PREVALENCE AND GENETIC CHARACTERIZATION OF BOVINE LEUKEMIA VIRUS FROM BEEF CATTLE IN THE TRADITIONAL SECTOR IN ZAMBIA

BY

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DECLARATION

I, **Phiri Mukonda Mundia,** computer number **2015130768,** hereby declare that this dissertation represents my own work and that it has not previously been submitted for a degree, diploma or other qualification at this or another University.

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APPROVAL

This dissertation of **Phiri Mukonda Mundia** is approved as fulfilling part of the requirements for the award of the degree of Master of Science in Molecular Biology by the University of Zambia.

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DEDICATION

This dissertation is dedicated to my sister, Sanana Akombelwa, my husband, Malumbe Shichilenge and my son Lubasi. Your love, encouragements, support and understanding have given me the strength to keep going. I thank God for your lives which are a blessing to me every day.

ABSTRACT

Bovine leukemia virus (BLV), the causative agent of enzootic bovine leucosis (EBL), is a member of the family *Retroviridae*, and genus *Deltaretrovirus*. Whilst BLV has been associated with considerable economic losses in the cattle industry worldwide, there is paucity of information on the molecular epidemiology of BLV infection in cattle in many African countries, including Zambia. Moreover, much of the work that has been conducted on BLV has focused on the dairy cattle sector where it has been identified to be a major constraint. Only limited studies have been conducted on the virus in beef cattle. This study was conducted to determine the prevalence and molecular epidemiology of BLV in beef cattle reared in the traditional sector in Zambia.

Archived whole blood samples from 880 animals (188 from Southern, 342 from Eastern and 350 from Western provinces of Zambia), were pooled, with each pool containing blood samples from 10 animals giving a total of 88 pools. Total genomic DNA was extracted from the pools and a segment of the *env* gene of BLV was amplified by nested polymerase chain reaction (PCR). Amplicons of positive samples were sequenced by the Sanger di-deoxy chain termination method and phylogenetic analysis was conducted to determine the evolutionary relationships of BLV isolated from the study areas within Zambia. The *env* gene of BLV was detected in 19.3% (17/88) of pooled whole blood samples and the overall estimated pooled prevalence was 2.1%. Out of these 17 positive pools, 10 were from Southern and Western provinces (i.e. five positive pools from each province) and seven were from Eastern Province. The pooled prevalence by province was estimated to be 3.0%, 1.5% and 2.2% for Southern, Western and Eastern provinces, respectively, there was no significant difference in the proportion of positive pools among the three provinces.

Phylogenetically, all the Zambian BLV detected from beef cattle in this study belonged to genotype I therefore only one genotype was detected. The BLV strains detected in the study formed a distinct cluster, suggesting long-term independent evolution within the country. Results from this study suggest that genotype I BLV is circulating in the major traditional beef cattle rearing regions of Zambia. This is the first study on the molecular detection and characterization of BLV from traditional beef cattle in Africa. Further studies are required to gain additional insights into BLV infections in other parts of the country to contribute to the development of prevention and control measures.

Keywords: Bovine Leukemia Virus, Traditional, Beef Cattle, Molecular Characterization.

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LIST OF ABBREVIATIONS AND ACRONYMS

AGID: Agar gel immunodiffusion

bp: Base pair

BLAST: Basic Local Alignment Search Tool

BLV: Bovine leukemia virus

DNA: Deoxyribonucleic acid

EBL: Enzootic bovine leucosis

EDTA: Ethylenediamine tetraacetic acid

ELISA: Enzyme Linked Immunosorbent Assay

HTLV: Human T-cell lymphotropic virus

LTR: Long terminal repeat

MEGA: Molecular Evolutionary Genetics Analysis

mg: Milligrams

ml: Milliliters

miRNA: Micro Ribonucleic acid

PCR: Polymerase chain reaction

RNA: Ribonucleic acid

RFLP: Restriction fragment length polymorphism

STLV: Simian T-cell lymphotropic virus

TAE: Tris Acetate Ethylenediamine tetraacetic acid

US: United States

USA: United States of America

UV: Ultra violet

˚C: Degrees Celsius

%: Percent

µl: Microliter

$: Dollars

CHAPTER ONE
INTRODUCTION

1.1 Background

Zambia is a landlocked tropical country located in the southern region of Africa with a total surface area of 752,614 square kilometers. The country has an estimated population of 16,405,229, of which an estimate of 48.9% depends on agriculture, predominantly through smallholder production for livelihoods (CSO, 2018; Chapoto *et al*., 2018).

Livestock farming is an important contributor to the agricultural Gross Domestic Product and national economy as a whole. Its contribution to the agricultural GDP as of 2015 was estimated at 35%. The majority of the population depends on livestock especially cattle for livelihood and therefore, the socio-economic impact of infectious livestock diseases on livestock production, trade and public health cannot be over emphasized (AGO, 2015).

A review on livestock viral diseases in Zambia (Mweene *et al.,* 1996) raised the need for research into various aspects of viral diseases in Zambian livestock including the need to develop robust diagnostic methods. Bovine Leukemia Virus (BLV) is one of the viral infections that have been implicated in economic losses to the cattle industry worldwide (Polat *et al.*, 2017b) and yet the infection remains under studied in Africa and Zambia in particular.

Together with the closely related simian T-cell lymphotropic virus (STLV) and human T-cell lymphotropic virus types I and II (HTLV-I and -II), Bovine Leukemia Virus (BLV) is an oncogenic member of the *Retroviridae* family, genus *Deltaretrovirus* (Juliarena *et al*., 2017). It is the causative agent of Enzootic bovine leucosis (EBL), a disease of cattle characterized by persistent lymphocytosis in approximately 30% of infected animals. It has been estimated that 5% of infections with the virus usually progress to lymphosarcoma and eventual death. EBL is responsible for major economic losses attributed to reduced reproduction efficiency, reduced milk production, weight loss, increased cost of diagnostic and veterinary care, reduced life span and reduced carcass value. Mostly due to management practices on dairy farms, BLV is more commonly detected in dairy cattle as compared to beef animals even though it has negative economic effects on both sectors (Juliarena *et al*., 2017).

Generally, BLV infection in cattle is mostly asymptomatic, with clinical disease, especially the malignant form of B-cell lymphoma, predominantly occurring in older animals of more than four years of age. Transmission mostly occurs horizontally through contact with fluids such as blood and milk. Congenital and mechanical transmission through blood sucking insects have also been reported (Ooshiro *et al*., 2013; Juliarena *et al*., 2017).

Before the 1960s, EBL was endemic in dairy cattle herds in Europe and North America but has since spread to all continents mostly through trade in breeding animals (EFSA AHAW, 2015; Polat *et al*., 2017b). Whilst serological evidence of BLV infection has been reported on all continents (EFSA AHAW, 2015; Polat *et al*., 2017b), implementation of prevention and control programs over the years has resulted in eradication or low infection levels in most European countries like Denmark, Finland, Switzerland, Estonia, the Netherlands and Poland (EFSA AHAW, 2015; Polat *et al*., 2017b). In the United States of America (USA), overall seroprevalence of 38.6% with positive rates being higher in dairy cattle than beef animals have been reported (Bauermann *et al*., 2017). Although prevalence levels of BLV may vary between and within countries, the virus still remains a cause of major economic losses in the cattle industry worldwide. For instance, in the USA, annual BLV associated economic losses in the dairy industry are estimated at $285 million (Bartlett *et al*., 2014).

Molecular characterization studies have revealed the existence of at least 10 genotypes (Polat *et al*., 2017b). Generally, there appears to be some geographical variation in the distribution of the genotypes. Genotypes 2, 5, 6 and 9 have been shown to occur mostly in South American countries, genotypes 7 and 8 in Russia and Eastern European countries, genotype 10 in Thailand, China and Myanmar and genotypes 1 and 4 have been detected on almost all continents (Matsumaru *et al*., 2011; Polat *et al*., 2016; Pandey *et al*., 2017; Polat *et al*., 2017b). Despite many studies showing widespread BLV infection worldwide, there is very little information on the status of BLV and genetic variations of the causative agent in Africa.

Serological evidence of BLV infection has been reported in several African countries including Botswana, Namibia, Ethiopia, Tanzania, Zambia and South Africa (Walrand *et al*., 1986; Mushi *et al*., 1990; Kaura and Hübschle, 1994; Heinonen and Assefa, 1995; Schoepf *et al*., 1997; Meas *et al*., 2004; Ndou *et al*., 2011). However, so far only Egypt and Zambia have reported molecular

detection and characterization of the virus in dairy cattle (Zaghawa *et al*., 2002, Pandey *et al*., 2017).

In Zambia, only two studies involving BLV have been conducted (Meas *et al*., 2004; Pandey *et al*., 2017). This study determined the prevalence and molecular epidemiology of BLV in beef cattle reared in the traditional sector in Southern, Western and Eastern provinces of Zambia.

1.2 Statement of the problem

Cattle farming is a major economic activity in the traditional agricultural sector in Zambia. The three major provinces where traditional cattle farming is practiced in the country are Eastern, Southern and Western provinces (CSO, 2018). Production is mainly constrained by infectious diseases (Mumba *et al*., 2018) and one of the neglected viral infections with considerable economic impact on cattle farming in Zambia is EBL caused by BLV. Although EBL has been identified as a major factor affecting the cattle industry worldwide, (Juliarena *et al*., 2017), very little is known about the prevalence and molecular diversity of the causative virus in Zambia.

Other than economic losses, there has been growing evidence that BLV could be zoonotic. BLV proviral DNA has been detected in human breast tissue and it has been hypothesized that BLV may be a contributing factor to human breast cancer (Buehring *et al*., 2017; Schwingel *et al*., 2019). Viral DNA has also been detected in human blood at least in one study by Buehring *et al*., 2019 and it was suggested that the virus may circulate through blood to various tissues and potentially infect and possibly lead to cancer development in the tissues (Buehring *et al*., 2019). BLV may thus be considered as a potential risk to public health particularly in traditional cattle farming areas where consumption of unpasteurized milk is a common practice.

1.3 Rationale

Through implementation of control and prevention programmes, most European countries have eradicated or reduced BLV infections in cattle to very low rates, the disease attributed to BLV infections is no longer considered to be a source of major economic losses in the cattle industry in these countries (EFSA AHAW, 2015; Polat *et al*., 2017b). Implementation of prevention and control programs require preliminary data with regards to the epidemiology of the infection which is what this study seeks to collect. The first evidence of BLV infection in traditional cattle herds in Zambia was produced in a study conducted fourteen years ago (Meas *et al*., 2004). Results from that study revealed the seroprevalence of BLV infection in traditional cattle to be at

5%. A recent study conducted on a single dairy cattle herd (Pandey *et al*., (2017), provided the first report of confirmed EBL in Zambia. Whilst these studies suggested that BLV could be an important infection among cattle in Zambia, they were very limited in geographical coverage and number of animals (herds) examined. Little is known about infection rates, prevalence and molecular diversity of BLV and how the virus infections affect traditional farmers in Zambia. The information on prevalence, molecular diversity of BLV and viral infection rates in traditional cattle sector will reduce this knowledge gap and contribute to national efforts aimed at developing prevention and control measures for BLV infection in the country.

1.4 Objectives

1.4.1 Main objective

The main objective of this research was to determine the prevalence and to genetically characterize Bovine leukaemia virus in selected traditional cattle farming areas of Zambia.

1.4.2 Specific objectives

The specific objectives were to:

(i) Determine the prevalence of BLV from beef cattle in the traditional sector in Southern, Western and Eastern provinces of Zambia based on the detection of the *env* gene.
(ii) Use the *env* gene to determine the genotype of the BLV strains prevailing in beef cattle in the traditional sector in the Southern, Western and Eastern provinces of Zambia.

1.5 Organization of the Dissertation

This dissertation is organized in five chapters. Chapter one provides a brief background on the study area, study population and the current information on BLV in the study area. It also highlights the purpose and potential benefits of the study and highlights the objectives. Chapter two endeavors to provide a foundation of knowledge on BLV, this includes the history and epidemiology of BLV worldwide, the impact of BLV infection on animal welfare, the economy and risk to public health, progression of disease, methods of diagnosis, treatment and control, existing genotypes and previous studies and findings conducted on BLV. It aims to identify gaps in knowledge on the virus within the study area and to provide information that guides the research. Chapter three explains the various methods used to conduct the research and achieve the objectives. Chapter four presents and analyses the findings of the research and shows how the objectives were met. Chapter five provides an interpretation of the research findings in relation

to previous studies on BLV as well as the current status and practices in the study area. It also explains the new information on BLV brought to light by the research and the possible implications on the study population. Chapter six summarizes the findings of this research and how the objectives were achieved. It highlights recommended follow up actions to fulfill and apply the benefits to the study population, it also further research required to close the knowledge gaps identified during this research.

CHAPTER TWO
LITERATURE REVIEW

2.1 Livestock farming and Bovine leukemia virus in Zambia

Zambia's cattle population and beef industry comprises traditional and commercial sectors with the traditional sector making up approximately 84% of the population. The leading provinces where traditional cattle farming is practiced are Southern, Western and Eastern provinces (CSO, 2018; Mumba *et al*., 2018). Commercial farmers mostly own large herds of cattle, the majority of which are exotic breeds, while traditional farmers keep local breeds of cattle alongside crop farming (Mumba *et al*., 2018). Traditional farmers keep cattle for various purposes including for draught power, as a form of financial savings to convert to cash when financial obligations arise and for production of manure. Traditional cattle is also used in local practices such as paying bridal price and in some cultures it serves as a symbol of high social status. In day to day lives, cattle-rearing takes secondary importance in income generation compared to crop farming which is considered as the primary economic activity. This results in less than satisfactory productivity of the traditional beef sector (Mumba *et al*., 2018, Randolph *et al*., 2008). Traditional cattle farmers report high disease burden as the cause of the insufficient productivity (Mumba *et al*., 2018), raising a need for improved veterinary care in the traditional beef sector.

Although there are several important virus livestock disease outbreaks in Zambia including the foot and mouth disease and swine fever, Bovine leukemia virus and its associated diseases was not among the notable viral infections in Zambia over two decades ago (Mweene *et al*., 1996). The first report on BLV in the country appeared over a decade and half ago (Meas *et al.,* 2004) in which a BLV seroprevalence of 5% in traditional cattle was observed. In an analysis of BLV infections in a single dairy cattle herd in the Central province of Zambia, the *env* gene was detected in seven cattle including in two that were symptomatic and five that did not show any disease symptoms (Pandey *et al*., 2017). Phylogenetic analysis of the *env* gene in the virus isolates indicated that all of them were closely related to genotype 4 BLV strains of Eurasian origin. It was hypothesized that, the isolates from this study may have been introduced into the country through importation of exotic cattle breeds.

Despite studies showing widespread BLV infection worldwide, there are very limited studies focusing on the molecular epidemiology of BLV in Africa and Zambia in particular. The prevalence and molecular diversity, pathogenesis, infection rates, genome sequence variations and other biological properties reviewed revealed that very little or no records exist on the status of BLV in traditional cattle farming areas of Zambia.

2.2 Enzootic Bovine Leucosis and molecular biology of Bovine Leukemia Virus

Bovine Leukemia Virus (BLV) causes enzootic bovine leucosis (EBL) a contagious lymphoproliferative disease. The disease is characterized by persistent lymphocytosis in 30-50% of cases and lymphosarcoma in approximately 5% of cases, it is otherwise asymptomatic (EFSA AHAW, 2015). BLV belongs to the family *Retroviridae,* subfamily *Orthoretrovirinae* and genus *Deltaretrovirus* (International Committee on Taxonomy of Viruses, ICTV, 2011; Polat *et al*., 2017b). The virion particle is enveloped and the capsid protein p24, encapsulates a single-stranded positive-sense RNA genome (Figure 2.1) that has been determined to be 8714 nucleotides long and is flanked by two identical long terminal repeats (LTR) (Aida *et al*., 2013; Polat *et al*., 2017b).

The BLV genome encodes structural and non-structural genes including *gag, pol, pro* and *env* typically found in members of the family *Retroviridae* (Polat *et al.*, 2017b). The main structural gene product is *gag* protein which is proteolytically processed into three polypeptides, p15, p24 and p12 from a precursor protein of 45 kDa (Polat *et al*., 2017b). The matrix protein (p15), a 15 kDa protein provides a structural scaffold between the virion core and the BLV envelope, p24 is the capsid protein that encloses the RNA genome and the host immune response usually targets this protein. Genomic RNA is wrapped by p12 protein creating the nucleoprotein complex in which the virus genome is tightly packaged (Polat *et al*., 2017b). The *pol* locus encodes reverse transcriptase enzyme responsible for viral replication while the *pro* gene encodes a protease enzyme.

The *env* locus encodes envelope glycoproteins (gp51 a surface protein component and gp30, a transmembrane glycoprotein) involved in the interaction of the virion particle and host cells bearing target receptor proteins during infection (Rice *et al*., 1987). The *env* gene product is a 72 kDa precursor polypeptide which is proteolytically cleaved to produce the two envelop proteins, gp51 and gp30 (Polat *et al.*, 2017b). The gene has been used in various studies analyzing genetic

diversity in the *Retroviridae* family including HIV and BLV (Qadri *et al*., 2016; Polat *et al*., 2017b). The choice for the *env* gene in retroviral phylogenetic studies has been justified by the fact that the gp51 glycoprotein component is involved in virion particle-cell interactions during the process of infection and has also been implicated in cytopathic effects such as synctium formation in infected animals (Polat *et al*., 2017b). In addition several regions including the CD4+ and G epitopic regions within the N-terminal segment of the gp51 gene product of the *env* gene have been identified as major epitopes that elicit host immune responses during infection (Polat *et al.,* 2017b).

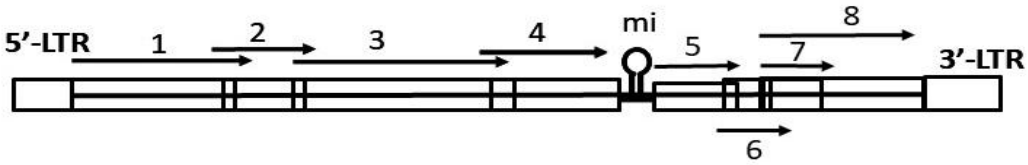

Figure 2.1. Schematic presentation of the BLV genome showing the major genome sequences representing genes as follows: 1, *gag*; 2, *pro*; 3, *pol;*4, *env*; mi, miRNA; 5, R3; 6, G4; 7, *rex*; 8, *tax* and LTR, long terminal repeats. Arrow positions depict known gene overlaps. (Adapted from Polat *et al.*, 2017b)

Like HTLV-1, BLV has a unique non-host origin sequence called the *pX* region, situated between the *env* gene and the 3'LTR. In BLV, this region encodes regulatory proteins Tax and Rex as well as R3 and G4 proteins (Aida *et al*., 2013). The Tax protein plays a critical role in virus induced leukomogenesis while the Rex protein is responsible for nuclear export of the viral RNA and accumulation and translation of viral messenger RNA in the host cell cytoplasm. The R3 and G4 proteins are responsible for high viral load maintenance (Aida *et al*., 2013).

Furthermore, the BLV genome has RNA polymerase-III-encoded viral microRNAs (miRNAs) located between the *env* and *pX* regions. These are expressed in both tumor and pre asymptomatic phase of infection. The miRNAs are not indispensable for viral infectivity, but they have been shown to alter at least six target genes associated with apoptosis, immunity, cell signaling and oncogenesis (Polat *et al*., 2017b; Juliarena *et al*., 2017).

Bovine leukemia virus attaches to host cells harboring CD5+, IgM+ and CD5− IgM+ B-cells; CD2+, CD3+, CD4+, CD8+, and γ/δT-cells receptors. In addition, monocytes, granulocytes in

peripheral blood and lymphoid tissues of cattle are also targeted by the virion particle but the main target cells are B lymphocytes (Polat *et al*., 2017b). Following initial infection, a high initial viral replication occurs by expression of virions and further infection of more target lymphocytes, this initial infection stage and immune response produces a self-resolving 'flu-like' syndrome in most cases. However, it is limited by the host immune system after about four to eight weeks, reducing the proviral load. The infection then continues to spread through clonal expansion of infected host cells. The proviral load is usually about 1% at this stage and about 70% of infected animals are asymptomatic carriers (EFSA, 2015; Juliarena *et al*., 2017).

The asymptomatic stage of BLV can last from a few months to several years after which, 30-50% of infected animals develop a persistent lymphocytosis due to polyclonal expansion of B lymphocytes. This phase is characterized by a perpetual increase in B cells circulating in the peripheral blood and it results in immune perturbation. This perturbation may affect productivity in several ways that may manifest in form of reduced milk production, reproduction inefficiency and susceptibility to infection (EFSA AHAW, 2015; Polat *et al*., 2017b).

Malignant lymphomas are observed in 5% of cases mostly in cattle over four to five years of age. The malignancies induce disruption of the spleen and enlargement of the lymph nodes, which can be visible under the skin. The malignant cells can penetrate into vital organs including right auricle of the heart, intestine, kidney, lung, liver, and uterus (EFSA AHAW, 2015; Polat *et al*., 2017b), The clinical signs of these malignancies involve digestive disturbance, weight loss, weakness, reduced milk production, loss of appetite, and enlarged lymph nodes (Polat *et al*., 2017b).

Enzootic bovine leucosis is transmitted horizontally or vertically through transfer of infected cells in fluids such as blood and milk (Sajiki *et al*., 2017; Juliarena, *et al*., 2017). Animals with persistent lymphocytosis are efficient in disease transmission due to the high number of infected cells (Rodriguez *et al*., 2011).

Horizontal transmission of BLV occurs mostly through iatrogenic procedures and other cattle management practices that involve transfer of infected blood. These procedures include reuse of unsterilized hypodermic needles, reuse of unsterilized tools for activities such as dehorning and ear tattooing as well as not changing examination sleeves during reproductive examination and rectal palpations (Bartlett *et al*., 2014; Rodriguez *et al*., 2011). Prolonged direct contact between

infected and healthy animals as well as natural breeding have also been considered as risk factors for BLV transmission (Rodriguez *et al*., 2011; Bartlett *et al*., 2014). Additionally, mechanical transmission through blood sucking insects has also been reported (Ooshiro *et al*., 2013), meanwhile movement of infected animals is said to be the major mode of transmission of BLV between herds (EFSA AHAW, 2015).

Vertical transmission occurs mostly through feeding of infected milk or colostrum to calves. It has been reported that specific antibodies obtained by natural passive transfer confer protection to calves (Juliarena *et al*., 2017). Other than feeding of infected milk to calves, intrauterine transmission of BLV in pregnant dams with high proviral load has also been demonstrated (Sajiki *et al*., 2017).

2.2.1 Economic implications of Enzootic Bovine Leucosis

Enzootic bovine leucosis is responsible for major economic losses to cattle farmers. In places where attempts have been made to quantify the losses, they have been estimated to be $412 per case and the mean annual cost of subclinical infection at 50% prevalence was estimated at $6,406 (Rhodes *et al.,* 2003). Economic losses incurred due to subclinical BLV are not easy to quantify as this state is associated with immune perturbation and susceptibility to opportunistic infections that all contribute to the resultant losses (Bartlett *et al*., 2014). The development of malignant lymphomas is the most obvious negative result of BLV infection but its incidence in an infected herd is usually low. As a result, the effects of subclinical infection are potentially more economically damaging even though the losses it causes are less obvious than those of lymphomas (EFSA AHAW, 2015; Rhodes *et al*., 2003). To determine the cost of BLV clinical and subclinical disease in mid Atlantic dairy herds in America, Rhodes, *et al.,* (2003) defined cost of clinical disease as the direct cost to the producer resulting from death or culling of cows due to Lymphosarcoma each year. These costs were estimated to include those related to diagnosis and treatment, loss of milk production due to culling, replacement of culled cows and fetal wastage in the case of culled pregnant cows. They defined the cost of subclinical infection as the direct cost to the producer due to reduced milk production and premature culling of BLV infected cows without Lymphosarcoma.

Enzootic bovine leucosis has been commonly associated with economic losses to the dairy cattle industry compared to the beef cattle industry, probably due to its prevalence being generally

higher in dairy cattle as well as due to the economic losses encountered due to reduction in milk production. However, the beef cattle industry also suffers economic impact attributed to BLV infection. In 2014, it was reported that malignant lymphomas are responsible for 13.5% and 26.9% of beef cattle and dairy cattle condemnations at slaughter plants respectively (Bartlett *et al*., 2014). Economic losses of BLV mostly affect the production traits of the industry and these include reduced milk production, reduced carcass value due to weight loss and reduced reproduction efficiency (Juliarena, *et al*, 2017), of which both dairy and beef herds are equally affected. Other factors associated with economic losses include cost of diagnostic and veterinary care, shortened lifespan and the inability to ship or sell animals, semen, embryos, and animal by-products to countries, regions or herds free of the disease. Added to these, the cost of eradication programs due to trade restriction measures were considered to be a significant factor (Juliarena *et al*., 2017; Gnad *et al*., 2004). Just like the case in dairy cattle, BLV in beef herds can also serve as a reservoir for the infection and can be transmitted to dairy herds or other beef herds (Bartlett *et al*., 2014) resulting in further losses.

Control measures are required to be implemented in order to prevent further spread and consequently further economic losses once prevalence is confirmed. There is no cure for the virus infection and so far there has not been a successful vaccine therefore the most effective method of BLV control is culling of infected animals. However, this method is bound to face resistance from cattle owners as it results in immediate losses (Juliarena *et al.*, 2017). An alternative method of control is maintenance of two herds i.e., BLV-free and BLV-infected herds that are kept and managed separately with separate equipment to ensure the negative herd stays negative, it also requires routine testing to note any new infections in the negative herd (Juliarena *et al*., 2017). BLV resistant alleles of the DRB3 Major Histocompatibility complex (MHC) gene has been a subject of research, animals that carry this allele have be hypothesized be resistant to BLV and thus may possibly offer a method of BLV control through genetic selection (Bartlett *et al*., 2014: Juliarena *et al*., 2017).

2.2.2 Epidemiology of Enzootic Bovine Leucosis

Natural BLV infection has been confirmed in domestic cattle, zebu and water buffalo while experimental transmission has been demonstrated in a number of species including sheep, goats, and rabbits (EFSA AHAW, 2015). There has been cumulative evidence that BLV could be

zoonotic as studies have shown that it has been detected in both healthy and cancerous human breast tissue and blood (Buehring *et al*., 2001; Buehring *et al*., 2017; Schwingel *et al*., 2019; Buehring *et al*., 2019). It has been hypothesized that BLV DNA may play a role in breast cancer development in humans (Buehring *et al*., 2017; Schwingel *et al*., 2019).

It has been suggested that BLV was present in Europe during the 19th century, from where it spread to America during the first half of the 20th century then back to Europe (OIE, 2018). Through the implementation of control and eradication programmes, the majority of European countries have successfully controlled and eradicated BLV (EFSA AHAW, 2015). The infection however remains a source of concern to the rest of the world including several countries in Eastern Europe. It has spread to all continents via trade of cattle breeds and is prevalent in cattle worldwide at varying infection levels (Polat *et al*., 2017b).

Prevalence of BLV varies with method of diagnosis, with geographical locations even within the same country or region, among herds, between dairy and beef cattle, between fattening beef herds and breeding beef herds and in different age groups of cattle. It can also be reported as herd level or animal level prevalence. A study conducted by LaDronka *et al*., (2018) in US dairy cattle revealed a herd level prevalence of BLV to be at 94.2% and within herd prevalence ranged from 0%-96.9% with an average of 46.5%. Bauermann *et al*., (2017) conducted a study that tested cattle presented for slaughter in the US for BLV. They reported prevalence of 46.7% from plants that processed mostly dairy cattle and 33.6% from plants that mainly processed beef cattle. Prevalence levels of BLV reported in South America range from 29.1% in Chile, 30.7% in Bolivia, 42.3% in Peru, 50% in Paraguay and 77.4% in Argentina with up to 90.9% herd level prevalence (Polat *et al*., 2016). A study conducted in China that sampled six provinces for dairy cattle samples and 15 provinces for beef cattle samples revealed prevalence of 49.1% in dairy cattle and 1.6% in beef cattle (Yang *et al*., 2016b). In 2011, a study in Japan reported a 34.7% in dairy cattle, 7.9% in fattening beef cattle and 16.3% in breeding beef cattle with an overall herd level prevalence of 68.1% (Murakami *et al*., 2011). Other reported prevalence levels include 9.1% in Myanmar and 58.7% in Thailand (Polat *et al*., 2017a; Lee *et al*., 2016).

Bovine leukemia virus is prevalent in Africa and several studies have been conducted that have reported prevalence at varying levels including prevalence rates of 37.7% and 72.8% in cattle under 2 years of age and those above the age of 2 years respectively in Egypt (Zaghawa *et al*.,

2002), 41% and 21.4% in dairy and beef cattle respectively in Tanzania (Schoepf *et al*., 1997), 12.6% in Botswana (Mushi *et al*., 1990), 12.3% in Namibia (Kaura *et al*., 2011), 5% in Zambia (Meas *et al*., 2004) and an average herd level prevalence of 96% across 5 provinces in South Africa (Maboe *et al*., 2017).

Using existing sequences in GenBank and newly generated sequences, Rodriguez *et al*., (2009) showed that BLV can be classified into six sequence clusters based on phylogenetic analysis of the *env* gene. They called the sequence clusters genotypes one to six. They also suggested that one of the identified strains belonged to a seventh group which they designated genotype seven. Their results aligned with the earlier classification of BLV into seven groups A to G based on restriction fragment length polymorphism (RFLP) (Fechner *et al*., 1997) and was further solidified by results from Moratorio *et al*., (2010) who confirmed the six genotypes and the existence of a seventh genotype. The study also supported the hypothesis that these genetic groupings correlate with geographical origin. Since then, more genotypes have been identified bringing the total number to 10 so far. These are genotypes 8 from Croatia, 9 from Bolivia and 10 from Thailand and Myanmar (Balic *et al*., 2012; Polat *et al*., 2016; Lee *et al*., 2016; Polat *et al*., 2017a).

Studies reveal that South America shows the most genetic diversity of BLV strains amongst all the continents with the presence of up to 8 genotypes (Rodriguez *et al*., 2009; Polat *et al*., 2017b), genotypes 1-7 have been reported on this continent, with genotype 1 mostly in Uruguay, Argentina, Brazil and Colombia, 2, 3 and 4 in Argentina, 3 in Colombia, 5 in Brazil and 6 in Brazil and Argentina. Additionally, genotype 7 has been reported in Chile and more recently genotype 9 has been reported in Bolivia (Rodriguez *et al*., 2009; Moratorio *et al*., 2010; Polat *et al*., 2016; Usuga-Monroy *et al*., 2018).

In North and Central America, genotypes 1, 3 and 4 have been detected in the USA, genotype1 and 5 in Costa Rica and genotype 1 in the Caribbean (Polat *et al*., 2017b; Yang *et al*., 2016a). On the Asian continent, genotype 6 has been reported in India, 1, 6 and 10 in Thailand, 10 in Myanmar, 1, 2 and 3 in Japan and 1 and 6 in Jordan (Gautam *et al*., 2018; Lee *et al*., 2016; Polat *et al* 2017a; Matsumura*et al*., 2011; Ababneh*et al*., 2012). In Europe, genotypes 1, 3, 4, 7 and 8 have been reported from various countries. Genotype 1 was reported in Germany, genotype 3 in France, 7 in Russia, Italy, Moldova and Poland and genotype 8 in Croatia, Russia and Ukraine.

On the other hand, genotype 4 has been reported in most affected countries including Belarus (Rola-Luszczak *et al*., 2013; Pluta *et al*., 2017; Polat *et al*., 2017). Genotype 1 has been reported in Australia (Coulston *et al*., 1990) whilst in Africa, genotype 4 has been reported in Zambia (Pandey *et al*., 2017).

2.3 Diagnosis of Bovine Leukemia Virus

In the past, bovine leukemia virus diagnosis was mainly dependent on clinical observations, hematological analysis and detection of lymphomas (EFSA AHAW, 2015). Clinical diagnosis involved observation of clinical manifestations of the infection that include lymphadenopathy, asthenia, weight loss, constipation, tachycardia, posterior paresis, exophthalmos, fever and palpable lymphomas (Polat *et al*., 2017b; Mammerickx *et al*., 1985). Hematological analysis involved the establishment of an increased absolute lymphocyte count due to persistent lymphocytosis. Due to persistent lymphocytosis being a non-specific symptom of BLV, hematological analysis is not always a reliable method of diagnosis for BLV, the fact that it only occurs in 30%-50% of infected animals, makes it useful as a herd level screening diagnosis but is not applicable for individual diagnosis (Mammerickx *et al*., 1985). Lymphomas are detected by observation of the presence of soft, grey-white lymphomas in multiple tissues on postmortem examination. Tissues that are normally affected include lymph nodes, heart, liver, spleen, abomasum and kidneys. Its limitation is that the approach cannot distinguish between EBL and sporadic lymphomas (EFSA AHAW, 2015). The percentage of infected animals that present with tumors is usually very low, therefore the detection of one or more tumors would indicate herd infection but would raise the need for more diagnostic tests to be conducted on all the animals of the herd (Mammerickx *et al*., 1985).

Clinical diagnosis of EBL needs to be confirmed by specific laboratory tests. Over the years, different techniques for detection of BLV have been developed and used and these can be divided into two main types i.e. serological and nucleic acid detection (Mammerickx *et al*., 1985; Polat *et al*., 2017b).

2.3.1 Serological diagnosis

Bovine leukemia virus infection leads to a lifelong immunological response in the host that produces antibodies against the p24 viral protein and the gp51 envelope glycoprotein. These antibodies can first be detected 2-12 weeks after infection in serum or milk. Maternally derived

antibodies last up to six to seven months and are indistinguishable from antibodies resulting from an active infection. Therefore, it is recommended that diagnosis during this time should be by proviral DNA detection (EFSA AHAW, 2015; Polat *et al*., 2017b; OIE, 2018; Mammerickx *et al*., 1985).

The first test to be used for BLV antibody detection was the agar gel immunodiffusion (AGID) test based on detection of the p24 antigen. However, it was later observed that the gp51 antigen was more efficient as antibodies to this antigen appear earlier and more regularly and reach higher titres than those against p24 antigen (Mammerickx *et al*., 1985). AGID is simple and relatively inexpensive and can be used to test a lot of serum samples at once but it is not sufficiently sensitive and is not suitable for milk samples (Mammerickx *et al*., 1985; Polat *et al*., 2017b).

Enzyme Linked Immunosorbent Assay (ELISA) has over the years replaced AGID for BLV routine diagnosis and several ELISA test kits are currently commercially available. It has been reported to have a higher sensitivity than AGID and it can be used for detection of antibodies in serum as well as milk. Both AGID and ELISA are unable to distinguish active infection antibodies and passive maternal antibodies (EFSA AHAW, 2015).

Other serological tests applied in the diagnosis of BLV include indirect fluorescent antibody test, indirect immunoperoxidase test, early polycaryocytosis inhibition test, complement fixation test, virus neutralization test, passive hemagglutination assay and radioimmunoassay (EFSA AHAW, 2015).

2.3.2 Diagnosis by Polymerase Chain Reaction

Being a retrovirus, BLV integrates its reverse transcribed genome at different positions of the host genome and remains part of the cellular genome where it exists as a provirus. This allows for detection of the proviral DNA using polymerase chain reaction (PCR) even in the absence of detectable antibodies (Qadri *et al*., 2016; Polat *et al*., 2017b). Nucleic acid detection methods broaden the range of samples that can be used and they are useful in circumstances where serological methods would be inapplicable as the cases are, for instance in young calves with maternal antibodies, differentiation between sporadic and infectious lymphoma tumour tissue from suspected cases collected at slaughter houses and before development of antibodies to

BLV. Nucleic acid detection tests are also valuable in cases of weak positive or uncertain results in serological tests as well as for research purposes (OIE, 2018; Polat *et al*., 2017b).

Various methods of nucleic acid detection using PCR have been applied worldwide, including standard PCR, semi-nested PCR, nested PCR and real time PCR. These methods target different segments of the BLV genome including the *env*, *gag*, *pol* and *tax* genes as well as the LTR region. The *env* gene is the most frequently targeted gene in PCR diagnosis as well as phylogenetic analysis as it is involved in virion particle-cell interactions during the process of infection and synctium formation, it is also known to have regions of variability as well as highly conserved regions (EFSA AHAW, 2015; Fechner *et al*., 1997; Polat *et al*., 2017b).

According to the OIE world organization for animal health manual of diagnostic tests and vaccine for terrestrial animals 2018, the most rapid and sensitive method of BLV diagnosis so far is real-time PCR. The manual recommends and describes two PCR methods i.e. nested PCR procedure described by Fechner *et al.*, (1996) and real-time PCR described by Rola-Luszczak *et al.*, (2013).

Nested PCR is said to be highly sensitive. However, its high sensitivity raises a risk of false positive results due to cross-contamination between samples during sample preparation (OIE, 2018, Polat *et al*., 2017b). For this reason, several protocols are usually adopted to minimize the risk of contamination (Mammerickx *et al*., 1985). These include the use of laminar air-flow hoods, separate rooms for different steps of the procedure, use of new gloves and inclusion of a negative control such as water (OIE, 2018).

This study aimed to use nested PCR to determine the prevalence of BLV in the traditional cattle rearing sector in Zambia, it also aimed to determine the genetic diversity of BLV strains isolated in the sector, hence reducing the existing knowledge gap on the BLV status in the country.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site

Archived whole blood samples were used in the study. These blood samples were obtained from the major traditional cattle rearing provinces in Zambia i.e. Western, Southern and Eastern provinces. The three provinces were selected for inclusion as they have the highest cattle population in the country the majority of which belongs to the traditional sector (CSO, 2018). Generally, farmers practicing traditional cattle rearing keep cross breeds of beef cattle and indigenous breeds, namely Tonga, Barotse and Angoni, predominantly found in Southern, Western and Eastern provinces of Zambia, respectively. The Tonga and Barotse breed belong to the *Bos Taurus africanus* (Sanga) while the Angoni belong to *Bos indicus* (Zebu) breeds of cattle. The samples were collected from four districts namely Senanga, Namwala, Mazabuka and Lundazi (Figure.3.1).

3.2 Study design

This study was based on laboratory experiments. It was conceived to determine prevalence and genetic diversity of BLV in traditional cattle rearing sector of Zambia. Archived whole blood from 880 apparently healthy adult animals was pooled in tens to give 88 samples before DNA extraction and examination by PCR. Pooled prevalence was estimated using EpiTools epidemiological calculator open source software (http://epitools.ausvet.com.au/content.php?page=PooledPrevalence). Amplicons of positive samples were sequenced by the Sanger dideoxy chain termination method and phylogenetic analysis was conducted to determine the evolutionary relationships of BLV strains isolated from the study areas.

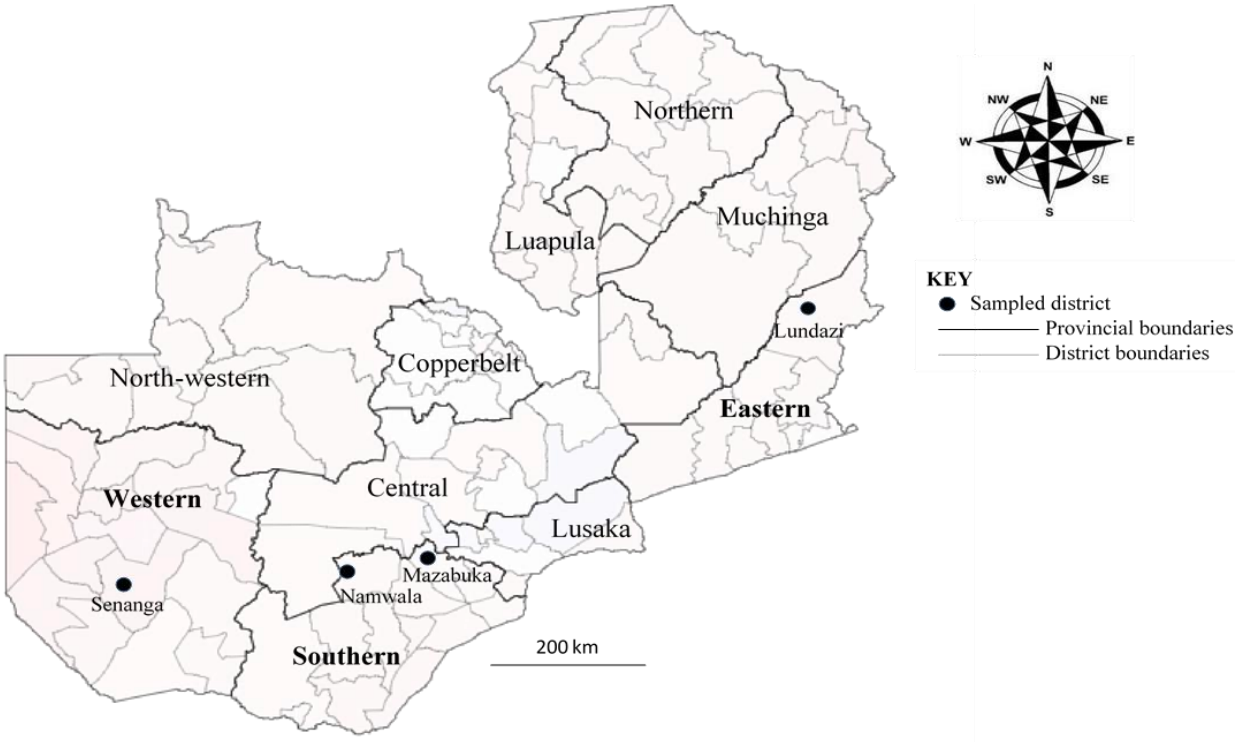

Figure 3.1. Map of Zambia showing sampling areas. The three provinces where the samples were collected are in bold text

3.3 Ethical considerations

The cattle whole blood samples were collected based on owner's willingness to participate. Ethical clearance waiver from the University Of Zambia Biomedical Research Ethics Committee (UNZABREC) has been granted. Ethical clearance reference number NASREC: 2019-AUG)-001.

3.4 Sample pooling

Archived whole blood from 880 apparently healthy adult animals was used. The blood was earlier collected in EDTA tubes over a period of 15months (March 2017 to May 2018) from three provinces (*n*=188, Southern; *n*=342, Eastern; *n*=350, Western). The samples were stored at -20^{o}C at the virology laboratory in the School of Veterinary Medicine, University of Zambia until analysis.

Pooled testing strategies are often used in estimation of prevalence of infections for programmes such as public health programmes, animal agriculture and vector surveillance programmes, and more so for rare infections (Cowling *et al*., 1999; Zhou *et al*., 2014; Sergeant *et al*., 1995; White,

2001). Such strategies offer a cost-effective alternative to testing samples from individual animals when the goal of a study is to estimate individual-animal prevalence (Cowling *et al*., 1999). The precision of estimates obtained with pooled testing may be comparable to individual-animal testing with the assumption that sensitivity of the test for the pool is approximately the same as it is for the individual sample (Cowling *et al*., 1999). This assumption is fulfilled in this study based on the high sensitivity of nested PCR. Here, the fixed pool size and perfect test option in EpiTools epidemiological calculator open source software which is based on method 3 described by Cowling *et al.*,(1999) was used. The method assumes perfect sensitivity and specificity as well as a binomial distribution for the number of positive pools. It notes a one to one relationship between the animal level prevalence and the positive pools. The lower confidence limit is at least zero and the upper is a maximum of one.

The samples were pooled in numbering order i.e. samples 1-10 made pool number 1 and so forth therefore, they were pooled according to region. The blood was pooled, with each pool containing 100 µl each of blood from 10 different animals giving a total of 88 pools with each pool containing a total of 1000µl of whole blood, the pools were stored at 80 ^{o}C until DNA extraction.

3.5 DNA extraction

Total DNA from whole blood samples was extracted using the QIAamp DNA Blood Mini prep Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. Briefly 20µl of QAIGEN proteinase K was pipetted into the bottom of labelled 1.5ml microcentrifuge tubes and 200µl of pooled sample was added to each of the tubes followed by 200µl of lysis buffer and the tubes were pulse-vortexed for 15 seconds. Tubes of these mixtures were incubated at 56^{o}C for 10 minutes followed by flash centrifugation at 6000×g. To each tube, 200µl of 100% ethanol was added and samples were again pulse-vortexed for 15 seconds and flash centrifuged at 6000×g. The sample mixture was each applied to an appropriately labelled QIAamp Mini spin column in a 2ml collection tube which was centrifuged at 20,000 ×g for one minute. The QIAamp Mini spin columns were transferred to clean 200µl collection tubes and the filtrate was discarded. To each spin column, 500µl of wash buffer AW1 was added, before centrifuging at 6000 ×g for one minute. The filtrate was discarded and columns were transferred to new collection tubes. To each column, 500µl of wash buffer AW2 was added and columns were centrifuged at 20,000 ×g for

three minutes. The filtrate was discarded and the columns were transferred to clean labeled collection 1.5 ml microtubes to which 50µl of elution buffer AE was added. These were left to stand at 25^{o}C for one minute after which they were centrifuged at 6000 ×g for one minute. The spin columns were discarded and the eluted DNA was stored at -30^{o}C until use. A total of 88 extracts were obtained.

3.6 DNA amplification by nested PCR

The BLV *env* gene was amplified by nested PCR using two sets of primers described previously (Fechner, *et al*., 1996, OIE, 2018).

The primer pair env5032 forward (5'TCTGTGCCAAGTCTCCCAGATA-3') and env5608 reverse (5'AACAACAACCTCTGGGAAGGGT-3') amplifies a 598-bp fragment in the first round and pair env5099 forward (5'-CCCACAAGGGCGGCGCCGGTTT-3') and env5521 reverse (5'-GCGAGGCCGGGTCCAGAGCTGG-3'), amplifies a 444-bp fragment. PCR was conducted using the One*Taq*® kit (New England BioLabs Inc, UK). The first round PCR mix was a total reaction volume of 25µl comprised of 12.5µl of One*Taq*®PCR master mix, 8µl of nuclease free water, 2.5µl of the DNA and 0.4µM of each primer. From the first round PCR product, 2.5µl was obtained and used as the sample in the second round PCR mix, with the same composition as first round PCR mix. A known BLV positive sample was used as a positive control and 2.5µl of nuclease free water was used in place of genomic DNA sample for the negative control. PCR reactions were carried out in a Veriti 96 well thermal cycler (Applied Biosystems) with the following thermal cycling conditions: one cycle of 95 ˚C for 2 minutes initial denaturation, followed by 40 cycles of 95 ˚C for 30 seconds, 55 ˚C for 30 seconds and 72 ˚C for 1 minute followed by one cycle of 72 ˚C for 5 minutes. PCR results were visualized using 1.5% agarose gel stained with ethidium bromide.

3.7 Prevalence analysis

Pooled prevalence was estimated using EpiTools epidemiological calculator open source software (http://epitools.ausvet.com.au/content.php?page=PooledPrevalence). The fixed pool size and perfect test option which is based on a method described previously by Cowling *et al.,* (1999) was used. The number of pools per province was taken as the total number of pools that contained at least one sample from that province. Table 3.1 shows the figures that were used as input data analyzed by the EpiTools epidemiological software.

Table 3.1. Input data used to calculate pooled BLV prevalence. Test method used =Perfect test & exact confidence limits, Pool size = 10 and confidence level= 95% for all inputs.

	Province			
	Southern	Western	Eastern	Overall
Number of tested pools	19	36	35	88
Number of PCR positive pools	5	5	7	17

3.8 DNA purification

Positive PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, positive samples that showed clean bands on gel electrophoresis were purified directly from the PCR products while those that showed multiple bands were excised from the gel prior to purification. For PCR amplification products, an equal volume of membrane binding solution was added to the products.

The excised bands were transferred to 1.5ml microcentrifuge tubes and 10µl of membrane binding solution was added for every10mg of gel slices. Tubes with agarose gel samples were vortexed and incubated at 56˚C until the agarose gel was completely melted after which they were processed the same way as the direct PCR product.

Both the melted gel solution and the direct PCR products were then added to labelled SV mini-columns with collection tubes and incubated for one minute at room temperature, then centrifuged at 16,000 ×g for one minute. The flow-through was discarded and the mini-columns reinserted into the collection tubes. Membrane wash solution of 700µl volume was added to the columns and columns were centrifuged at 16,000 ×g for one minute, the flow-through was discarded, the mini-column were reinserted and the wash step was repeated with 500µl of wash solution with centrifugation for five minutes. The collection tubes were emptied and the mini-columns reinserted, and were then centrifuged with the lids open to allow evaporation of any residual ethanol contained in the membrane wash solution. The mini-column was transferred to a clean 1.5ml microcentrifuge tube and 50µl of nuclease-free water was added to the mini-column

and these were incubated for one minute at room temperature. They were thereafter centrifuged at 16,000 ×g for one minute to elute the DNA and the mini-columns were discarded. Purified DNA was stored at -20˚C till use.

3.9 DNA sequencing

The purified PCR products were sequenced by the Sanger dideoxy chain termination method using BigDye™ Terminator v3.1 Cycle Sequencing Kit. To each PCR tube, 3µl of DNA along with 0.5µl of BigDye terminator, 3.7µl of 5× sequencing buffer, 12.5µl of nuclease free water were added to give a total volume of 20µl with a primer concentration of 0.15µM. Each DNA sample had 2 tubes, one with a forward primer and the other with a reverse primer. The tubes were then vortexed and flash centrifuged and were loaded into the thermocycler and reactions were conducted with the following temperature profiles: an initial run at 96˚C for 1 minute, 25 cycles of 96 ˚C for 10 seconds, 50 ˚C for 5 seconds and 60 ˚C for 4 minutes.

The DNA was precipitated by adding 2µl of EDTA, 2µl sodium acetate and 50 µl of 100% ethanol to the BigDye reaction mix. Care was taken not to expose the tubes to light. The tubes were vortexed and incubated at room temperature for 15 minutes and centrifuged at 10,000 × g for 15 minutes, the supernatant was discarded and 70µl of 70% ethanol was added to the tubes. The tubes were centrifuged again at 10,000 × g for 15 minutes, the supernatant was discarded and the tubes were dried in a vacuum for 5 minutes. To each tube, 15µl of formamide was added before vortexing and flash centrifuging, they were then loaded in the thermocycler for two minutes at 95˚Cfor denaturation. The tubes were transferred on ice and the contents were carefully pipetted into loading tray loaded on a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) sequencer and the reaction was run.

The sequence data in form of ab1 files was assembled and edited using the software Genetics ATGC version 7.5.1 (GENETYX Co., Tokyo, Japan). The sequences were deposited in DNA Data Bank of Japan (DDBJ) GenBank under accession numbers LC440653-LC440666.

3.10 Phylogenetic analysis

Phylogenetic analysis was inferred using the Maximum Likelihood method based on the K2 + G (Kimura 2-parameter model) (Kimura, 1980). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5306)). Phylogenetic

tree topological robustness was assessed by bootstrapping 1000 replicates. Model selection and phylogenetic tree reconstruction was performed in MEGA6 (Tamura, *et al*., 2013) and the output data was an unrooted phylogenetic tree which clearly depicts the evolutionary relationships among genotypes as seen in figure 4.2. Amino acid sequence alignment was conducted using BioEdit sequence alignment editor software version 7.0.5.3 (Hall, 1999) giving the clearly aligned amino acid sequences of the 13 BLV strains used for sequence alignment.

CHAPTER FOUR

RESULTS

4.1 Prevalence of Bovine Leukemia Virus

Out of the 88 pools of blood samples tested, a total of 17 pools were positive. Figures 4.1 A and B (34 out 88 pools are shown).

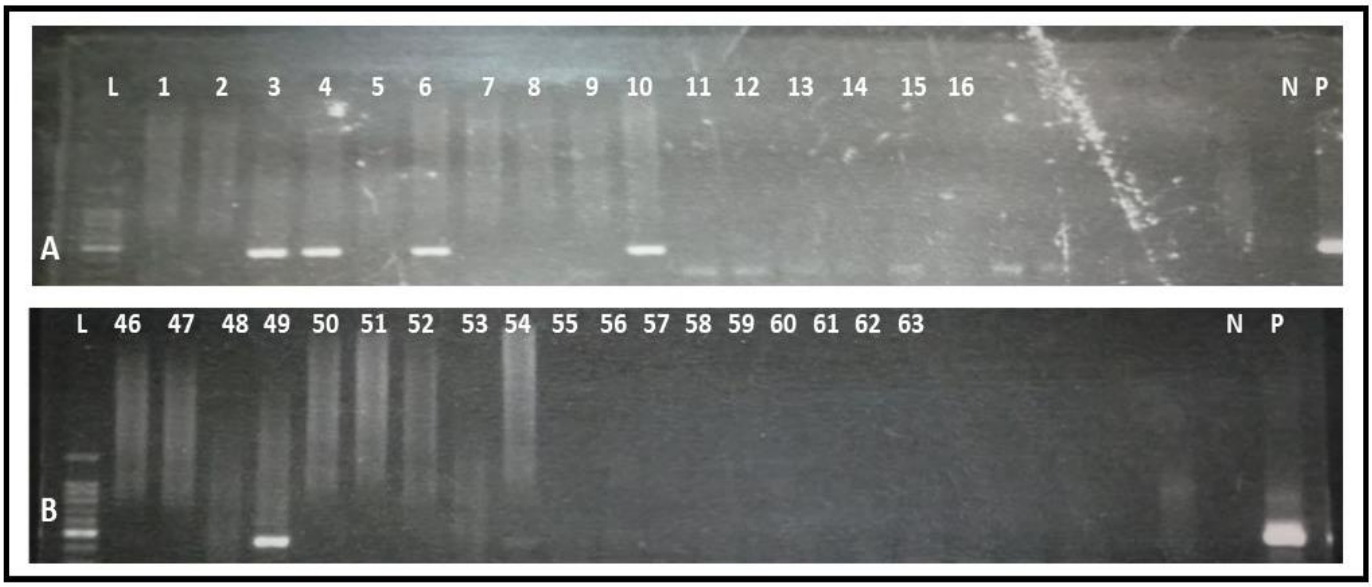

Figure 4.1(A) and (B). Representative gel photograph of PCR products showing the *env* gene fragment in whole blood. The *env* gene fragment is evident in pools 3, 4, 6, 10, 49 and54 as 444bp bands. N=negative control, P=positive control and L= 100bp ladder.

Of the 88 pools tested, a total of 17 pools were positive (19.3%) for BLV. The EpiTools epidemiological calculator open source software was used to calculate the prevalence of the BLV using the numbers of the pools from the three provinces under study (Table 3.1).

The BLV prevalence analysis data is recorded in Table 4.1. The pool size indicates the total number of pools for each province, Number +ve shows the number of positive pools for each province, confidence level shows at which confidence level each prevalence was calculated in the EpiTools epidemiological calculator, Est Prevalence shows the estimated prevalence output from the EpiTools calculator, 2.5% CL shows the prevalence estimate at 2.5% confidence level, 97.5% CL shows the estimated prevalence at 97.5% confidence level, Std. Error shows the

standard error and Percentage prevalence shows the Prevalence of BLV for each province expressed in percentage.

Table 4.1. Input and output data for bovine leukemia virus prevalence analysis.

	Province			
	Southern	Western	Eastern	Overall
Pool size	19	36	35	88
Number +ve	5	5	7	17
Confidence level	95%	95%	95%	95%
Est. Prevalence	0.0221	0.0148	0.0301	0.0212
2.5% CL	0.0088	0.0048	0.0095	0.0123
97.5% CL	0.0451	0.0343	0.0692	0.0338
Std. Error	0.0083	0.0066	0.0133	
Percentage prevalence	2.2%	1.5%	3.0%	2.1%

The estimated overall pooled prevalence was calculated to be 2.1%. Out of the 17 positive pools, 10 were from Southern and Western (i.e. five positive pools from each province) and seven were from Eastern Province. The pooled prevalence by province was estimated to be 3.0%, 1.5% and 2.2% for Southern, Western and Eastern provinces, respectively.

Statistical analysis was conducted to determine whether there was any significant differences in the proportion of BLV positive sample pools i.e. prevalence among the three provinces.

The chi square test of homogeneity determines if different populations have the same distribution of a categorical variable. Accessed at (https://courses.lumenlearning.com/wmopen-concepts-statistics/chapter/test-of-homogeneity/) 20/11/19.

Here we used the chi square test of homogeneity to determine if the three provinces under study have the same distribution or proportion of BLV positive sample pools.

Null hypothesis: There is no significant difference in the proportion of BLV positive sample pools among the three provinces

A chi square test of homogeneity calculator accessed at (https://www.socscistatistics.com/tests/chisquare2/default2.aspx) 20/11/19 was used in this study with input and output data as shown in Table 4.2. The test was conducted at 0.05 significance level and 2 degrees of freedom. The table shows the total number of positive and negative pools

per province as input into the calculator software. The numbers in brackets are the expected values as determined by the calculator and the numbers in parentheses are the chi square values for each cell.

Table 4.2. Input and output data for the chi-square calculator

	Eastern	Southern	Western	Row Totals
Positive	7 (6.61) [0.02]	5 (3.59) [0.55]	5 (6.80) [0.48]	17
Negative	28 (28.39) [0.01]	14 (15.41) [0.13]	31 (29.20) [0.11]	73
Column Totals	35	19	36	90 (Grand Total)

The chi-square statistic was determined to be 1.2997 and the *p*-value was 0.522131.

Since the p-value 0.522131 is greater than the significance level 0.05, we accept the null hypothesis i.e. the proportion of BLV positive sample pools did not differ among the provinces

4.2 Phylogenetic analysis

Nucleotide sequences of fourteen Zambian BLV strains were determined in this study. Nucleotide sequence analyses revealed that the *env* gene of Zambian BLV strains in our study shared 99.32-100% similarity. Analysis using the Basic Local Alignment Search Tool (BLAST) algorithm showed that the *env* gene of most of the BLV strains detected in Zambia were highly similar (99%) to a genotype 1 virus (pvAN015, accession no. AP018032), detected in Japan (Murakami *et al*.,2018). However, the *env* gene of one strain (BLV-ZAMB75-2018) showed 99% nucleotide similarity to that of a different genotype 1 BLV strain (Pt12-G4, accession no. KU233559), which was isolated in Thailand in 2014 (Lee *et al*., 2016).

Phylogenetically, although all the Zambian BLVs characterized in this study belonged to genotype 1, they formed a well-supported (95% bootstrap value) distinct cluster as shown in Figure 4.2.

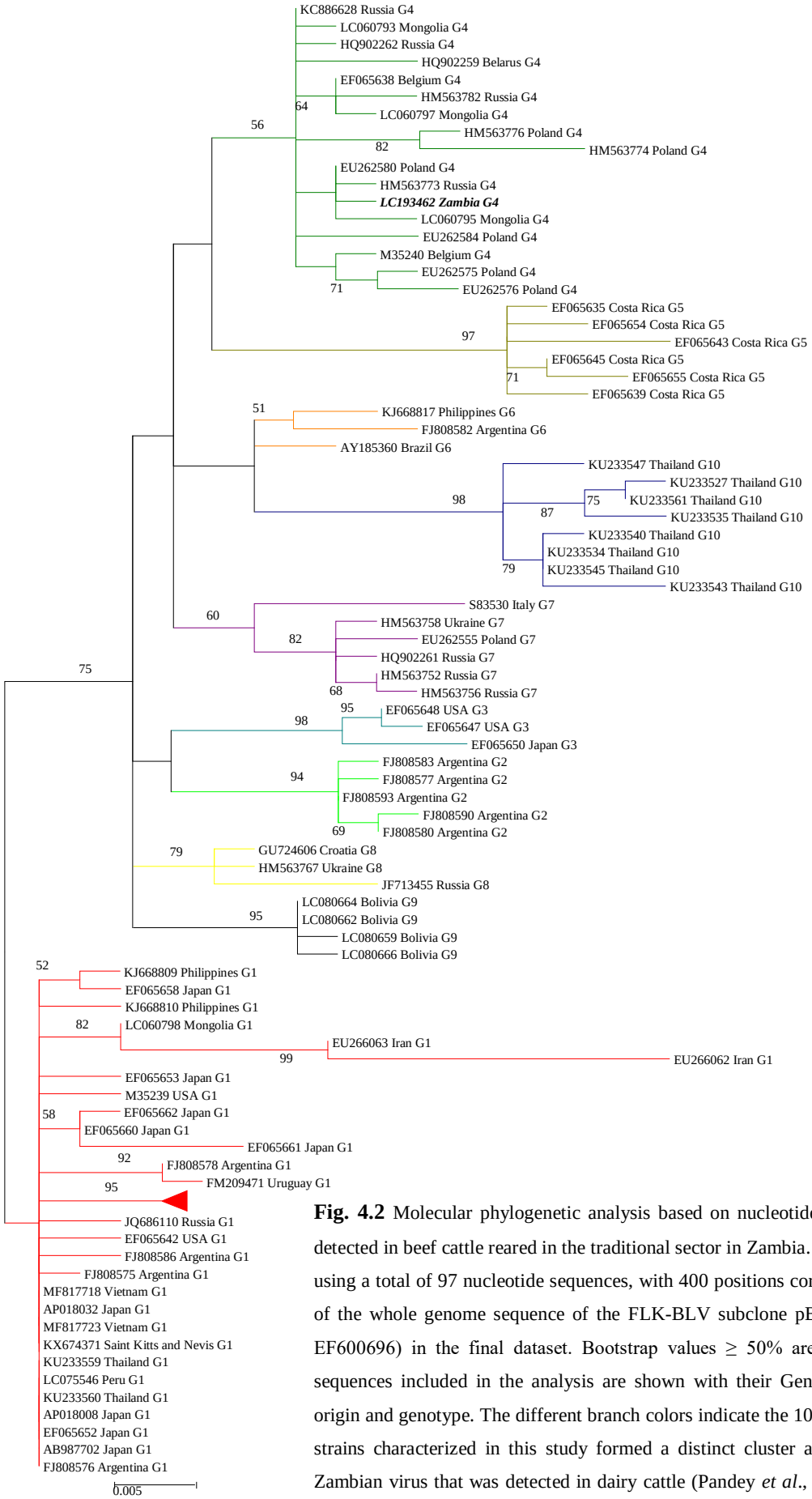

Fig. 4.2 Molecular phylogenetic analysis based on nucleotide sequences of the *env* gene of BLV detected in beef cattle reared in the traditional sector in Zambia. The evolutionary history was inferred using a total of 97 nucleotide sequences, with 400 positions corresponding to nucleotides 5126–5525 of the whole genome sequence of the FLK-BLV subclone pBLV913 (GenBank accession number EF600696) in the final dataset. Bootstrap values ≥ 50% are shown at branch nodes. Reference sequences included in the analysis are shown with their GenBank accession numbers, country of origin and genotype. The different branch colors indicate the 10 distinct genotypes of BLV. The virus strains characterized in this study formed a distinct cluster and are compressed (14 strains). The Zambian virus that was detected in dairy cattle (Pandey *et al*., 2017) is italicized and is in bold text. Scale bar, number of substitutions per site.

4.3 Amino acid sequence analysis

At the amino acid level, the predicted residues of the *env* gene of BLVs characterized in this study shared 96.6-100% similarity. Amino acid sequence alignment of genotype 1 viruses revealed that the Zambian BLV strains had several amino acid differences. All Zambian strains shared a unique I140F amino acid difference in the B-epitope (Figure. 4.3). Individually, BLV-ZAMB65-2018 had D35G, I69T and W105R amino acid differences in the vicinity of the G-epitope and in the $CD8^+$ and near the E-epitope, respectively. Meanwhile, BLV-ZAMB75-2018 had a unique D30N amino acid difference near the G-epitope (Figure. 4.3).

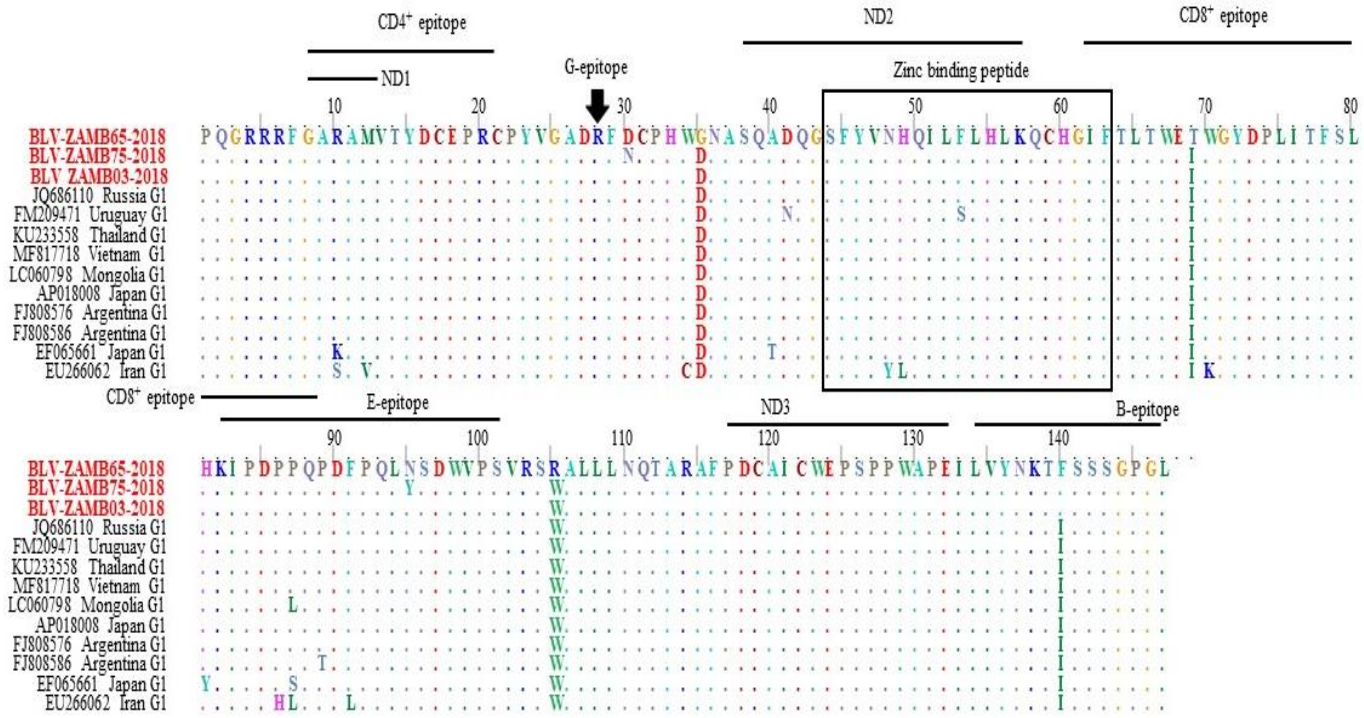

Figure. 4.3 Alignment of translated partial sequences of deduced amino acids of the Env protein of genotype 1BLV detected in Zambia. From 14 amino acid sequences, three Zambian strains representing sequences with at least one amino acid difference were included in the analysis and are shown in red bold text. Reference sequences included in the alignment are shown by GenBank accession numbers, country of origin and genotype. The first, second and third neutralizing domain (ND1-ND3), as well as other antigenic determinants (epitopes), are indicated by solid lines and a downward arrow at the top of the alignment. The black frame on the alignment refers to the Zinc-binding peptide region. Dots indicate amino acid sequence identical to the Zambian strain BLV-ZAMB65-2018.

CHAPTER FIVE
DISCUSSION

Bovine Leukemia Virus has over the years gained significance in agriculture and livestock farming and several countries have embarked on eradication and control programs of which most European countries have successfully attained BLV free status (Juliarena *et al*., 2017). Currently there is limited information on BLV in Africa despite the majority of the population being dependent on agriculture and livestock farming, this study has provided the first molecular evidence of BLV infection among beef cattle reared in the traditional sector in Africa, particularly, in Zambia.

BLV prevalence varies widely among different geographical locations and different cattle herds, a wide range of prevalence rates have been reported worldwide. In this study three different provinces where sampled and an estimated overall prevalence rate of 2.1% was recorded. Prevalence was found to not vary very significantly in the areas of study with Southern province having the highest BLV prevalence rate (3.0%), followed by Eastern (2.2%) and Western having the lowest prevalence rate of 1.5%.

The prevalence rates reported here are lower than those reported in a previous study conducted in Zambia which reported 5% prevalence. These rates were also found to be lower than those reported elsewhere in Africa including Botswana (7.7%) and Tanzania where a prevalence rate of 36% was reported (Meas *et al*., 2004; Mushi *et al*., 1990; Schoepf *et al*., 1997).

The relatively low prevalence rates reported here could possibly be attributed to type of cattle herds sampled i.e. beef cattle. Studies have shown that BLV infection rate is generally higher in dairy cattle compared to beef cattle (Polat *et al*., 2017a; Bauermann *et al*., 2017). As was also reported in a study conducted in China by Yang *et al* in (2016b), where a prevalence rate of 49.1% in dairy cattle and a considerably lower prevalence of 1.6% in beef cattle was seen within the same study and sampling area. Another possible attribute could be the difference in location from the study that was earlier conducted in beef cattle in Zambia by Meas *et al* (2004) as it was conducted in Central and Lusaka provinces of Zambia as opposed to Southern, Western and Eastern provinces sampled in this study. Further studies are thus required to better clarify the prevalence trends of BLV in Zambia, particularly in the diary and commercial beef sectors.

Animals sampled in our study were from the traditional sector composed of exotic and indigenous breeds. The sector is characterized by practices such as open communal grazing, farmers making their own disease diagnosis without consulting veterinarians, and rearing cattle for the purpose of social status and draught power (Mumba *et al*., 2018). Open communal grazing potentially exposes livestock to prolonged risk of transmission of BLV from herd to herd through blood-sucking insects (Ooshiro *et al*., 2013) as animals share pasture. When farmers conduct their own disease diagnosis the chances of identifying herds and animals that are BLV infected are reduced, thus potentially facilitating spread of infection within and amongst herds. In addition, when the focus of livestock rearing is merely for social status, the farmer's willingness to invest more time, financial resources and effort in disease diagnosis, prevention and control is reduced. As a result, the traditional sector may act as a possible reservoir for infectious diseases like BLV, which are usually asymptomatic.

Phylogenetic analysis of the partial *env* gene sequences obtained demonstrated that the BLV strains detected in this study belong to genotype 1, which is widely distributed globally (Polat *et al*., 2017a). In some instances, genotype 1 strains have been found to co-circulate with other genotypes, while in others the genotype occurs on its own (Lee *et al*., 2016; Moratorio *et al*., 2010; Ababneh *et al*., 2012; Yang *et al*., 2016a; Benavides *et al*., 2017). The previous detection of genotype 4 on a dairy farm (Pandey *et al*., 2016) and our detection of genotype 1 in beef cattle in this study may indicate that different genotypes may be circulating in distinct sectors of the cattle production industry in Zambia, although further studies are needed to substantiate this observation. The BLV genotype 1 detected in this study formed a distinct cluster with high bootstrap support, suggesting possible independent evolution of genotype 1 within the country. This is further supported by the observation of a unique I140F amino acid difference in the B-epitope as seen in the amino acid sequence analysis. It has been suggested that over time attainment of homogeneity of such mutations as observed in the B-epitope of the Zambian BLV strains which is associated with genetic drift play a role in BLV diversification and its association to geographical locations (Rola-Luszczak *et al*., 2013). It has also been suggested that BLV has spread worldwide through trade in breeding stock (Polat *et al*., 2017a; EFSA AHAW, 2015). Initial introduction of BLV into the country may have happened several years ago as suggested by the suspected long term independent evolution of the virus within the country. This may imply that BLV has become more established and prevalent in the country

than has been demonstrated in this study thereby justifying the need for additional BLV prevalence studies. In Zambia, traditional livestock farmers mostly keep local breeds of cattle while commercial farmers keep large herds of mostly exotic breeds (Mumba *et al*., 2018). To improve their breeds, traditional farmers buy exotic breeds, especially bulls, from commercial farmers, a practice which may facilitate introduction and spread of infectious diseases such as BLV into traditional cattle herds. As such, there is a need to determine the BLV infection status of cattle on commercial beef farms in order to better understand the epidemiology of BLV in the country.

Amino acid comparisons of predicted partial sequences of the Env protein of Zambian BLVs showed a number of amino acid differences. Two of these (D30N and D35G) amino acid differences occurred in the vicinity of the G-epitope, which has been speculated to play a role in the viral evolution of escape mutants (Pluta *et al*., 2017). One mutation, I69T, which was found in BLV-ZAMB65-2018, occurred in the region that stimulates a CD8+ cytotoxic T-cell response and overlaps with the zinc-binding peptide which is critical for viral fusion and infectivity in vivo (Dube *et al*., 2000; Gatot *et al*., 2002). Only BLV-ZAM65-2018 had a W105R mutation located near the E-epitope and all the Zambian strains possessed I140F substitution in the B-epitope. In concordance with other studies (Murakami *et al*., 2018; Pluta *et al*., 2017), the amino acid differences observed in this study occurred within or near functional domains and thus may be associated with viral properties such as antigenicity, pathogenicity and viral infectivity. Further studies are required to elucidate the potential impact of these mutations on viral properties and disease progression in the host.

CHAPTER SIX
CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Results from this study have demonstrated that BLV is present in the major traditional cattle rearing areas of Zambia and only genotype 1 was detected in all three provinces. This study has provided the first molecular evidence of circulation of genotype 1 BLV in beef cattle reared in the traditional sector in the major cattle producing regions of Zambia. Phylogenetically, the distinct clustering of Zambian BLV together with the presence of unique mutations in the predicted Env protein sequence suggest possible long-term independent evolution of the isolates in circulation in the three provinces studied. These results suggest that BLV could be a noteworthy problem in the traditional cattle sector in Zambia.

6.2 Recommendations

There is need to raise awareness on the presence of BLV in the country and in the traditional sector in particular. This would help traditional farmers to be more alert towards infections and seek the right diagnostic and veterinary care. Additionally, there is need to create awareness in traditional livestock farmers to avoid practices that could facilitate widespread transmission of the pathogen.

Traditional farmers should also be sensitized about the need to boil or pasteurize milk as BLV has been shown to have zoonotic potential and has been associated with breast cancer in women. As evidence of BLV's zoonotic potential accumulates, there is need to take action to investigate and manage the risk that the virus poses to public health.

There is need for further studies on BLV and its risks to human and animal health in the country. Further studies are required to better clarify the epidemiology of BLV in wildlife, commercial and dairy cattle sector in the country to guide in the development of preventive and control measures for the virus.

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APPENDIX I: Ethical approval copy

THE UNIVERSITY OF ZAMBIA

DIRECTORATE OF RESEARCH AND GRADUATE STUDIES

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Approval of Study

5th August, 2019

REF. NO. NASREC: 2019-AUG- 001

Ms. Mundia P. Phiri
The University of Zambia
School of Natural Sciences
Department of Biological Sciences
P.O. Box 32379
LUSAKA

Dear Ms. Phiri,

RE: "PREVALENCE AND GENETIC CHARACTERIZATION OF BOVINE LEUKEMIA VIRUS FROM BEEF CATTLE IN THE TRADITIONAL SECTOR IN ZAMBIA"

The University of Zambia Natural and Applied Sciences Research Ethics Committee IRB has approved the study noting that there are no ethical concerns.

On behalf of The University of Zambia Natural and Applied Sciences Research Ethics Committee IRB, we would like to wish you all the success as you carry out your study.

In future ensure that you submit an application for ethical approval early enough.

Yours faithfully,

Dr. E. Mwanaumo
CHAIRPERSON
THE UNIVERSITY OF ZAMBIA NATURAL AND APPLIED SCIENCES RESEARCH ETHICS COMMITTEE IRB

cc: Director Directorate of Research and Graduate Studies
Assistant Director (Research), Directorate of Research and Graduate Studies
Assistant Registrar (Research), Directorate of Research and Graduate Studies
Senior Administrative Officer (Research), Directorate of Research and Graduate Studies

Excellence in Teaching, Research and Community Service

APPENDIX II: Copy of first page of manuscript published based on this work.

Archives of Virology
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BRIEF REPORT

Molecular detection and characterization of genotype 1 bovine leukemia virus from beef cattle in the traditional sector in Zambia

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Abstract
Whilst bovine leukemia virus (BLV) causes considerable economic losses to the dairy industry worldwide, information on its molecular epidemiology and economic impact in beef cattle is limited. Here, blood from 880 animals from Zambia's major cattle-rearing provinces was screened for BLV by nested PCR. Positive pools were sequenced and phylogenetically analyzed. The estimated pooled prevalence was 2.1%. All strains belonged to genotype 1 and formed a distinct phylogenetic cluster. The study suggests circulation of genotype 1 BLV in beef cattle in these regions. This is the first report on molecular detection and characterization of BLV from beef cattle in Africa.

Enzootic bovine leucosis (EBL) is a disease of cattle caused by bovine leukemia virus (BLV). It is characterized by persistent lymphocytosis in approximately 30% of infected animals, of which about 5% will progress to lymphosarcoma and eventual death. Generally, BLV infection in cattle is mostly asymptomatic, with clinical disease, especially the malignant form of B-cell lymphoma, predominantly occurring in older animals of more than 4 years of age. Transmission mostly occurs horizontally through contact with fluids such as blood and milk, particularly through iatrogenic procedures and feeding contaminated milk to calves, respectively. However, congenital and mechanical transmission through blood-sucking insects has also been reported [1, 2]. EBL is responsible for major economic losses attributed to reduced reproduction efficiency, reduced milk production, weight loss, increased diagnostic and veterinary care, reduced lifespan, and reduced carcass value. Mostly due to management practices on dairy farms, BLV is more commonly detected in dairy cattle than in beef animals, but it has negative economic effects on both sectors [2].

Together with the closely related simian T-cell lymphotropic virus and human T-cell lymphotropic virus types I and II, BLV is an oncogenic member of the genus *Deltaretrovirus*, family *Retroviridae*. Several regions of the BLV genome are targeted for nucleic-acid-based diagnosis, and these include the LTR region and the genes *gag*, *pol* and *tax* [3]. Serological diagnosis is also important and is usually conducted through detection of antibodies against the p24 viral capsid protein encoded by the *gag* gene and the gp51 protein encoded by *env-gp51*, using techniques such as agar gel immunodiffusion, enzyme-linked immunosorbent assay, and radioimmunoassay [3].

Historically, EBL was endemic in dairy cattle herds in Europe and North America before 1960, and it has since spread to all continents, mostly through trade in breeding animals [3, 4]. Whilst serological evidence of BLV infection has been reported on all continents, implementation of prevention and control programs over the years has resulted in eradication or low infection levels in most European

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