarcoma were detected: Nodular (60.7%), plaque (22.6%) and patch (16.7%). The nodular lesion was the most common and was identified mostly in males (40.5%, 34/84) than females (20.2%, 17/84) (p=0.041). Human Herpes virus 8 DNA was detected in 53.6% (45/84) and mostly in the nodular KS lesions (60%, 27/84) (p=0.035).

Conclusion: These findings indicate that Human Herpes virus 8 is detectable in Kaposi's sarcoma tissues, and, as previously reported in other settings, is closely associated with Kaposi's sarcoma.

Dedication

I dedicate this work to my family, for their love, kindness, patience and encouragement during the time of study.

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List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
AAKS	AIDS- associated Kaposi's Sarcoma
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded Deoxyribonucleic Acid
EBV	Epstein - Barr Virus
ELISA	Enzyme-linked Immunosorbent Assay
FLICE	Fas-associated Death Domain Interlukin-1B
FFPE	Formalin-Fixed Paraffin Embedded
HAART	Highly Active Antiretroviral Therapy
H and E	Haematoxylin and Eosin
HHV-8	Human Herpes Virus-8
HIV	Human Immunodeficiency Virus
IFA	Immunoflourescent Assay
IHC	Immunohistochemistry
KS	Kaposi's Sarcoma
KSHV	Kaposi's Sarcoma Associated Herpes Virus
LANA	Latency Associated Nuclear Antigen
LYVE1	Lymphatic Vessel Endothelial Receptor 1
MiRNA	Micro RNA
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction

v-cyc	Viral Cyclin
VEGFR3	Vascular Endothelial Growth Factor Receptor 3
vFLIP	Viral Fas-associated Inhibitory Protein
vIL-6	Viral Interlukin 6
vGPCR	Viral G-Protein Coupled Receptor
UNZA	University of Zambia
UNZABREC	University of Zambia Research Ethics Committee
UTH	University Teaching Hospital
USA	United States of America

Chapter 1

Introduction

1.1 Background

Human herpesvirus-8 (HHV-8), a γ2-herpesvirus, also known as Kaposi sarcoma-associated herpesvirus (KSHV), is the aetiological agent of Kaposi sarcoma(KS) (Azadmanesh *et al*, 2012; Veetil *et al*, 2014; Gramolelli and Schulz, 2015), and is also aetiologically linked to two other lymphoproliferative disorders, Primary Effusion Lymphoma (PEL) and Multicentric Castleman's disease (MCD) (Kouri *et al*, 2012; Veettil *et al*, 2014; Avey *et al*, 2015), which have rarely been reported in Africa (Tumwine *et al*, 2011). KS is a mesenchymal tumour involving blood and lymphatic vessels and was first described in Eastern Europe in the late 19th century and classically considered as an indolent disease of elderly men (Johnston *et al*, 2009; Jalilvand *et al*, 2011). This malignancy is predominantly seen in people with acquired immunodeficiencies, including acquired immunodeficiency syndrome (AIDS) and iatrogenic immunosuppression in the setting of organ transplantation, but can also develop in the elderly (Valdarchi *et al*, 2007; Azadmanesh *et al*, 2012). KS is most frequent in regions with high HHV-8 seroprevalence, such as sub-Saharan Africa and some Mediterranean countries (Dal Maso *et al*, 2009; Ghaninejad *et al*, 2009) The global seroprevalence of HHV-8 is uneven, it is low in the United States and Western Europe, moderate in the Mediterranean and as high as 80% in sub-Saharan Africa (Newton *et al*, 2003; Mbulaiteye *et al*, 2004; Mwakigonja *et al*, 2008).

KS is now the most frequently reported malignant skin tumour in some areas of Africa and was endemic in Africa even before the advent of Human Immunodeficiency Virus (HIV) infection (Ahmed, 2013; Olp *et al*, 2013). It remains the most frequent HIV-associated malignancy and major scourge in sub-Saharan Africa, especially in the so-called "KS" belt, which covers Kenya, Uganda, Tanzania, Zambia, Zimbabwe and South Africa (Mwakigonja *et al*, 2008; White *et al*, 2008; Dollard *et al*, 2010; Kumar *et al*, 2013). Detection of HHV-8 provides important epidemiological data which can be used to determine the occurrence, prevalence and spread of KS (Azadmanesh *et al*, 2012).

1.2 Statement of the Problem

Zambia is one of the major countries in sub-Saharan Africa affected by the high HHV-8 and KS prevalence and coupled with the HIV pandemic, a drastic increase in the number of KS cases diagnosed has been reported (Minhas *et al*, 2011; Kumar *et al*, 2013). At the largest referral hospital in Zambia, the University Teaching Hospital (UTH) located in Lusaka, the diagnosis of these KS cases is based solely on clinical appearance of the lesions and by Haematoxylin and Eosin (H and E) staining of biopsies. This poses a risk of misdiagnosis, resulting in improper patient care as the causative agent, HHV-8, is not sought and KS lesions may be mistaken for other reactive and neoplastic vascular proliferations (Dittemer *et al*, 2012).

1.3 Justification of the Study

It is necessary to confirm the presence of HHV-8 DNA using molecular techniques such as Polymerase Chain Reaction (PCR) in tissue biospies to enhance the diagnosis of HHV-8-

associated diseases, such as KS, as this will lead to better treatment options for affected patients (Endo *et al*, 2003). PCR can also be used for confirmation of histopathological examinations, especially in cases of early vascular lesions, in which histopathological diagnosis is difficult (Machado *et al*, 2014). Furthermore, targeting aetiological agents in tumours is vital in disease control and prevention (Rohner *et al*, 2014). This may also be useful for clinical assessment of patients with KS and in monitoring disease progression which is important for clinical practice. This study also provides the basis for developing further studies on HHV-8 and KS in Zambia and for future development of HHV-8 vaccines. To the best of our knowledge, this is the first study of its kind to be conducted in Zambia.

1.4 Literature Review

1.4.1 Basic Biology of HHV-8 and its Transmission

HHV-8 is a large double-stranded DNA virus of approximately 165kb, and 140kb of this DNA contains coding information, flanked on either side by tandem terminal repeats of 1.4kb of highly GC-rich non-coding sequences (Endo *et al*, 2003; Sasco, 2010). It is classified as a member of the γ2-hepersvirus closely related to herpesvirus saimiri (HVS), Rhesus monkey rhadinovirus (RRV), and murid herpesvirus 68 (MHV-68), which are important model systems for the study of γ-herpesviruses (Young and Murray, 2003; Raab-Traub, 2012). Herpesviral particles are composed of a linear double-stranded DNA (dsDNA) genome enclosed by an icosahedral protein capsid, which is enveloped by a glycoprotein-studded lipid bilayer (Veettil *et al*., 2014). An intermediate layer between the capsid and envelope is referred to as the tegument.

The genome contains at least 100 open reading frames (ORFs), of which 4 to 75 are homologous to those of other herpesviruses (Fouchard *et al*, 2000; Cai *et al*, 2010; Ganem, 2010; Raab-Traub, 2012). The genome consists of conserved gene blocks overlapping with other herpesvirus family members, as well as more than 15 genes (K genes) unique to HHV-8, and these ORFs are designated with the prefix K, from K1 through K15 (Ouyang *et al*, 2014). This region of the HHV-8 genome is particularly heterogenous and variable, and is used to define the 7 subtypes and more than 20 clades of HHV-8 (Zhang *et al*, 2008; Tornesello *et al*, 2010).

The possible routes of HHV-8 transmission include horizontal, sexual, vertical, blood-borne, and through organ transplantation (Edelman, 2005; Crabtree, 2013). In Zambia, a large prospective study conducted on a cohort of children from birth to 48 months to estimate annual incidence of HHV-8, showed that transmission in children was mainly horizontal, through maternal mastication of food (Minhas *et al*, 2011; Olp *et al*, 2013). HIV-1 infection has also been demonstrated to increase the risk of acquiring HHV-8 in endemic areas (Butler *et al*, 2011; Stefan *et al*, 2011). Post allograft medical immunosuppression has also been associated with increased risk of HHV-8 infections, as well as other viruses associated with malignancies (Sunil *et al*, 2010; Butler *et al*, 2011; Crabtree, 2013).

HHV-8 can infect a variety of cell types, and enter cells either through fusion or receptor-mediated endocytosis (Chandran, 2010; Chakraborty *et al*., 2012; Veettil *et al*., 2014). Once the genome is delivered into the host nucleus, HHV-8, like all herpesviruses, is capable of entering into one of two alternative life cycles, latency or lytic replication (Ganem, 2010).

1.4.2 Epidemiology of Kaposi' Sarcoma

Worldwide KS accounts for only 0.02 - 0.07% of all malignancies in the general population (Dal Maso *et al*, 2009; Jalilvand *et al*, 2011). The region with the highest incidence is Africa, where KS represents 3% to 9% of all cancer cases (Jalilvand *et al*, 2011). Classic KS occurs as a rare and indolent form in elderly Mediterranean men, with particularly high incidence in Italy, Greece, Turkey, and Israel (Lyubomir *et al*, 2003). Historically, KS incidence in the Mediterranean is up to 10-fold higher than in the rest of Europe and the United States (Iscovich *et al*, 2010).

Before the HIV pandemic, KS was very infrequent in Western countries, with an incidence rate of 0.3 per 100,000 from 1978 to 1993 in the United States of America (Eltom *et al*, 2002). However, KS has become the most common neoplasm in AIDS patients since it was first reported among homosexual men in the United States at the beginning of the AIDS epidemic (Borbolla *et al*, 2010; Butler *et al*, 2011). In the past, the incidence of KS was over 20,000 times higher in patients with AIDS than in the general population (Engels *et al*, 2006). However, the discovery and widespread use of highly active antiretroviral therapy (HAART) in developed countries such as the United States and Europe, has led to a substantial decrease in the incidence of AIDS-associated Kaposi's sarcoma (AAKS) (Mocroft *et al*, 2003; Krown *et al*, 2006; Maskew *et al*, 2011).

The burden of HIV infection and AIDS is greatest in the developing world and neoplastic complications are increasingly encountered (Mbulaiteye *et al*, 2003; Asuquo *et al*, 2009; Sasco *et*

al, 2010; Maskew *et al*, 2011). The incidence of KS has been steadily increasing in parallel with AIDS epidemic in Sub-Saharan Africa (Parkin *et al*, 2008; Sasco *et al*, 2010; Maskew *et al*, 2011). Although KS was endemic in Central and East Africa before the AIDS epidemic, AAKS has become the most frequently diagnosed tumour in several African countries (Wabinga *et al*, 2000; Ahmed *et al*, 2013). The incidence has been steadily increasing, partly because of limited access to antiretroviral drugs and other preventive or curative therapies for AIDS-associated cancers (Krown, 2006; Ganem, 2010). In countries with long-standing AIDS epidemic such as Uganda and Zimbabwe, the incidence of KS has increased twenty-fold to become the most common cancer in men and the second most common in women (Marimo, 2010; Ahmed *et al*, 2013). Before the advent of the AIDS pandemic in South Africa, approximately 100 new cases of histologically diagnosed KS cases were reported to the National Cancer Registry each year, accounting for 0.3% of female and 0.7% of male cancer patients (Mosam *et al*, 2008; Maskew *et al*, 2011). Since 1993, the incidence of KS in South Africa has doubled in men and increased 7-fold in women (Mosam *et al*, 2008). A study conducted in Rwanda found a clear association between HIV infection and KS with odd ratios ranging from 21.9 (95% CI 12.5-38.6) to 47.1 (95% CI 31.9-69.8) (Stein *et al*, 2008).

In Zambia, there has been an increase in KS co-incidental with the HIV pandemic and high incidence of HHV-8 infection, with seroprevalence reported to range from 20-48% (Endo *et al*, 2003; Newton, 2003; Olp *et al*, 2012). In children, KS was relatively rare prior to 1980, but by 1990 to 1992, post the emergence of the HIV epidemic, the incidence in children had risen to constitute 20-25% of all paediatric malignancies in Zambia (Chintu *et al*, 1995; Olp *et al*, 2012;

Minhas *et al*, 2010). In a recent study, the incidence of paediatric KS at the UTH in Lusaka was shown to have increased from 3.2% to 19% in just one decade, demonstrating the enormity of the problem of KS (Crabtree, 2013). However, no studies to determine the incidence in adults have been done in Zambia.

1.4.3 Pathogenesis of HHV-8

Circulating blood mononuclear and endothelial progenitor cells are believed to be the source of early KS lesions (Radu and Pantanowitzi, 2013). Infection with HHV-8 reprogrammes the host's blood endothelial cells so that they resemble lymphatic endothelium, up-regulating several lymphatic-associated genes such as lymphatic vessel endothelial receptor-1 (LYVE-1), podoplanin, and vascular endothelial growth factor receptor-3 (VEGFR-) (Hong *et al*, 2004). However, HHV8 infection alone appears to be insufficient for the development of KS. KS progression relies also on some degree of host immune dysfunction and the local inflammatory milieu (Douglas *et al*, 2007; Pantanowitz *et al*, 2009). Once the HHV-8 virus enters the host's nucleus, it is capable of entering either the latency or lytic life cycle (Ganem, 2010; Sunil *et al*, 2010). The latent phase which is the default phase of HHV-8, helps with immune evasion and in establishing a persistent viral infection.The major latent viral proteins include latency-associated nuclear antigen (LANA1), viral cyclin (v-cyc), and the viral Fas-associated death domain interleukin-1B converting enzyme (FLICE) inhibitory protein (vFLIP). Kaposin, viral interferon regulatory factors 3 (vIRF3), and KSHV-encoded microRNA (miRNA) are among the other latent viral proteins (Sunil *et al*, 2010). Viral G-protein coupled receptor (vGPCR), ORF5, ORF59, ORF65, K8 and viral interleukin-6 (vIL-6) are lytic-phase proteins, although vIL-6 is

also often found in latently infected cells (Sunil *et al*, 2010). LANA is the main protein expressed by HHV-8,and its function is to maintain the HHV-8 episome (McLaughlin *et al*, 2009; Radu and Pantanowitz, 2013). LANA inhibits the transcriptional activity of the tumour suppressor protein, p53, resulting in the development of malignancy (Mc Laughlin *et al*, 2009). HHV-8 latency genes include LANA-1, v-FLIP and Kaposin ABC (Borbolla *et al*, 2004; Radu and Pantawitz, 2013). HHV-8 persists in latent form in infected cells, and its genome replicates extra-chromosomal DNA (Borbolla *et al*, 2004). Studies have shown that a potent transcriptional activator of viral cellular gene is responsible for the switch from latency to lytic cycle in HHV-8 infection (Borbolla *et al*, 2004). Like other herpesviruses, HHV-8 remains latent within cells and has developed a variety of mechanisms to evade the host immune system (Radu and Pantanowitz, 2013).

1.4.4 Clinical Presentation of Kaposi's Sarcoma

In the most immunocompetent individuals with chronic HHV-8 infection, the infection is usually asymptomatic (Luppi *et al*, 2000). Acquisition of HHV-8 in immunocompetent children and organ transplant recipients has been associated with a primary infection syndrome consisting of fever, rash, lymphadenopathy, bone marrow failure, and occasional rapid progression to KS (Luppi *et al*, 2000; Andreoni *et al*, 2002). KS manifestations vary widely, but most patients have non-tender, purplish, indurated skin lesions. Intraoral lesions are common and visceral dissemination can occur, occasionally without the presence of skin lesions (Krown, 2006).

When KS occurs in the immunocompromised, for example in AIDS associated KS, there is a wide range in the distribution and clinical manifestations (Eltom *et al*, 2002). The disease usually presents initially as violaceous skin lesions, but oral, visceral, or nodal KS may precede cutaneous involvement (Krown *et al*, 2006). Biopsy for definitive diagnosis is recommended to distinguish KS from other pigmented skin conditions, which may include bacillary angiomatosis, non-Hodgkin lymphoma, and cutaneous fungal or bacterial infections (Krown, 2006; Dittmer *et al*, 2012).

1.4.5 Diagnosis of Kaposi' Sarcoma

Presently, the diagnosis of KS requires clinical and histological evaluation. However, the increasing documentation of its association with HHV-8 has raised the important possibility of being able to predict disease occurrence by demonstrating the HHV-8 infection (Edelman, 2005; Whitby *et al*, 2008; Crabtree, 2013).

1.4.5.1 Histological Detection

Primary histological diagnosis of KS is achieved by morphological characterisation using the routine Haematoxylin and Eosin (H and E) staining technique (Mwakigonja *et al*, 2008; Tumwine *et al*, 2010). KS lesions on microscopic examination, after Hand E staining, appear to be characterised by angiogenesis, proliferation of spindle cells, oedema and occasional dissemination into visceral organs (Kumar *et al*, 2013). The inflammatory infiltrate containing lymphocytes, plasma cells and macrophages is abundant and surrounded by abnormal vessels. At early stages, KS lesions appear as flat red patches in which the main component is the

inflammatory infiltrate and few spindle cells are present with extravasation of red blood cells (RBCs) (Speicher *et al*, 2015). The patches develop into plaques and spindle cells become predominant. During the advanced stage, the nodular stage, spindle cells growth leads to the formation of macroscopically visible nodules. Spindle cells are either arranged around vascular spaces containing RBCs or can form fascicles streaming in different directions (Orenstein, 2008 Gramolelli and Schulz, 2015). The histological technique does not, however demonstrate the presence of aetiological agents, and pathologists sometimes find it difficult to differentiate this neoplasm from other cancers and KS mimics conditions such as bacillary angiomatosis, spindle cell haemangioendotheliomas, kaposiformhaemangioendotheliomas, fibrohistiocytic tumours, spindle cell melanoma and granuloma annulare (Tumwine *et al*, 2010; Speicher *et al*, 2015).

1.4.5.2 Serological Detection

Currently, four methods have been used to demonstrate antibodies to HHV-8: enzyme-linked immunosorbent assay (ELISA), immunofluorescent assay (IFA), Western blot, and immunohistochemistry (IHC) (Edelman, 2005; Crabtree, 2013). Detection of infection and determination of seroprevalence can be dependent upon which test is selected (Hudnall, 2004). ELISA methods vary according to the HHV-8 antigens used and whether they are recombinant antigens, viral lysates, or synthetic peptides (Edelman, 2005).

IFA methods incorporate virally-infected cell lines, either latently infected with expression of LANA-1, or cells that express lytic antigens following chemical induction (i.e., those representing viral replication) (Edelman, 2005; Crabtree, 2013). The Western blot technique

utilises electrophoretically separated virally infected cell lysates or whole viral lysates, which are transfered to nitrocellulose with subsequent detection of reactive antigens. It has the advantage of detecting the presence of antibodies to specific antigens (Hudnall, 2004). IHC, on fixed cells and tissue, allows the determination of which cell types harbour the virus *invivo* and semi-quantitative analysis of infected cell type for understanding viral pathogenesis. IHC is also useful for confirming or ruling out clinical diagnosis of KS (Hudnall, 2004). However, all these methods have the disadvantage of having low specificity and sensitivity (Edelman, 2005).

1.4.5.3 Molecular Detection of HHV-8

HHV-8 infection can be identified by Polymerase Chain Reaction (PCR) in tissues (Biggar *et al*., 2000). PCR-based methodology of HHV-8 DNA detection gives the greatest specificity for diagnosis compared to all other tests currently used for determining HHV-8 exposure or infection (Crabtree, 2013). PCR of KS lesions from known HHV-8 positive patients is detected in approximately 95% of all cases (Crabtree, 2013). However, amplification methods are expensive, time consuming, and have been shown to be lacking in sensitivity for easily accessible diagnostic specimens such as plasma and peripheral blood mononuclear cells (PBMCs). This has limited the clinical application of viral DNA detection in population screening (Biggar *et al*, 2000; Edelman, 2005; Crabtree, 2013). The most common DNA sequence used for detection of HHV-8 in clinical samples by PCR is the small ORF26 locus and is known to give positive results with most samples that come from the acknowledged HHV-8-associated disease conditions including all forms of KS (Zong *et al*, 2007).

1.4.6 Treatment of Kaposi's Sarcoma

Although the course of AAKS is variable, most patients would eventually develop progressive and disseminated disease requiring active therapy (Crabtree, 2013). The choice of treatment is determined by the stage of KS disease, its rate of progression, the degree of immune competence and HIV associated diseases (Krown, 2004). Several therapeutic options are available for AAKS, but the optimal therapy is still unclear. Highly active antiretroviral therapy, including protease inhibitors, may be the first treatment step for indolent slowly progressive disease (Martellotta *et al*, 2009). Following treatment with HAART, there may be complete remission in patients with good immunological response and limited disease (Martellotta *et al*, 2009). However, recent studies indicate that there is no significant regression when patients with advanced, symptomatic AAKS are treated with HAART without simultaneous chemotherapy (Krown, 2004; Martin-Carbonero, 2004).

A wide variety of chemotherapeutic agents used individually and in combination, have been evaluated for the treatment of AAKS. In high income countries, a combination of vincristine, doxorubicin and bleomycin (VAB) that was considered the standard chemotherapy regimen for AAKS has been supplanted by liposomal anthracylines due to their higher efficacy and reduced toxicity (Ashish *et al*, 2007; Cooley, 2007). In addition, the angiogenic nature of KS makes it particularly suitable for therapies based on targeted agents such as metalloproteinase inhibitors (e.g batimastat), angiogenesis inhibitors (e.g thalidomide) and tyrosine kinase inhibitors (e.g imatinid) (Koon *et al*, 2011). In low income countries, the choice of therapeutic agents is limited to the combination of vincristine, doxorubicin and bleomycin or even more toxic drugs such as

thalidomide because liposomal anthracyclines are not available or affordable (Makombe *et al*, 2009).

1.5 Research Question

Is HHV-8 DNA detectable in paraffin-embedded Kaposi's sarcoma tissues at the University Teaching Hospital?

1.6 Objectives

1.6.1 General Objective

To detect Human Herpes Virus-8 DNA in Kaposi's sarcoma tissues at the University Teaching Hospital in Lusaka.

1.6.2 Specific Objectives

1.6.2.1 To identify the histological types of KS using Haematoxylin and Eosin staining.

1.6.2.2 To determine the presence of HHV-8 DNA in Kaposi's sarcoma tissues using Polymerase Chain Reaction.

Chapter 2

Materials and Methods

2.1 Study Design

This was a laboratory-based retrospective study on 120 archival formalin-fixed paraffin-embedded (FFPE) KS tissues.

2.2 Study Site

The study was conducted at the University Teaching Hospital (UTH) in the Department of Pathology and Microbiology, Histopathology Laboratory, in Lusaka. The UTH is a tertiary referral and teaching hospital with a bed capacity of about 1,664. The hospital has about 11 departments namely: Obstetrics and Gynaecology, Paediatrics, Surgery, Community medicine, Pathology, Radiology, Physiotherapy, Pharmacy and Blood bank. It is the biggest referral hospital and the centre for all histopathology diagnostic work in Zambia.

2.3 Sampling Frame

Archived FFPE tissue blocks of KS were used. These included oral, skin and lymph node biopsies from patients diagnosed with KS. One hundred twenty tissue blocks, stored over a 2 year period from January 2013 to December 2014, were collected. These included cases from all age groups.

2.3.1 Inclusion Criteria

All Archived paraffin wax-embedded tissue blocks of KS cases from patients of all age groups stored from the January 2013 to December 2014 were included in the study.

2.3.2 Exclusion Criteria

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

2.4 Sample Size Determination

Prevalence of HHV-8 infection in Zambia is estimated to be about 25% (Olp *et al.*, 2013). In order to estimate the prevalence within 5% (or 0.05) and considering 95% confidence level, a minimum sample size of 285 was used, as shown by the calculation:

$n = \frac{z^2 p(1-p)}{e^2}$, where $p = 25\%$ (or 0.25) is the sample proportion and

$e^2 = 0.0025$

n= 285

Applying correction for finite population size formula (Israel, 2013), the new sample size was

$$n_0 = \frac{n}{1 + \frac{n-1}{N}},$$

Where n= sample size 285 as calculated above, N=Total number of KS blocks stored from January 2013 to December 2014 (population size).

The calculated new $n_0 = \frac{n}{1 + \frac{n-1}{N}}, = \frac{285}{1 + \frac{285-1}{200}} = 118$

Therefore, the minimum sample size (n) was 118.

To obtain this, tissue blocks were selected by systematic random sampling. First, a list of all suspected tissue blocks stored from January 2013 to December 2014 was made. The k^{th} tissue block in the sampling frame was calculated by dividing 200, the total number of tissue blocks (K) stored over a 2 year period by the sample size, 118. Selection of the first isolate from the first K tissue blocks was done using simple random sampling. Then every k^{th} (2^{nd}) tissue block on the list was selected (Crossman, 2012).

2.5 Identification of Histological types of KS using H and E staining

2.5.1 Paraffin Block Retrieval

One hundred and twenty formalin-fixed paraffin embedded (FFPE) KS blocks from the UTH Histopathology Laboratory specimen archive were retrieved. All the blocks contained tissues which were previously diagnosed as KS based on clinical appearance and H and E staining. Each tissue block was given a new identification code and number for easy identification.

2.5.2 Section Cutting

Sections of tissue were cut at 6µm on Shandon Finesse 325 microtome (Thermo Scientific-Shandon, USA). Briefly, tissue blocks were first placed face down on an ice-cold plate for 20 minutes and cut on a microtome to generate serial sections. Separate blades were used to cut each tissue to avoid contamination during PCR amplification of the viral targets. Each ribbon was picked by forceps and transferred to a water bath set at 37°C in order to float out the

sections. The ribbons were laid on the water bath to allow the sections to stretch for a few seconds. The sections were then carefully separated and each section was picked on a glass slide at an angle. The slide sections were allowed to drain for a few minutes before transferring them to a hot plate for drying.

2.5.3 Haematoxylin and Eosin Staining

Before H and E staining, sections first deparaffinised in two changes of xylene (2 minutes each), washed in two changes of absolute alcohol (2 minutes each), 2 minutes in 95% alcohol, 2 minutes in 90% alcohol, 2 minutes in 80% alcohol, 2 minutes in 70% alcohol and then in water for 2 minutes. The slides were stained with Ehrlich's Haematoxylin solution (Hopkins and William, England) for 25 minutes. After staining, the slides were washed in running tap water for 5 minutes and then differentiated in 1% acid-alcohol (1% hydrochloric acid in 70% alcohol) for 15-30 seconds. The slides were further blued in running tap water for 10 minutes and counter stained in 1% aqueous eosin for 5 minutes. Finally, the slides were rinsed in water, and dehydrated in 70%, 80%, 90%, 95% alcohol and in two changes of absolute alcohol for 2 minutes at each stage. The slides were then cleared in 2 changes of xylene for 2 minutes in each jar, cleared in DPX mountant (Electron Microscopy Sciences, Hatfield), covered with a cover-slip and allowed to dry for 2 hours.

2.5.4 Microscopic Examination of Slides

The slides were observed under the microscope to confirm the diagnosis of KS. The histopathological criteria for diagnosing KS included spindle cell proliferation, erythrocyte-filled

vascular slits and proliferation of small vessels, with vessels showing evidence of extracellular haemorrhage and haemosiderin deposition. The KS lesions were further classified into patch, plaque and nodular stages.

2.6 Detection of HHV-8 DNA in Kaposi' Sarcoma Tissues

2.6.1 Section Cutting

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

2.6.2 DNA Extraction

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

2.6.3 Detection of HHV-8 DNA

DNA extracted from Section 2.6.2 was used for the detection of the HHV-8 sequences. Nested PCR was performed using two sets of primers, KS-1and KS-2, KS-4 and KS-5 (primer sequences shown in Table 2.1 below) amplifying the *ORF26* gene of the HHV-8 genome. The 2 sets of primers were used for screening the DNA for HHV-8. The first round PCR reactions were

performed in a final volume of 25µl of which 2µl was genomic DNA, 1X PCR buffer, 3.5 mM $MgCl_2$, 0.2mM of deoxynucleoside triphosphates, and 0.2 U of Invitrogen *Taq* DNA polymerase (Thermo scientific co Ltd, USA), 2.5µl of each primer on a Gene amplification 2700 Thermocycler (Applied Biosystems, CA, USA). Primers were combined at a final concentration of 0.2µM. For the second-round PCR, 2µl of the first-round PCR products was used as the template DNA. The cycling procedure for the KS-1, KS-2 primer pair was 94^{o}C for 5 minutes, then 35 cycles of 94^{o}C for 30 seconds, 58^{o}C for 30 seconds, 72^{o}C for 30 seconds and 1 final extension cycle of 7 minutes at 72^{o}C. For the KS-4 and KS-5 primers, the amplification conditions were similar except the annealing temperature was at 53^{o}C. The PCR products were analysed by electrophoresis on a 1.5% agarose gel containing ethidium bromide (10ug/ml in distilled water) and were visualized under ultraviolet light alongside a 50bp DNA ladder using the Biotop SC-645 Gel Documentation system (Biotech Co. Ltd, Shanghai, China). A known KS case was used as a positive control and a negative control containing nuclease-free without DNA was always included. The expected product size was 233bp.

As an internal control, the extracted DNA was subjected to PCR using primers for human β-actin (Table 2.1) with the following conditions: initial denaturation of 94^{o}C for 1 minute, 35 cycles of 95^{o}C for 30 seconds, 62^{o}C for 1minute, 72^{o}C for 1 minute and 1 final extension cycle of 10minutes at 72^{o}C. The PCR products were also analysed by electrophoresis on a 1.5% agarose gel containing ethidium bromide (10µg/ml) and visualised under ultraviolet light alongside the 50bp DNA ladder. The expected product size was 200bp.

Table 2.1: Primer sequences used in the detection of HHV-8 and the expected PCR amplicon sizes

Primer	Primer sequence	Amplicon size	Reference
HHV-8 detection			
KS-1	AGCCGAAAGATTCCACCAT		
KS-2	TCCGTGTTGTCTACGTCCAG	233bp	Olp *et al*, 2013
KS-4	CGAATCCAACGGATTTGACCTC		
KS-5	CCCATAAATGACACATTGGTGGTA	233bp	Olp *et al*, 2013
Internal control- β actin			
Forward GCC ATG TAC GTT GCT ATC C			
Reverse CCG CGC TCG GTG AGG		200bp	Olp*et al*, 2013

2.7 Data Analysis

GraphPad Prism Software Version 6 (San Diego, California, USA) was used for data analysis. Logistic regression was used to determine associations among HHV-8, histological types of KS, gender and age. Chi-square and Fisher's exact test were used to determine associations among histological types of KS, gender and age. A p-value of less than 0.05 was chosen to indicate statistical significance.

2.8 Ethics Considerations

This study was laboratory-based with no direct contact with patients. Permission to use the archived histopathology tissues was sought from the Head of the Department of Pathology and Microbiology at the University Teaching Hospital. To ensure confidentiality and anonymity tissue blocks were assigned project identification numbers. Ethics approval for the project was sought from the University of Zambia Biomedical and Research Ethics Committee (UNZABREC)(Ethics clearance number: 002-04-14/B; Appendix 1).

Chapter 3

Results

3.1 Identification of Histological Types of Kaposi's Sarcoma

Out of the 120 suspected FFPE KS tissues analysed, 70% (84/120) were confirmed KS cases in mostly males (64.4% , 56/84) than females (33.3%, 28/84) (Figure 3.1).

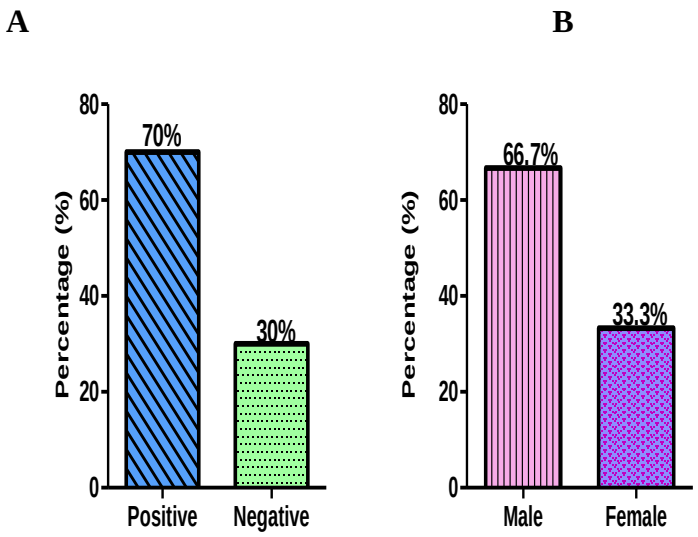

Figure 3.1 Determination of KS cases (A) and (B) KS cases based on gender

The KS lesions were grouped into three histological types: Patch (early vascular lesion), plaque (intermediate lesion) and nodular (advanced lesion) based on the features seen under the microscope as shown in Figure 3.2 below.

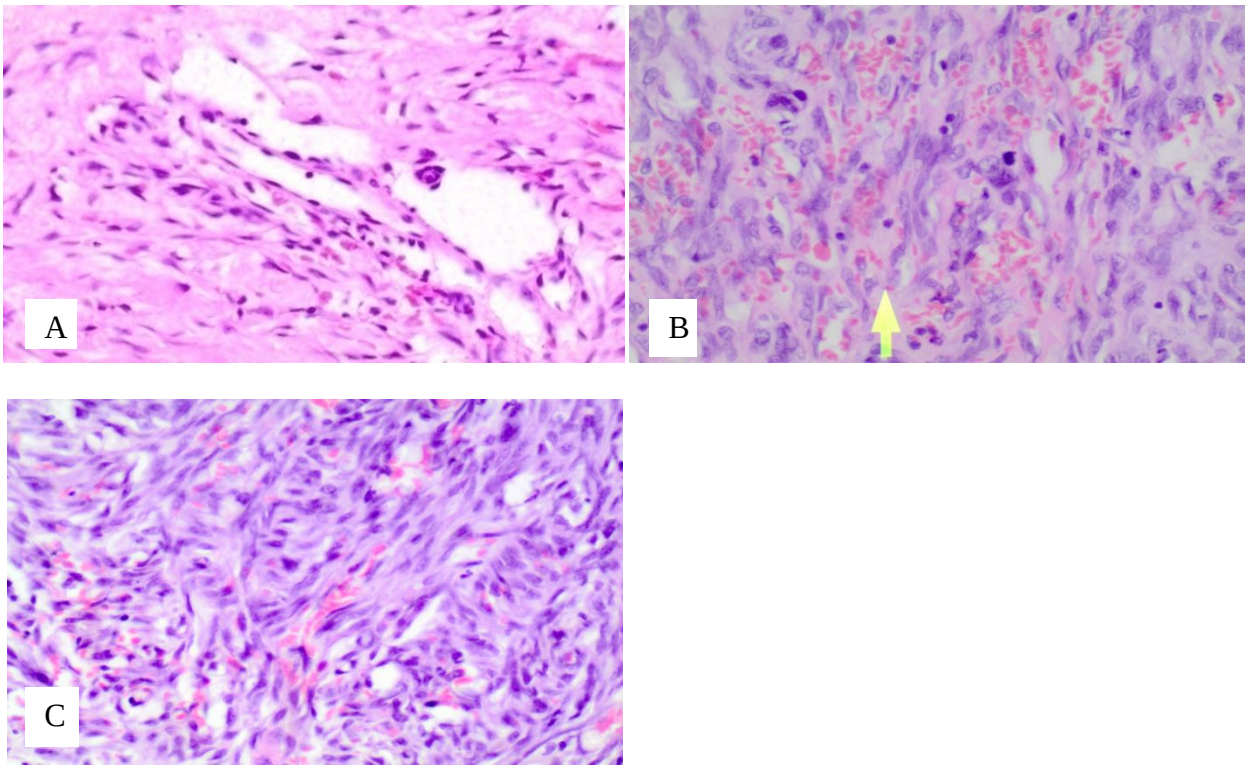

Figure 3.2: Histopathological features of KS lesions (H and E staining). (A) shows the Patch stage: dissection of collagen bundles by slit-like vascular channels lined by a monolayer of relatively flattened endothelial cells, variable degree of erythrocyte extravasation; (B) Plaque stage: proliferation of spindle cells and extravasation of erythrocytes in slit-like vascular channels; and (C) Nodular stage of KS with formation of well-defined nodules, more spindle cell proliferation and erythrocyte extravasation, disappearance of all normal features of the skin.

The histological type distribution was as follows: Nodular (60.7%, 51/84), Patch (22.6%, 19/84), and Plaque (16.7%, 14/84) (Figures 3.3 below). The nodular type was the commonly diagnosed histological lesion.

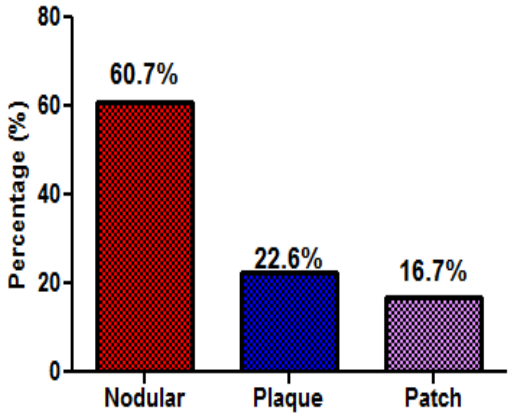

Figure 3.3: Histological Types of KS identified

The nodular histological type of KS was the commonly identified histological type of KS and affected mostly males (40.5%, 34/84) than females (20.2 %, 17/84) and this was statistically significant (p=0.041) (Figure 3.3A). The age group 21-40 years was the most affected by nodular lesions of KS compared to the other age groups. However, this difference was not statistically significant (p=0.199) (Figure 3.4B).

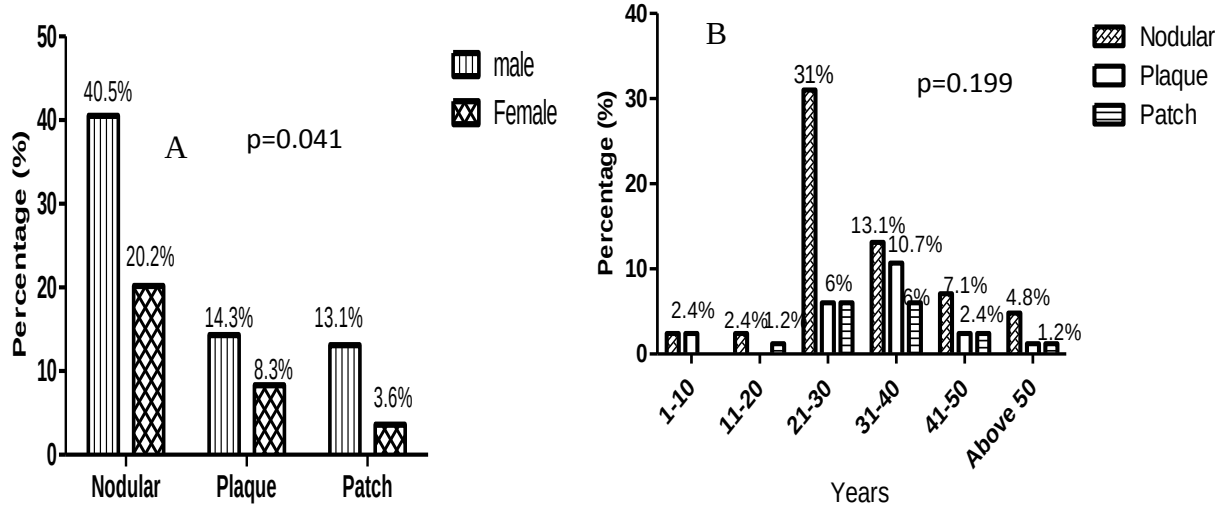

Figure 3.4: Identification of Histological Types of KS based on gender and B) Age

3.2 Detection of HHV-8 DNA in Kaposi's Sarcoma Tissues

As an internal control, the 200bp region of human β-actin gene, was used to confirm the efficiency of DNA extraction from KS biopsies (Figure 3.5A).This was performed on all tissues analysed. The presence of HHV-8 DNA was confirmed by running nested PCR products on a 1.5% agarose gel and a product size of 233bp was observed as shown in Figure 3.5B below:

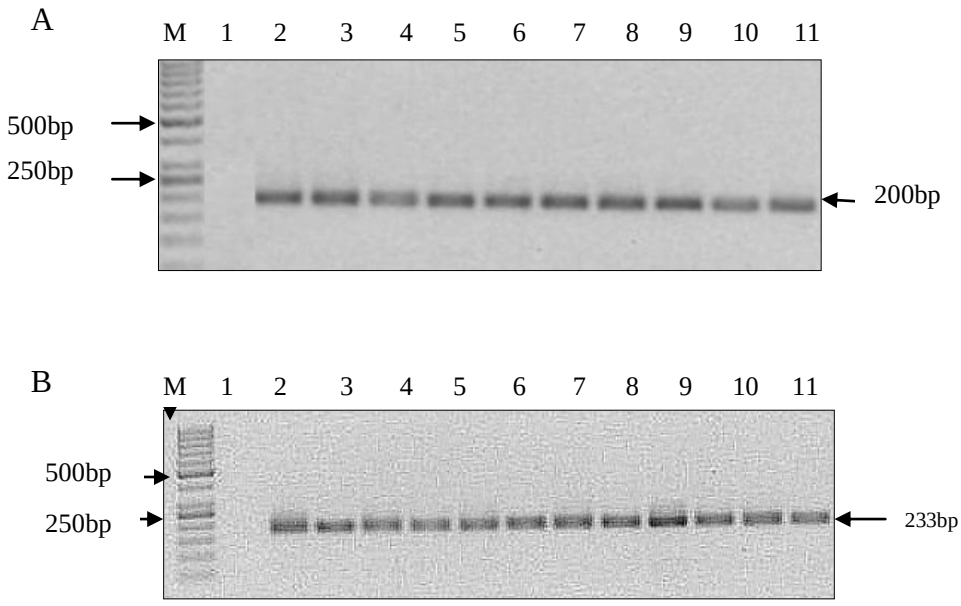

Figure 3.5 Detection of PCR products: A): Human β- actin internal DNA extraction control-PCR detection of β-actin. Lane M: 50bp DNA marker, Lane 1, Negative control; Lanes 2-11 Representative KS tissue samples. B) PCR detection of HHV-8Lane M: 50 bp DNA marker, Lane 1: Negative control, Lane 2: Positive control, Lanes 3(RT058), 4(RT060),) 5(RT87), 6(RT161), 7(RT169), 8(RT173), 9(RT176) 10 (RT206) 11 (RT207): Positive for HHV-8.

Out of the 84 confirmed KS tissues, HHV-8 DNA was detected in 53.6% (45/84) of the tissues (Figure3.6 A). HHV-8 DNA was detectable in the tissues with the following histological distribution: Nodular (60%, 27/45), Plaque (31.1%, 14/45) and Patch (8.9%, 4/45). The nodular

stage was more likely to harbour HHV-8 DNA than the other histological types of KS (OR=1.43, 95%CI 0.06 -2.04, p=0.035) (Figure 3.6B).

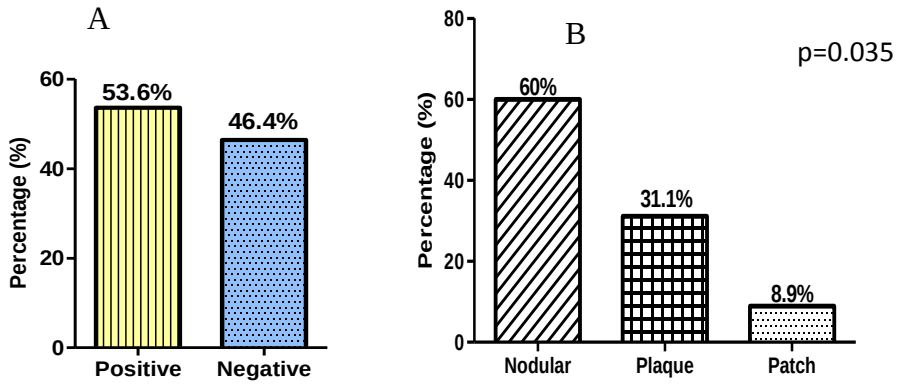

Figure 3.6. Detection of HHV-8 in KS tissues. A) Total detection of HHV-8 DNA in KS Tissues. B) HHV-8 detection in various KS histological types

HHV-8 DNA was mostly detected in males (71.1%, 32/45) (p=0.493) than in females (28.9%, 13/45) (Figure 3.7A) and mostly affecting the age group 21-30 years (53.3%, 24/45) (p=0.359) (Figure 3.7B).

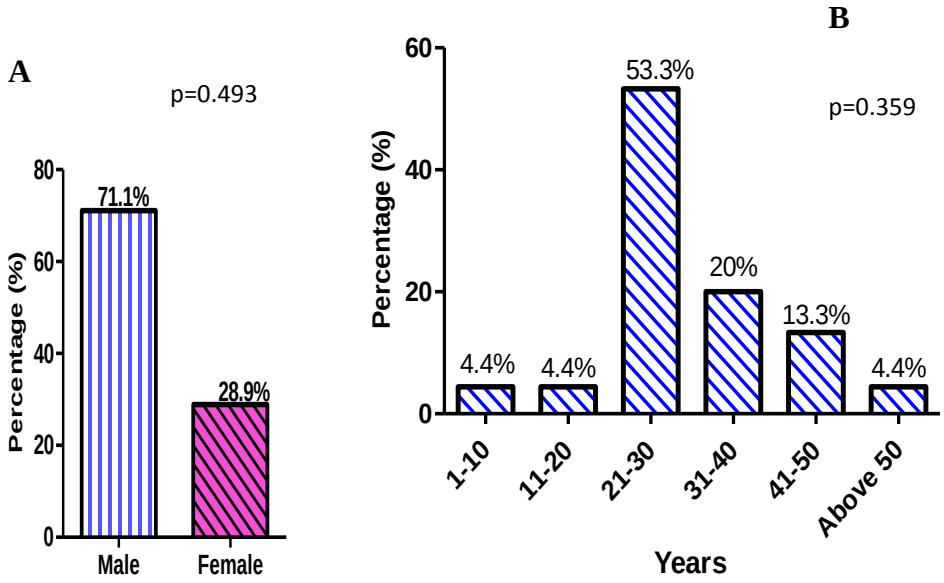

Figure 3.7 Detection of HHV-8 DNA based on (A) Gender and (B) Age

Chapter 4

Discussion

4.1 Discussion

HHV-8 has been proven to be the causal agent of all forms of KS (Betsem *et al*, 2014; Mancuso *et al*, 2011). The higher (93%) HHV-8 prevalence among KS cases compared to non-KS tumours and reactive lesions support a causal relationship between the virus and primary KS (Mwakigonja *et al*, 2008). Detection of HHV-8 by means of PCR is important for making the differential diagnosis of KS, and can be used for the confirmation of histopathological diagnosis especially in cases of early vascular lesions which are difficult to diagnose (Machado *et al*, 2014). Epidemiological studies have indicated that HHV-8 seropositivity in various populations is strongly correlated with the population's risk of developing KS (Machado *et al*, 2014; Rohner *et al*, 2014).

In this study, three histological types of KS were identified: patch, plaque and nodular types. Patch is an early vascular lesion which mostly presents diagnostic challenges to the pathologists as it may be misdiagnosed because of its resemblance to other vascular lesions such as haemangiomas, haematomas and purpuras (Dittmer *et al*, 2012). Plaque is the intermediate stage in which there is moderate proliferation of spindle-shaped cells while the nodular type is an advanced stage of the disease which, in most cases, poses no diagnostic challenges. Data in this study also demonstrated that the nodular type of KS was the commonly diagnosed histological type. These findings are consistent with those in a study conducted in Tanzania in which most of

the KS cases (68.7%, 82/120) were identified at this stage (Mwakigonja *et al*, 2008). The similarities in the two studies can be attributed to the fact that both countries are in the endemic region of KS where such results are expected. It could also be attributed to misdiagnosis at an early stage of the disease as the lesions are easily identified at an advanced stage or it may be that patients report to the hospital when the disease reaches an advanced stage (Machado *et al*, 2014).

This study also showed that the occurrence of KS was proportionally higher in men than in women. Men presented with more nodular lesions of KS compared to women. These findings are similar to studies carried out in other sub-Saharan countries (Ahmed and Muktar, 2011; Tornesello *et al*, 2014). A Ugandan report showed that women were less likely to have nodular lesions of KS (OR=0.33, 95% CI 0.16-0.69 p=0.003) as compared to men (Phipps *et al*, 2010). The reduced rate of KS among females may be due to gender related factors that include hormonal, environmental or genetic factors which normally protect women against the disease. Human gonadotrophin has been hypothesised to be a protective factor in KS development based on its inhibition of the growth of KS cell lines *in vitro* and oestrogen has also been known to have direct effects on KS cell proliferation or by its direct effect on anti-tumour immune response (Phipps *et al*, 2010).

Data presented in this study also demonstrates the presence of HHV-8 DNA in more than half of the KS tissues analysed. Tumour biopsies are a convenient source of viral DNA as they have a high viral load compared to peripheral blood (Kouri *et al*, 2012). A recent study employing

nested PCR to evaluate the frequency of HHV-8 infection in HIV infected patients with and without KS manifestations in Brazil detected HHV-8 DNA in 100% (13/13) of the tissues analysed (Machado *et al*, 2014). This was attributable to the pathogenic role of HHV-8 in KS. An Iranian study reported the presence of HHV-8 DNA in 83.3% (30/36) of the KS tissues (Azadmanesh *et al*, 2012). Several other studies have also reported high detection rates of HHV-8 DNA in KS tissue biopsies (Ramo-da-silva *et al*, 2006; Zhang *et al*, 2008; Ouyang *et al*, 2014). Our findings along with those from other studies emphasize the pathogenic role of HHV-8 in the development of KS (Bhutani *et al*, 2015; Vincenzi *et al*, 2015). The differences with this study in which a lower detection rate was obtained, may be attributed to formalin fixation in the tissues used. Formalin fixation results in widespread cross linkage between nucleic acids and proteins, with the result that DNA extracted from fixed tissues is fragmented into sequences of variable size, making it difficult to amplify the viral DNA (Ryan *et al*, 2002). It is noteworthy that several authors have observed the detectable presence of HHV-8 to be intermittent, perhaps contributing to the overall lack of sensitivity of PCR in detecting HHV-8 infection (Edelman, 2005). It has also been observed that the sensitivity of PCR depends on the accuracy and location of the excised biopsy. However, most biopsies are obtained in sites where there is less bleeding and this compromises the quality of the samples collected as the most aggressive lesion is not excised (Dittmer *et al*, 2012).

In this study, HHV-8 detection was higher in the nodular lesions of KS than other lesions. The nodular lesions were more likely to harbour HHV-8 DNA (OR=1.43 95% CI 0.06-2.04 p=0.035) than the other histological types of KS. These findings were consistent to those in a USA study

in which higher levels of HHV-8 DNA were also reported in the nodular stage compared to the patch and plaque stages (Mendez *et al,* 1998). Nodular lesions are the commonly diagnosed KS lesions, and semi-quantitative analysis has established that the nodular stage is associated with a higher viral load than the two other stages (Mendez *et al*, 1998; Leão *et al*, 2002). The higher viral load in these lesions is attributed to the fact that these lesions are an advanced form of KS and therefore contain more endothelial spindle cells than the other lesions, the cells that harbour the HHV-8 virus. This makes the virus to be readily detected and hence more likely to be found in these lesions (Reed *et al*, 1998). Data obtained in this study strongly suggest a pathogenic role of HHV-8 in KS. Therefore HHV-8 detection has potential applications in the early diagnosis, staging and monitoring of KS lesions.

This study also demonstrated that HHV-8 DNA detection was high in the 21-40 age group and in male patients, with no statistical significance with regard to age and sex. Similar results were obtained in a study conducted in Germany in which HHV-8 DNA was mainly detected in the middle-aged group ($p>0.05$) and was mostly seen in males ($p>0.05$) than in females (Martinez *et al,* 2013). High HHV-8 detection in the middle aged group in the two studies may be attributable to the high HIV risk due to high risk behaviour in this age group (Ahmed and Muktar, 2011). However, we could not attribute our findings to HIV infection due to lack of clinical data. The high detection rate in women may be attributed to gender- related factors such as hormonal, environmental and genetic factors which protect women against KS (Phipps *et al,* 2010). Studies conducted in Cameroon, China and Brazil have shown that HHV-8 prevalence increases significantly with age and is not related to sex (Cunha *et al*, 2005; Zhang *et al*, 2012; Betsem *et*

al, 2014). Local risk factors, geographical locations or ethnic makeup of the study population could also influence results obtained in the different studies (LaDuca *et al*, 1998; Kumar *et al*, 2012).

4.2 Conclusion

Data generated in this study shows that KS lesions consist of three histological types: patch, plaque and nodular lesions. Of the three, the nodular lesion was the most common (60.7%) and was mainly identified in males than in females, supporting the notion that KS is a male-associated disease. Another significant finding was the detection of HHV-8 DNA in more than half (53.6%) of the KS tissues analysed, and this was mostly detected in the nodular lesions. Our data corroborate those from other studies suggest a role of HHV-8 in KS pathogenesis. We propose detection of HHV-8 in tissues by PCR as a way of making a definitive diagnosis for KS, especially in early vascular proliferations when the characteristic histopathological features are not present.

4.3 Limitations of the Study

Although previous epidemiological studies in Zambia have identified factors such as HIV infection and other opportunistic infections to be strongly associated with the risk of developing KS (Olp *et al*, 2013), it was not possible to link our data with the incidence and severity of these infections as we did not have access to patient clinical data. An attempt was made to collect this data but we could not obtain it due to poor record keeping at the UTH. In some cases, patients

are allowed to carry their files home. Even though some files could be found, most of them had incomplete data.

Initially, it was envisioned that we could genotype the HHV-8 strains detected. However, this could not be done as the targeted gene, K1 could not be amplified in the tissues analysed. This may be attributed to formalin fixation which is known to fragment DNA into variable sizes, thereby making amplification of large fragments such as the K1 gene difficult.

Findings in this study cannot be generalised to the whole country because of the small sample size used.

4.4 Future Directions

Investigations described in this study warrant future research. With the potential that HHV-8 infections are associated with HIV infection, and other opportunistic infections, it would be important to collect data for these infections. This can be achieved by conducting a prospective study so that clinical information is collected just as the patients report to the hospital. This would help in correlating HHV-8 infections with HIV, and would help in determining whether KS cases in Zambia are AIDS- associated, endemic or iatrogenic.

It would also be of interest to genotype HHV-8, and this can be achieved by amplifying the viral hypervariable region, K1 gene. This would provide insights into the different subtypes of HHV-8

circulating in Zambia, as such information would be important for informing future vaccine design for HHV-8 infections.

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Appendices

Appendix I: Ethics Approval Letter

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

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Lusaka, Zambia

Assurance No. FWA00000338
IRB00001131 of IORG0000774

8th April, 2014.

Our Ref: 002-04-14/B.

Ms. Rabecca Tembo,
University of Zambia,
School of Medicine,
Department of Pathology and Microbiology,
P.O Box 50110,
Lusaka.

Dear Ms. Tembo,

RE: SUBMITTED RESEARCH PROPOSAL: **"MOLECULAR CHARACTERISATION OF HUMAN HERPES VIRUS 8 IN KAPOSI'S SARCOMA TISSUES AT THE UNIVERSITY TEACHING HOSPITAL, LUSAKA"**
(REF. NO. 002-04-14/B)

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 18th March, 2014. The proposal is approved.

CONDITIONS:

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

Yours sincerely,

Dr. J.C. Munthali
CHAIRPERSON

Date of approval: 8th April, 2014. **Date of expiry**: 7th April, 2015.

Appendix II: Reagents

10x TBE buffer

108g of Tris

55g Boric acid

7.5g EDTA

Up to 1000ml of distilled water

1xTBE buffer

1 volume of 10x TBE buffer

Agarose Gel (1.5%)

1.5 g of agarose powder

1x TBE buffer

Dissolve 1.5g of agarose powder in 100ml of 1x TBE buffer. Heat the mixture until all the agarose powder dissolves. Allow to cool for your skin to tolerate the heat, and then pour into an appropriate tray used during gel electrophoresis.