

**SEROPREVALENCE OF BOVINE LEUKOSIS INFECTION IN SELECTED FARMING  
SYSTEMS IN KENYA**

**EMILY KATHAMBI KIUGU (BVM)**

**UNIVERSITY OF NAIROBI**

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## DECLARATION

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

Signature \_\_\_\_\_ Date \_\_\_\_\_

Emily Kathambi Kiugu, BVM (J56/82947/2015)

This thesis has been submitted for examination with the approval of the supervisors

Signature \_\_\_\_\_ Date \_\_\_\_\_

Dr. Gerald M. Muchemi (BVM, MSc, PhD)

Department of Public Health, Pharmacology and Toxicology

Signature \_\_\_\_\_ Date \_\_\_\_\_

Prof. George K. Gitau (BVM, MSc, PhD)

Department of Clinical Studies

## **DEDICATION**

This research is dedicated to my great mum Catherine Mataria for her support while undertaking this project and for simply being awesome

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## **LIST OF ABBREVIATIONS**

AGID	-	Agar Gel Immuno-Diffusion
AVPC	-	Average Positive control
BLV	-	Bovine Leukemia Virus
DNA	-	De-oxyribose Nucleic Acid
EBL	-	Bovine leukosis infection
EEC	-	European Economic Community
EFSA	-	European Food Safety Authority
ELISA	-	Enzyme-Linked Immuno-Sorbent Assay
EU	-	European Union
IGAD	-	Inter governmental Authority on Development
NAHIS/AHA	-	National Animal Health Information System-Animal Health Australia
NAHMS/USDA-	-	National Animal Health Monitoring System- United States Department of Agriculture
NC	-	Negative Control
OIE	-	World Organization on Animal Health
PC1	-	Positive Control 1
PC2	-	Positive Control 2
PCR	-	Polymerase Chain Reaction
PL	-	Persistent Lymphocytosis
RT	-	Reverse Transcriptase- Polymerase Chain Reaction
S.P	-	Sample Positivity
TMB	-	Tetra methyl benzidine

## **ABSTRACT**

Bovine leukosis infection (EBL) is a worldwide occurring disease of cattle caused by the bovine leukemia virus and is clinically characterised by occurrence of multiple lympho-sarcomas in about 10% of the infected cattle. In Africa, prevalence of Bovine leukosis infection has been reported in Namibia, Egypt, South Africa, Uganda and Tanzania. In Kenya, cases of bovine lympho-sarcomas have been reported but the incidence, prevalence and distribution of the disease in the country remains unknown. In addition to this, the risk factors associated with the occurrence of bovine leukosis infection in Kenya have not been established. The economic losses associated with the disease include; death of the cattle, decreased milk production, decreased reproductive efficiency and condemnation of infected carcasses and restriction of trade of livestock to other countries. The two objectives of this study were to determine the seroprevalence of bovine leukosis infection and the risk factors associated with the occurrence of the disease in selected farming systems in Kenya.

The sera samples used in the study were collected in a cross-sectional study under surveillance for sensitive trade diseases project that was carried out jointly by the ministry of livestock in Kenya and the Intergovernmental Authority on Development (IGAD) across the 47 counties of Kenya between July and October 2016. In this study, 1383 bovine sera samples were collected from 14 Counties in Kenya that were conveniently selected in consideration to the common livestock farming system in the Counties and tested for the presence of antibodies against Bovine leukemia Virus using the IDEXX anti –BLV indirect ELISA test (IDEXX Leukosis Serum Screening, 06-02110-17). The Microsoft® Excel 2013 spreadsheet was used to enter the raw data which was analysed using the Stata® 14 statistical package. Seroprevalence of bovine leukosis infection (expressed as a percent positivity) was described in the different categories of the risk

factors which were: Age, breed, sex, farming systems and County of origin. Using a binary outcome, a univariate logistic regression model was used to determine risk factors associated with occurrence of bovine leukosis infection significantly ( $p \leq 0.1$ ). These and confounders were fit in a multivariate mixed logistic regression model with County being a random effect due to the clustering of the data at this level and the other risk factors as fixed effects.

An overall prevalence of 7.6% was observed with 105 out of the total 1383 bovine sera samples testing positive for antibodies against bovine leukemia virus. On accounting for clustering at the county level, age ( $p=0.001$ ) was significantly associated with occurrence of EBL in Kenya while farming system was associated with occurrence of EBL marginally ( $p=0.063$ ) at the 5% significance level. The odds of EBL occurring in cattle less than one year old in a given County were 0.36 times less than older cattle in the same county. The odds of EBL occurring in cattle in pastoral farms were 19 times higher than in zero grazed cattle in the same County. The odds of EBL occurring in ranches was 10 times higher than zero grazed cattle in any of the 14 Counties in Kenya. Given that a cattle in a given County tested positive for EBL, the probability of a randomly selected cattle from the same County testing positive was 64.2%.

Bovine leukosis infection is present in cattle in Kenya and cattle kept in Pastoral farming systems have a higher probability of testing positive for bovine leukosis infection. Awareness about the occurrence and spread of the disease should be created in the country with emphasis put in the Counties with predominantly pastoral systems as they had the highest prevalence. Control measures against occurrence and spread of EBL especially in Counties with high prevalence of the disease should be undertaken using this information and further research carried out to determine the frequency of bovine lympho-sarcoma cases in slaughterhouses in Kenya and quantify the economic losses from Bovine leukosis infection in Kenya.

# CHAPTER ONE

## INTRODUCTION

Bovine leukosis infection caused by bovine leukemia virus affects cattle of all ages, breeds and sex (Gillet *et al.*, 2007). The infections are mostly subclinical. Persistent lymphocytosis develops in about 30-70% of the affected animals while about 10% of the animals develop tumors.

The signs observed on the cattle include; enlarged lymph nodes, in-appetence, and loss of weight, general weakness and neurological signs. At post mortem, lymphomas are seen on the lymph nodes and other affected organs. Some of the organs involved are; abomasum, the heart, spleen, intestines, liver, kidney, and uterus (World organization on animal health, 2012).

At present Bovine Leukemia Virus affects cattle herds globally. For instance bovine leukosis infection was observed in 38% of beef herds and 84% of dairy herds in the United States (Buehring *et al.*, 2014). Some countries like Belgium, Denmark, Germany, Ireland, Spain and United Kingdom are recognized as officially free from bovine leukemia virus (European Food Safety Authority, 2013).

The bovine leukemia virus is found in the tumor cells and body fluids including; nasal fluids, saliva and milk. Natural transmission of the virus is dependent on transfer of infected cells from an infected animal to a susceptible one e.g. during parturition. Lateral artificial transmission occurs in the presence of contributory factors such as; blood contaminated needles, surgical equipment and rectal gloves (Monti *et al.*, 2005). Mechanical transmission of the virus may occur in the presence of large numbers of blood-sucking insects especially tabanids (Koyabashi *et al.*, 2014). Pro-viral DNA can be isolated in semen and milk of infected animals. However transmission through these secretions has not been clearly demonstrated (OIE 2012).

Natural infection with bovine leukemia virus occurs only in cattle, water buffaloes and capybaras. Sheep and goats have been successfully infected experimentally.

Bovine leukosis infection can be diagnosed in various ways that include; hematology, post-mortem, serology and DNA analysis. The world organization for animal health has recommended agar gel immuno-diffusion and enzyme linked immune-absorbent assay tests as the prescribed serological tests for diagnosis of BLV. There is no established treatment for the disease. However, control and eradication programs have been designed and implemented in various countries e.g. Finland, Ireland, Belgium and United Kingdom.

The economic losses associated with BLV positivity include; increased heifer replacement, condemned carcasses, decreased reproductive efficiency, decreased milk production, cattle deaths and in ability to export cattle and their products to countries with strict bovine leukosis infection control measures like EU (Ott.*et al.*, 2003; Rhodes *et al.*, 2003). In Michigan, a lymphoma case was estimated to cost > \$400 including veterinary and diagnostic costs (Rhodes *et al.*, 2003).

### **1.1 General objective**

The overall objective of the study is to determine the seroprevalence of bovine leukosis infection in selected farming systems in Kenya.

### **1.2 Specific objectives**

1. To estimate the seroprevalence of bovine leukosis infection in selected farming systems in Kenya
2. To determine the risk factors associated with occurrence of bovine leukosis infection in selected farming systems in Kenya.

### **1.3 Problem statement**

Bovine leukosis infection is a disease that naturally affects cattle causing persistent lymphocytosis and malignant lymphomas. The disease occurs worldwide and mostly affects dairy herds compared to beef herds (Buehring *et al.*, 2014). A total of 35 cases of bovine lympho-sarcoma were encountered at the department of pathology, faculty of veterinary medicine in the University of Nairobi over a duration of 28 years (Wandera *et al.*, 2000). Incidence, prevalence, distribution and economic losses associated with Bovine leukosis infection in Kenya remain unknown. The losses associated with the disease include; increased costs of replacement heifers, cattle deaths, condemned carcasses, decreased reproductive efficiency and decreased milk production and in ability to export affected animals and their products to countries that have strict bovine leukosis infection control measures.

### **1.4 Justification**

Livestock is a key contributor to the Kenyan economy having contributed Ksh. 318.971 Billion of the agricultural GDP in 2009. In Kenya, livestock provides about 45% of the output of the agricultural and forestry sector. Dairy cattle kept in high potential areas produce milk that contributes about 70% of the total gross value of livestock contribution to the Kenyan agricultural sector. In the arid and semi- arid areas, pastoralists produce 80% of the meat consumed in Kenya (ICPALD 4/CLE/8/2013). In addition, the cattle produce manure used to fertilize agricultural land (Liu *et al.*, 2010) and use the livestock for savings and insurance purposes (Abegaz *et al.*, 2008). Therefore there is need to establish the prevalence, distribution and economic impact of bovine leukosis infection in livestock in Kenya to enable the designing of control programs, decrease economic losses and eventually eradicate the disease.



## CHAPTER TWO

### LITERATURE REVIEW

#### 2.1 Introduction

Bovine leukosis infection is a lympho-proliferative disease of cattle caused by the bovine leukemia virus and is notifiable to the world organization for animal health. The condition where lymphomas are found in young animals or the skin and thymus of adult animals is called sporadic bovine leukosis and its cause has not been established. Some lymphomatous conditions in cattle are not categorized as sporadic or bovine leukosis infection because their aetiology remains unknown and thus are termed adult multi-centric lymphomas (Gillet *et al.*, 2007). Irrespective of the age at infection in cattle, lympho-sarcomas are seen in animals older than three years due to its slow spread and manifestation (OIE 2012). After introduction of infected animals, the first lymphomas may appear after five years while high prevalence and impact of the disease may be seen decades later (EFSA 2015). The specific international trade standards for bovine leukosis infection are provided for in chapter 2.4.11 of the OIE manual of diagnostic tests and vaccines for terrestrial animals (OIE 2013).

#### 2.2 Etiology of Bovine leukosis infection

Bovine leukosis infection is caused by an exogenous oncogenic RNA virus called Bovine Leukemia Virus (BLV) which is a delta retrovirus in the retroviridae family. It is closely related in structure and function to the human T-cell leukemia Virus HTLV-1 (Buehring *et al.*, 2014) and primate T-lympho-tropic virus 1,2 and 3 (PTLV-1, -2, -3) (EFSA 2015). Retroviruses cause persistent infections in cattle by reverse transcription of the viral genome to generate pro-viral DNA which becomes an integral part of the host cell genome and is passed to all offspring of the infected cell during mitosis (EFSA 2015; OIE 2012). BLV has five genome regions, four of these are typical in all retroviruses and they include: the long terminal repeat (*LRT*) also called

the promoter region; the group specific antigen (*gag*) or capsid region; the polymerase, reverse transcription region (*pol*) which synthesizes a DNA copy of the BLV RNA genome; the envelope region (*env*). The fifth region is specific to delta retroviruses and is called the trans-activating region of the X gene (*tax*). It has regulatory functions and causes malignant transformation of host cells by inhibiting DNA repair and trans-activating disruption of cellular growth control mechanisms (Gillet *et al.*, 2007). This leads to deregulation of the immune system followed by a chronic, progressive lympho-proliferative disease (Rodriquez *et al.*, 2011). There are eight BLV genotypes that have been described but no clinical difference has been observed among the different genotypes (Rodriquez *et al.*, 2009; Rola-kuszczyk *et al.*, 2013).

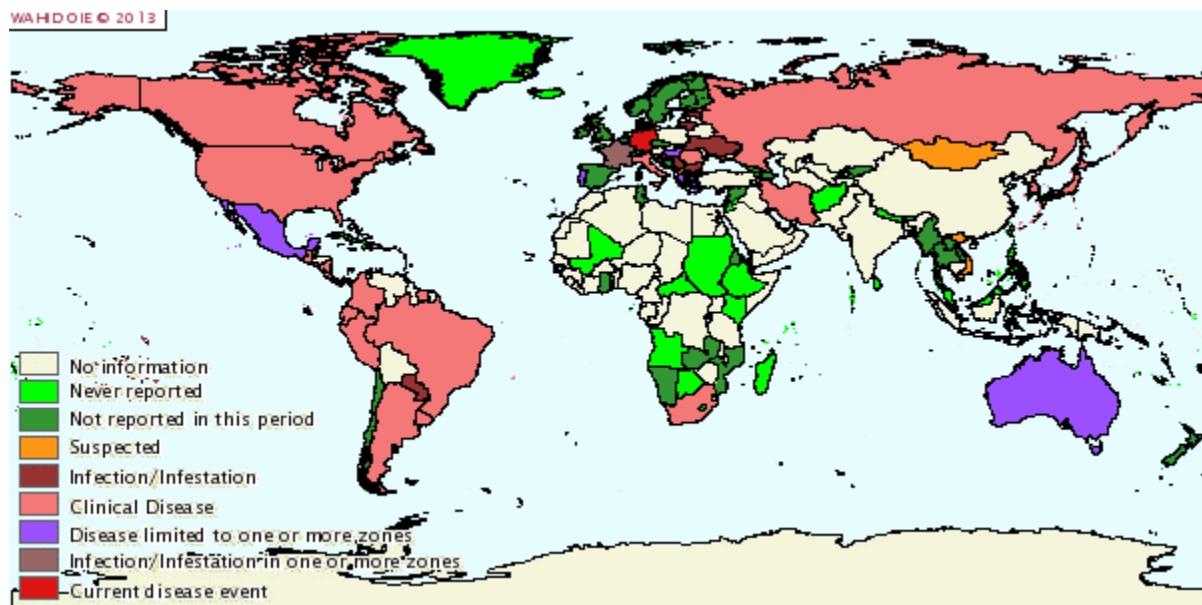
### **2.3 Epidemiology of Bovine leukosis infection**

Bovine leukosis infection is prevalent in all continents with exception of some countries that are declared free of the disease (OIE 2012). The disease was first described in Lithuania and spread into other countries through introduction of infected breeding cattle. In the UK, BLV was introduced by breeding cattle imported from Canada in 1968 and 1973 (Davies *et al.*, 1980). BLV prevalence estimates given as herd prevalence or within herd prevalence are mainly based on antibody testing in serum and/or milk (EFSA 2015). BLV infection tends to become endemic in countries that do not have strict eradication or control programs of the disease. In Japan, prevalence at the individual level increased from 3% in the 1980s to 35% in 2011 (Murakami *et al.*, 2013). In Canada, the prevalence was 87% in Alberta (Scott *et al.*, 2006) and 60% in Manitoba (Van Leeuwen *et al.*, 2006) as of 2006 compared to a 45% prevalence that was reported in in the 1980s (Reed, 1981). The proportion of slaughtered animals in Quebec-Atlantic Canada, condemned due to lymphomas decreased from 0.5% in 2012 to about 0.1% in 2013 and 2014 (Agriculture and Agri-Food Canada 2014).

A number of studies reported that 83.9% of U.S. dairy herds had enzootic bovine leucosis (NAHMS-USDA 2007). Approximately 0.8% of the BLV infected cattle in the USA had been condemned post-mortem due to lymphomas between 2005 and 2007 (White and Moore 2009). In Colombia, Chile, Venezuela and Uruguay prevalence of between 34% and 50% has been reported at individual levels (Rama *et al.*, 2011; Nava *et al.*, 2011). Several studies done in Brazil indicate the individual prevalence is over 50% (Rodriguez *et al.*, 2011). The individual and herd prevalence in Argentina was reported at 32.8% and 84% respectively (Trono *et al.*, 2001). About 2-5% of infected adult cattle in high prevalence herds in Argentina developed lymphomas after the first infection (Gutierrez *et al.*, 2011).

In Cambodia and Taiwan an individual prevalence of 5% was reported from various studies (Meas *et al.*, 2000). The seroprevalence in Japan was 28.6% in individual animals and 68.1% in herds while 86.8% of dairy herds in Korea were infected (Murakami *et al.*, 2011; Suh *et al.*, 2005; Yoon *et al.*, 2005). Studies in the Middle East have shown that the prevalence of BLV is about 20% in most countries except Turkey and Iran where the herd prevalence was 48.3% and 64.7% respectively (Burgu *et al.*, 2005; Suh *et al.*, 2005; Haghparast *et al.*, 2008; Brujeni *et al.*, 2010). In a serological survey in South Africa, it was seen that up to 10% of animals in Jersey herds had antibodies against BLV (Vorster & Mapham 2008). Other African countries like South Africa, Namibia, Nigeria, Uganda and Tanzania reported prevalence of 12.6%, 12.3%, 4.2%, 36% and 17% respectively (Adul & Olson 1981; Azuba *et al.*, 1994; Kaura & Hbschule 1994; Ndou *et al.*, 2011; Schoepf *et al.*, 1997). In Kenya, a total of 35 cases of bovine lympho-sarcoma were encountered at the department of pathology, faculty of veterinary medicine in the University of Nairobi over a duration of 28 years (Wandera *et al.*, 2000). Some countries with strict control and eradication programs have successfully eradicated bovine leukosis infection.

Following a voluntary eradication program in Australia, 5354 of the total 5356 dairy herds were declared free of EBL in 2013 (NAHIS, 2013). A control program was set up in New Zealand in 1997 and dairy herds declared free of EBL in 2008 (Anonymous, 2012). Twelve European countries had eradicated BLV by 2003 and another eight by 2011 (EFSA 2015). In other countries like the Russian Federation, EBL remains endemic irrespective of the compulsory eradication programs put in place (Rosselkhoznador, 1999). The prevalence of EBL in the federation has declined from 12.3% in 1996 to 7.5% in 2010 (Gulyukina, 2011). The frequency of lymphomas in the former USSR in slaughtered animals was 58 cases per 100,000 in 1980 (Abakin 2004). Over a period of 12 years, the number of BLV positive cattle in Ukraine decreased from 359,598 to 2,316 (Aranci and Rudyashko, 2013).



**Figure 2.1: Worldwide prevalence and distribution of BLV**

Source: World Animal Health Information Database, OIE 2013.

## **2.4 Transmission of Bovine leukosis infection**

Natural transmission occurs when infected cells are transferred from an infected animal to a susceptible one, for example during parturition. Trans-placental and peri-partum transmission has been reported to be responsible for about 10–25% of the infections (Meas *et al.*, 2002). The most important mode of transmission for BLV is iatrogenic and occurs through; blood contaminated needles, instruments for dehorning or tattooing (Lassauzet *et al.*, 1990), contaminated rectal gloves (Mekata *et al.*, 2015), contaminated vaccines and other immunological products (DiGiacomo *et al.*, 1987). Large numbers of blood sucking insects like Tabanids have been shown to transmit the virus by mechanically transferring lymphocytes through biting. Having these flies in a stable that is in close approximation to a cattle farm, has been considered a risk factor for higher within herd BLV prevalence (Koyabashi *et al.*, 2014; Kobayashi *et al.*, 2010; Erskine *et al.*, 2012). Pro-viral DNA can be isolated in milk of the infected animals but transmission through the fluids has not been confirmed (Dus Santos *et al.*, 2007). Nagy *et al.* (2007) observed that feeding colostrum from BLV infected cattle to new born calves provides them with protection through passive BLV antibodies in some cases. In a different study, it was reported that colostrum and milk from BLV- positive cattle was a risk factor if fed untreated to calves (Romero *et al.*, 1983; Lassauzet *et al.*, 1989). Whether infected milk or colostrum is a risk or protective factor is dependent on the timing of feeding i.e. if the milk is fed while uptake of passive antibodies is ongoing it is protective while it's a risk factor if fed after closure of gut uptake of antibodies (EFSA 2013). Kanno *et al.* (2013) reported that BLV virus can be eliminated from the milk and colostrum using a freeze-thaw cycle. Natural mating using infected bulls may lead to transmission of infected cells to susceptible animals due to the intense contact during mating (Erskine *et al.*, 2012) while insemination in an infected herd

without adhering to strict biosecurity measures may be followed by transmission through contaminated rectal sleeves or instruments (Hopkins and DiGiacomo, 1997). Spread of BLV within a herd may be influenced by herd level management factors like housing system, biosecurity measures and calf management (Koyabashi *et al.*, 2010; Koyabashi *et al.*, 2015). In addition to this, Fernandes *et al.* (2009) deduced that milking cattle using machines was a risk factor of spread of the virus within the herd in comparison to hand milking. This conclusion was based on the fact that the milking machines have been shown to transmit pathogens within herds if they are not well cleaned and managed especially mastitis causing micro-organisms (Edmondson, 2001).

## **2.5 Infection and persistence of Bovine leukosis infection**

Natural BLV infection has only been confirmed in three species: *Bos Taurus* (domestic cattle), *Bos Indicus* (zebu) and *Bubalus bubalis* (water buffalo) (EFSA 2015). In water buffaloes, EBL has been reported in Philippines (27.6%), Cambodia (16.7%), Pakistan (10.3%) and Brazil (4.2%) (Mingala *et al.*, 2009; Meas *et al.*, 2000a; Meas *et al.*, 2000b; Chaves *et al.*, 2012). Presence of BLV antibodies has been reported in Zebu (*Bos indicus*) in three separate studies (Marin *et al.*, 1982; Singh *et al.*, 1988; Jimenez *et al.*, 1995). Bovine leukemia virus infection has been reported in sheep in some occasions. In Venezuela, one flock was sero positive for BLV over a period of one year (Marin *et al.*, 1982) while infection was detected in 5 out of a total of 16 sheep farms in Stavropol region in Russia (Abakin 2004). A prevalence of 20.5% was described in a flock of merino sheep kept in the same farm with BLV infected cattle in South Africa (Green *et al.*, 1988). In a different study in Japan, all the sheep tested for BLV infection were negative (Giangaspero *et al.*, 2013). In 5 studies done to investigate the presence of BLV infection in wild animals, only two reported infection with BLV. One seropositive animal was

identified in a study done among wild European bison (*Bison bonasus*) in Poland (Kita and Anusz, 1991). Free-ranging red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*) in six national parks in Germany tested negative for BLV in 2006 (Frolich *et al.*, 2006). There is no conclusive evidence to demonstrate that other species apart from cattle are significant reservoirs of BLV (EFSA 2015). Bovine leukemia virus is not released in its free form into the environment but survives for a short time in blood and milk (EFSA 2015). The infected cells can be inactivated by freezing, high temperatures and Ultra Violet light (Baumgartener *et al.*, 1976; Graves and Jones, 1981; Kanno *et al.*, 2013). Monti *et al* (2007a) and Tsutsui *et al* (2010) estimated the time between introduction of BLV in a free herd and detection of lymphomas caused by the infection using a basic age structured model. It was noted that, it would take about 5 years after introduction to detect the first case of lympho-sarcoma and about 10 years to detect secondary clinical cases in the same herd. Third generation infection was estimated to be detected 10-15 years after introduction of the infection in the herd with longer delays being expected.

## **2.6 Pathogenesis and diagnosis of Bovine leukosis infection**

Following infection, three stages in the progression of BLV infection can be identified as;

### **2.6.1 Primary and persistent infection**

The Bovine Leukemia Virus infects B-lymphocytes. An infected cell that has a copy of the virus integrated into the host genome is transferred to a susceptible animal. Here the virus multiplies and infects new target lymphocytes which proliferate through mitosis. The flu-like syndrome observed at this stage is a result of the replication of BLV and initiation of the immune response. The persistent infection can last for months to several years. At this stage, BLV- infected cells transcribe large numbers of viral micro RNAs but they do not express high levels of viral

proteins. On the other hand, oncogenic viral protein (Tax) in BLV activates expression of viral structural proteins and stimulates cell proliferation. Typically, cytotoxic and humoral immune responses eliminate cells expressing viral antigens from the blood but by-pass infected cells showing very insignificant levels of viral antigens. In BLV infected cells, there is a continuous opposing cycle where cells are stimulated to proliferate and then destroyed almost immediately by immune responses due to activation of viral proteins. This feature of BLV infection is called immune dysregulation and is associated with abnormal expression of cytokines e.g. IL-2, IL-6, IL-10 and IL-12. After a prolonged duration of time, the cytotoxic and helper –associated immunity of the infected animals is weakened reducing the spontaneous recovery time from secondary and opportunistic infections like mastitis. The typical pro-viral load is about 1% at this stage. At this stage, seroconversion occurs from two weeks to three months after infection with Bovine Leukemia Virus About 70% of the infected animals act as carriers of the virus. These animals do not manifest clinical signs or a change in the circulating lymphocyte counts (OIE 2012; EFSA 2015).

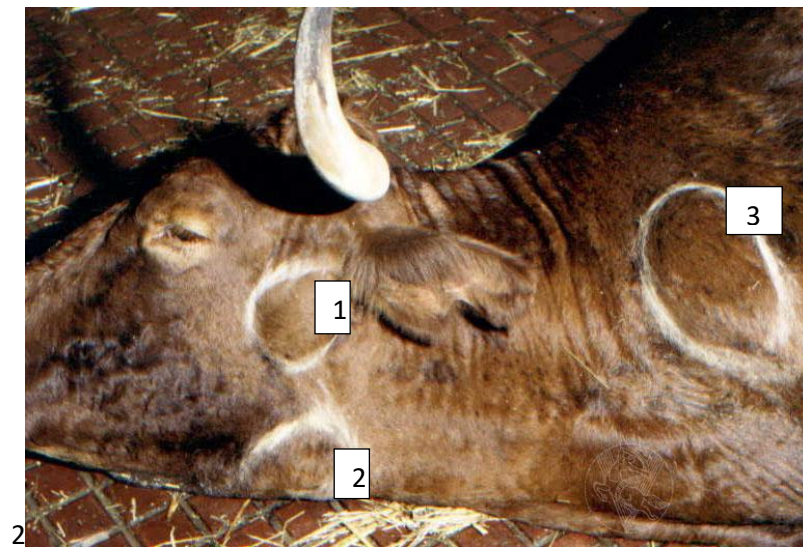
### **2.6.2 Persistent lymphocytosis**

After a given period of time, about 30 to 50% of the affected animals develop persistent lymphocytosis (PL) which is a polyclonal proliferation of B cells. The number of blood circulating B lymphocytes increases to above 10,000/mm<sup>3</sup> which is the main feature of this infection phase in addition to the weakness of the animal and presence of opportunistic secondary infections like mastitis. Persistent lymphocytosis can be latent for several years then progress to the lymphoma stage. This leads to the consideration of persistent lymphocytosis in cattle as a pre-tumor stage (OIE 2012; EFSA 2015).



### 2.6.3 Lymphoma development

The final stage of BLV infection is characterized by the development of malignant lymphomas in organs especially the lymph nodes. About 5% of the infected animals that are older than 3–5 years manifest with the fatal lymphomas. The clinical signs that accompany development of lymphomas depend on the organ involvement and the stage of progression (EFSA 2015). Neoplastic lesions in the heart cause heart- diseases like signs including; tachycardia, dyspnea, jugular distension arrhythmias or heart failure. Lymphomas in the bronchial, mediastinal and cervical lymph nodes cause dyspnea and partial or complete tracheal obstruction. In the abomasum, the tumors may cause abdominal pain, diarrhea and constipation. Lesions in the uterus and liver cause reproductive failure and liver failure respectively. Lymphomas in the spleen lead to rupture and sudden death while spinal lesions compress the spinal cord or nerves causing pelvic limb paresis (Merck, 2014). The most commonly encountered clinical signs are lymphadenopathy, asthenia, weight loss, constipation, tachycardia, posterior paresis, exophthalmia and fever (OIE 2012; EFSA 2015).



**Figure 2.2: Hypertrophy of the parotid (1), sub-mandibular (2) and pre-scapular (3) lymph nodes.**

Source: Faculty of Veterinary Medicine, University of Lisbon.

## **2.7 Zoonotic aspects of Bovine leukosis infection**

In regard the close relation of the BLV virus with the human T-cell leukemia virus HTLV-1, concerns on whether BLV can infect man were raised and several studies done establish its zoonotic importance. In the 1970s, 10 studies were carried out independently to detect BLV in humans and no antibodies were found against Bovine leukemia virus prompting Burrige (1981) to conclude that there was no evidence that the virus infected man. However, in 2003, Buehring et al reported the presence of BLV antibodies in 39% of the 257 humans tested for the infection while Baltzell et al (2009) found Bovine leukemia virus sequences in human breast cancer tissue. A report by Buehring et al (2014) stated that BLV was localized in the secretory epithelium of the human breast which suggested that the virus could be involved in the development of various types of cancers in humans. In a different study, Matsumoto et al (2007) theorized that human cancer could be associated with dietary exposure to Bovine Leukemia Virus. BLV infection was absent in all the farm workers who drank raw milk from seropositive cattle. With no conclusive evidence of transmission of the virus to humans, bovine leukosis infection is not considered zoonotic.

## **2.8 Diagnostic Techniques**

The bovine leukemia virus that primarily targets B-lymphocytes (Gillet *et al.*, 2007), consists of a single stranded RNA, nucleoprotein (p12), capsid protein (p24), transmembrane glycoprotein (gp30) envelope glycoprotein (gp51), reverse transcriptase and other enzymes (OIE 2012). Four techniques can be used to detect BLV infection at the different stages of infection.

### **2.8.1 Hematology**

This technique can be used to detect an increase in the absolute number of peripheral B lymphocytes in animals with persistent lymphocytosis and lymphomas. On examination of blood, the number of circulating lymphocytes is above 10,000/mm<sup>3</sup> (EFSA 2015). In the 1960s, the method was used to establish herd diagnosis in control and eradication programs (Bendixen, 1960a). The success of these programs was dependent on whether individual or herd level testing was carried out (Flensburg and Streyffert, 1977).

### **2.8.2 Post mortem examination**

At post mortem, presence of grey-white solid lymphomas in peripheral and visceral lymph nodes, uterus, abomasum, heart, liver, spleen and kidneys indicate neoplastic proliferation of lymphoid tissue. Lymphomas in the spleen may lead to rupture and sudden death and histological examination shows masses of lymphoblasts in the red pulp of the spleen (Kirkland and Rodwell, 2005). Cross- section of the enlarged lymph nodes shows hyperplastic follicles and lymphoid cells infiltrating medullary sinus and accumulating in the cortical region (Ishino *et al.*, 1990). Histological examination of the lymphomas which shows densely packed monomorphic lymphocytic cells (EFSA 2015). The diffuse large cleaved lymphoid cells occurred in 38% of enzootic lymphomas compared to 14% of sporadic lymphomas (EFSA 2015). This is not enough to distinguish between enzootic and sporadic lymphomas thus Polymerase chain reaction is required to test for the presence of BLV genome specifically before the suspect lymphomas are examined histologically in surveillance for freedom from EBL using Post mortem (EFSA 2015).

### **2.8.3 Detection of antibodies against bovine leukemia virus**

Sero-conversion in BLV infected cattle occurs two weeks to three months after infection and several methods can be used to detect the antibodies formed against BLV. The first method that

was used in the early 1970s was the agar gel immunodiffusion test (AGID) which was further improved to increase its sensitivity by identification of antibodies against the envelope glycoprotein (gp51) which appear earlier than the capsid protein p24 (Miller and Olson, 1972; Onuma *et al.*, 1975). Enzyme linked immunosorbent assays (ELISA) many of which have similar sensitivity (97% -100%) and close specificity (78%-100%) are more commonly used than AGID due to their higher sensitivity (Klintevall *et al.*, 1991; Trono *et al.*, 2001; OIE 2012). ELISA can be used to detect antibodies against gp51 and p24 proteins of BLV in both serum and milk samples while AGID can only be used in serum samples due to its lower sensitivity and specificity (OIE 2012). Indirect ELISA sensitivity will determine the number of animals that can be included in a bulk milk sample (OIE 2013). Serological tests especially AGID and ELISA cannot distinguish between antibodies produced from an active infection and those passed passively from dam to calves (Ballagi- Pordany *et al.*, 1992) neither are they sensitive enough to identify all BLV infected cattle (Eaves *et al.*, 1994; Klintevall *et al.*, 1994). Both AGID and ELISA are recommended by the World Organization for Animal Health as suitable tests for serological diagnosis of bovine leukosis infection (OIE 2013). Other immune assays that can be used to detect these antibodies include; indirect immuno-peroxidase test, indirect fluorescent antibody test, early polycaryocytosis inhibition test, complement fixation test, virus neutralization test and radio-immunoassays (OIE 2012).

#### **2.8.4 Detection of BLV provirus**

Reverse transcription of the BLV viral genome generates the Pro-viral DNA of the virus that integrates into the host cell (OIE 2012). The pol and env gene contain sequences in the BLV genome are targeted for provirus detection (Rola-Luszczak *et al.*, 2013). Several studies have shown successful use of Polymerase Chain reaction (PCR) to detect the BLV pro virus (Rola and

Kuzmak, 2002; Teifke and Vahlenkamp, 2008). Nested PCR followed by gel electrophoresis is the most sensitive and rapid method of detection (OIE 2012). Modifications such as PCR-ELISA (Rola and Kuzmak, 2002) have also been applied to detect pro-viral DNA. Recently, an in situ PCR that allows detection of very small quantities of BLV DNA in cells or tissues without extracting the DNA have been developed (Duncan *et al.*, 2005; Kubis *et al.*, 2007). Real time PCR that can be used to detect pro-viral DNA in infected cattle with undetectable or very low levels of antibodies during early phases of infection has been developed (Heinemann *et al.*, 2012). Use of PCR to detect the BLV pro virus is vital in differentiating between enzootic and sporadic lymphomas in suspect tumor cases as well as active and passive antibodies in seropositive calves. In addition to this, PCR is used to confirm weak positives or uncertain serological results. Infected animals that have not produced antibodies against BLV can be detected using PCR to identify the pro viral DNA. PCR is also the most sensitive method to screen cattle used for vaccine production to ensure they are certainly BLV free (OIE 2012; EFSA 2015).

## **2.9 Effects of Bovine leukosis infection on productivity**

With BLV infection and milk production being greatly influenced by age of the cattle, the size of the herd, lactation number and genetic potential of the animal, there arises a difficulty in assessing the impact of BLV infection on milk yield using observational studies (EFSA 2015). In a well-designed study of Ott et al (2003) the reduction of milk was estimated to be 9.5 kg per cattle (total herd) per year for each percentage-point increase in the within-herd prevalence of BLV-infected cattle. In a corresponding study Ott et al (2003) reported that BLV positive herds produced 3% less milk than BLV free herds. In a study by Erskine et al. (2012) the corresponding figure was estimated to be 10.5 kg per cattle (total herd) per year. This would

correspond to a reduction of milk production in BLV-infected cattle of 950–1050 kg/cattle per year.

Studies comparing calving intervals in BLV positive cattle and BLV negative cattle have indicated insignificant differences between the two (Heald *et al.*, 1992). However, Tiwari *et al* (2003) established a statistically significant association between enzootic bovine leucosis seropositivity and calving interval after the first lactation, but not from the second lactation onwards. In this study, there was an increase of 1.97 log days (95% CI 0.29; 3.65) in BLV seropositive cattle compared to seronegative cattle. In one study by Bartlett *et al* (2013) the hazard ratio of pre-mature culling for seropositive animals compared to seronegative animals was 1.23 (p=0.00).

### **2.10 Economic impact of Bovine leukosis infection**

Some of the losses to the dairy producer include; high replacement costs, condemned carcasses, cattle deaths, decreased reproductive efficiency, reduced milk production and the inability to export cattle and their products to countries with strict bovine leukosis infection control measures (Rhodes *et al.*, 2003; Ott *et al.*, 2003). In a study done in Virginia, a lympho-sarcoma case was costed at \$400; with a lympho-sarcoma rate of 2 cases per 300 milking cattle in a herd with 50% seropositive cattle, the annual cost was averaged at \$6,400 per 100 milking cattle (Rhodes *et al.*, 2003). In a study done on dairy herds in the United States, it was observed that herds with BLV positive cattle yielded 3% less milk than herds with BLV negative cattle. In conclusion, BLV seropositivity was linked to a loss in economic surplus of \$285 million to producers and \$240 million to consumers (Ott *et al.*, 2003).

## **2.11 Management, Control and Eradication of Bovine leukosis infection**

A number of preventive and therapeutic strategies against Bovine Leukemia Virus infection have been developed over time and include; segregation, elimination or corrective management of infected animals, vaccination, genetic selection against BLV and competitive infection with attenuated pro-viruses.

### **2.11.1 Segregation or elimination of infected animals**

This strategy employs three different approaches which involve testing of all the animals to identify the BLV infected which are eliminated, segregated or managed. In the early years, identification of the BLV positive animals was done using hematology (Bendixen, 1960a) and later more sensitive and specific techniques such as ELISA and AGID were adapted to become the official tests used for international trade (OIE 2012). While using serological tests, presence of anti-BLV antibodies indicates presence of an infection. However, there are some exceptions to this such as, presence of anti-BLV antibodies in calves passed passively from the dam during parturition (Burrige *et al.*, 1982) or in colostrum (Ferrer *et al.*, 1981; Lassauzet *et al.*, 1991; Van Der Maaten *et al.*, 1981). In this regard, the most accurate technique that can be used to identify BLV infected animals for segregation or elimination is PCR (Jimba *et al.*, 2010). BLV pro-viral load of infected animals in the peripheral blood can be used to determine the transmission risk of the virus and identify highly infected animals (Juliarena *et al.*, 2007; Esteban *et al.*, 2009; Gutierrez *et al.*, 2011).

#### ***Testing and elimination***

Diagnostic techniques like serology, hematology and PCR are used to identify BLV positive animals which are promptly removed from the herd and slaughtered. This method has been used to eradicate BLV in several countries in Western Europe e.g. Finland, Belgium and Lithuania

(Knapen *et al.*, 1993; Nuotio *et al.*, 2003; Acaite *et al.*, 2007). Although efficient, this methodology has the limitation of high economic cost that is brought about by diagnostic procedures, compulsory premature culling and replacement of the culled animals. In addition to this, a high initial prevalence results to very high economic costs that cannot be justified. The exception to this can be use of the strategy in pedigree breeds with very high genetic potential and animals for export to BLV-free countries (Rodriquez *et al.*, 2011). For the benefits of this approach to outweigh the costs, governmental economic compensation policies must be used. Countries such as USA, Argentina, Canada and Japan that do not have financial compensation policies in place have tried unsuccessfully to use these programs for eradication of Bovine Leukemia Virus in their herds (Trono *et al.*, 2001; Asfaw *et al.*, 2005; Monti *et al.*, 2005; Scott *et al.*, 2006; Van Leeuwen *et al.*, 2006; Murakami *et al.*, 2011).

### ***Testing and Segregation***

This strategy involves identification of BLV positive animals and separating them from the healthy ones (Johnson *et al.*, 1985; Brenner *et al.*, 1988; Shettigara *et al.*, 1989). The infected animals are confined in a separate area with at least 200 metres distance between them and the seronegative animals (Shettigara *et al.*, 1989). In cases where the space for confinement is available, the seropositive animals are kept in the same farm but managed separately. To ensure that the virus is not transmitted to the healthy animals, separate equipment should be used for the two groups of animals. For non-disposable equipment, strict hygiene and disinfection should be done. The economic cost associated with this approach is significantly low because premature culling and replacement of these animals is prevented. Control and eradication of BLV has been done using this approach (Van Der Maaten *et al.*, 1979; Kaja *et al.*, 1984) but more time is



required to achieve it due to the risk of re introducing the virus in the herds (Otachel-Hawranek J 2007).

### ***Testing and corrective management veterinary practices***

This approach aims to reduce the iatrogenic transmission of BLV among animals by reducing the transfer of BLV infected cells in blood, secretions, contaminated surgical instruments and rectal gloves. The main management practices that should be carried out to control the spread of the virus include; Use of single-use needles during vaccination and treatment, use of single –use rectal sleeves and replacement of sleeves between examination of BLV-positive and BLV-negative cattle, use of colostrum from non-infected dams to feed calves, elimination of insects to limit mechanical transmission of the virus, use of BLV-negative bulls for natural mating or artificial insemination and avoiding gouging equipment during dehorning. In addition to this, testing and isolating new animals before introducing them to the herd is vital, while minimizing movement of animals between milking or feeding groups limit transmission of BLV by direct contact. Other biosecurity measures such as limited access to visitors and housing calves in individual clutches may reduce the risk of transmission and spread of the virus in the herd. This control methodology is very cost effective but labour intensive (Rodriquez *et al.*, 2011). The efficacy of this program is highly dependent on the strict implementation and compliance of the control measures outlined in the program. The successful use of this management strategy exclusively to control and eradicate BLV in animals is contradictory at its best (Ruppanner *et al.*, 1983; Sprecher *et al.*, 1991; Gutierrez *et al.*, 2011).

The table below gives the summary of the segregation or elimination approaches that can be used to control and eradicate Bovine leukosis infection (Rodriquez *et al.*, 2011).

**Table 2.1 Approaches used to control and eradicate Bovine leukosis infection**

<b>Approach</b>	<b>Basis of the control program</b>	<b>Advantages</b>	<b>Disadvantages</b>
<b>Test and Eliminate</b>	Identify the BLV-infected cattle & slaughter them	Highly Efficient  Requires very few facilities  Eradication may be achieved within a short time	May be expensive & impractical depending on prevalence levels.  Requires constant surveillance  Needs compensation policies to be in place
<b>Test and Segregate</b>	Detect BLV-infected herds and isolate in from BLV- free herds	Requires no replacement of slaughtered BLV-positive cattle	Requires housing facilities for BLV-positive & BLV-negative cattle  Permanent surveillance needs to be carried out  Prolonged commitment required
<b>Test and Manage</b>	Biosafety & management measures are taken	Inexpensive  Few facilities required  No replacement of slaughtered cattle is required.	Labour intensive  Implemented measures should be strictly adhered to  Prolonged commitment needed  Susceptibility to various human and environmental factors is high  Personnel require intensive training

### **2.11.2 Vaccination**

Over the years, different trials have been carried out in an attempt to develop a vaccine against bovine leukemia virus (Kettman *et al.*, 1994; Willems *et al.*, 2000). In regard to studies that ascertained the preventive ability of anti-BLV antibodies in colostrum against BLV infection in calves; inactivated virus vaccines, Cell-derived vaccines and Viral Subunit vaccines were developed with the aim of inducing an optimal humoral response in the vaccinated animals (Nagy *et al.*, 2007). Inactivated BLV vaccine was prepared by treatment of the virus using chemical agents such as formaldehyde, 0.1% formalin and N-acetylythylenimine. The vaccines induced a neutralizing humoral response and provided partial protection in cattle and sheep when low doses of the viral challenge were used. On the other hand, if high challenge doses on the vaccinated animals, they became infected with BLV (Fukuyama *et al.*, 1993). Several of these vaccines showed partial protection for a limited period of time. Different trials were carried out to develop cell derived vaccines from plasma membranes or cell extracts from BLV lymphosarcomas (Ristau *et al.*, 1987) and BLV infected SF-28 cells fixed with 3% glutaraldehyde (Onuma *et al.*, 1984). Some of the vaccines provided partial protection to the animals but the risk of transmitting BLV infection was very high (Rodriquez *et al.*, 2011). The gp51 surface envelope glycoprotein that is a component of the BLV virus carries at least three neutralizing epitopes (Portetelle *et al.*, 1989 Mamoun *et al.*, 1990); a characteristic that has been exploited in the development of viral subunit BLV vaccines (Onuma *et al.*, 1984; Miller *et al.*, 1984; Kabeya *et al.*, 1996). In addition, the gp51 sequence is very well conserved among BLV isolates (Monti *et al.*, 2005; Carmargos *et al.*, 2007; Rodriquez *et al.*, 2009; Moratorio *et al.*, 2010; Matsumura *et al.*, 2011). The developed gp51 subunit vaccines were immunogenic but did not protect the animals both adults and calves fully against BLV viral challenges (Onuma *et al.*, 1984; Burkhardt *et al.*, 1989). An attempt to use the p24 protein was unsuccessful in protecting the

vaccinated animals against BLV infection (Onuma *et al.*, 1984). The three vaccines mentioned above showed the significance of the humoral response in protection against BLV infection. However, the major limitation of the vaccines was the rapid decrease of the antibody titers in vaccinated animals which is undesired in an effective vaccine (Rodriquez *et al.*, 2011). It was concluded that the humoral response did not provide full protection against BLV infection and thus the vaccines could not be used efficiently in herds (Gatei *et al.*, 1993; Burny, 1996). In the following years, emphasis was given to the cellular component of the immune response. Several trials were done on Recombinant Vaccinia Virus (RVV) which is a live recombinant vector with the ability to induce humoral and cell-mediated immunity and is used as a vehicle for immunization against BLV antigens (Rodriquez *et al.*, 2011). Trials done with RVV coding for surface glycoprotein gp51 alone did not protect sheep and rabbits against BLV or induce a humoral response in the vaccinated animals (Kumar *et al.*, 1990). In different studies, RVV carrying the env gene that encodes gp51 and gp30 glycoproteins (RVV-env vaccine) induced neutralizing specific antibodies against BLV in sheep and rabbits (Ohishi *et al.*, 1996). In two separate studies, RVV-env vaccines elicited a strong humoral and CD4+ T cell response and protected sheep and rabbits against BLV infection (Gatei *et al.*, 1993; Kumar *et al.*, 1996) but were inefficient in cattle (Burny *et al.*, 1996; Cherney *et al.*, 1996). A DNA vaccine with the env gene stimulated cell-mediated immunity thus partial protection in vaccinated calves (Brillowska *et al.*, 1999). A different DNA vaccine expressing the Tax trans-activator protein decreased BLV replication in immunized sheep (Usui *et al.*, 2003) while another Tax DNA vaccine induced a cytotoxic response at first but not later in the infection (Van den Broeke *et al.*, 2010). Use of BLV DNA vaccines is questionable due to the contrasting results from different studies.

### **2.11.3 Selection of BLV-Resistant cattle**

The major histocompatibility complex that influences immune responses, resistance and susceptibility to infection in bovines is called the Bovine Lymphocyte Antigen (BoLA) (Gogolin-Ewens *et al.*, 1990; Lamont, 1998). In a different study, Van Eijik *et al.* (1992) indicated that the development of subclinical persistent lymphocytosis was closely related to class II DRB2 genes while Xu *et al.* (1993) contradicted this by showing a strong association between resistance or susceptibility and class II DRB3 gene. It was concluded that genetic resistance is controlled by multiple genes which contribute slightly to the phenotype individually (Lander & Schork 1994) while choosing one allele over others as a marker for selecting BLV resistant cattle is hard because the infection is dependent on many factors (Glass *et al.*, 2011). Selecting BLV resistant cattle reduces the genetic pool of the population with negative effects on productivity characteristics (Williams *et al.*, 2005) and resistance to other pathogens (Glass *et al.*, 2011).

### **2.11.4 Competitive infection by attenuated pro-viruses**

Attenuated derivatives of BLV proviruses harbor viral actors that stimulate an immune response permanently in the vaccinated animals thus are considered optimal vaccines (Kerkhofs *et al.*, 2000; Reichert *et al.*, 2000; Willems *et al.*, 2000; Florins *et al.*, 2007). A BLV hybrid derivative lacking the tax, rex, R3 and G4 genes while containing promoter cis-acting regulatory sequences of spleen necrosis virus stimulated production of specific antibodies against BLV in vaccinated rats (Boris-Lawrie *et al.*, 1997). Another virus containing the spleen necrosis virus regulatory sequences in addition to the *gag*, *pol* and *env* genes induced specific antibodies against BLV in vaccinated rats and rabbits (Altanerova *et al.*, 2004). Several studies showed that the 6073-mutated provirus impaired replication of the bovine leukemia virus but not its infectivity

(Reichert *et al.*, 2000; Willems *et al.*, 2000; Florins *et al.*, 2007) and induced a wild-type immune response in the vaccinated animals (Kerkhofs *et al.*, 2000). The pathogenicity and conversion of these vaccines to wild-type are the main factors to consider in the development of the attenuated vaccines (Reichert *et al.*, 2000). Trials to evaluate the safety and efficacy of this strategy in real herd conditions are ongoing (Rodriquez *et al.*, 2011).

## CHAPTER THREE

### MATERIALS AND METHODS

#### 3.1 Study design

The sera samples used in the study were collected in a well- designed surveillance project for sensitive trade diseases that was carried out jointly by the ministry of livestock in Kenya and the Intergovernmental Authority on Development (IGAD) between July and October 2016. Multi stage sampling was adopted with the 47 Counties of Kenya being sampled. Being part of this bigger project, the study was cross sectional with 1383 bovine sera samples collected from 14 Counties that were conveniently selected based on the common livestock farming system in the Counties. The Livestock farming systems considered were; Zero- grazing, ranching and pastoral systems and their characteristics are shown in the table below.

**Table 3.1 Characteristics of farming systems used for the Bovine leukosis infection study**

<b>Farm system</b>	<b>Land size</b>	<b>No. of animals</b>	<b>Breed</b>	<b>Feed type</b>	<b>Inputs</b>	<b>Ecological zone</b>
<b>Zero grazing</b>	Small	1-30	Exotic	Forage & concentrates	High	High altitude
<b>Ranching</b>	Large	>30	Cross & exotic	High quality pasture	Low	Moderately high altitude plains
<b>Pastoral</b>	Vast	>30	Indigenous	Pasture	Very Low	Arid and semi-arid areas

Source: Omore *et al.*, 1999

### 3.2 Sample size

The required sample size was determined using the formula described by Dohoo *et al.*, 2009:

$$N = \frac{Z^2(pq)}{L^2}$$

Where N= Minimum sample size, Z =1.96 (standard deviation score at 95%), p= not known, therefore assumed to be 50%, q= (1-p) and L= Precision (5%).

The least required sample size was 384 sera samples per farming system which gave a total minimum sample size of 1052 sera samples. Of the 14 Counties selected, nine predominantly practiced zero grazing farming system; four had most cattle in the Pastoral system while in one of the Counties ranching was the most common farming system. The numbers of samples from each of the farming systems are shown in the table below.

**Table 3.2 Number of samples per farming system and their Counties of origin**

<b>Farming system</b>	<b>Counties</b>	<b>Number of samples</b>
Zero Grazing	9 (Homabay, Kakamega, Kiambu, Machakos, Murang'a, Nakuru, Nandi, Nyamira and Nyeri)	638
Pastoral	4 (Garissa, Kwale, Marsabit and Narok)	492
Ranching	1 (Laikipia)	253
Total	14	1383



### **3.3 Sample collection**

The blood was collected in sterile vacutainer tubes using sterile needles and labelled with information that included; the animal ID, herd number, age, sex, breed, village, location and the County. On clotting, the tubes were kept in a standing rack and chilled overnight. The samples were stored in portable cool boxes and transported to the Central Veterinary Investigation Laboratories where they were stored, they were then centrifuged and the serum harvested. The serum was divided and put into cryovials that were stored in cold chain to be tested for bovine leukosis infection.

### **3.4 Laboratory Analysis**

All the samples were tested at the serology laboratory at the central veterinary investigation laboratories in Kabete, Nairobi, Kenya. The sera samples were tested for anti-BLV antibodies using an indirect antibody Elisa kit (IDEXX Leukosis Serum Screening, 06-02110-17) that had a sensitivity and specificity of 100%. Each kit had five micro plates with 96 wells each and three of the wells were used for controls leaving 93 wells per micro plate for the samples. Fourteen micro plates tested 93 samples each and one plate tested 81 samples which totalled to 1383 sera samples.

#### **3.4.1 Test principle**

The samples to be tested are diluted and incubated in wells that are coated with the BLV antigen. On incubation of the test samples, anti-BLV specific antibodies form immune complexes with the BLV antigens on the wells. After washing of the unbound material, an anti-bovine antibody enzyme conjugate is added which binds to any antigen-antibody immune complex. On washing away of the unbound conjugate, a TMB substrate is added which is oxidized in the presence of the enzyme to become a blue colored compound which turns to yellow on addition of the stop

solution. Colour development and intensity is directly correlated to the amount of antibodies against BLV that are present in the test sample. The optical density is measured using a microplate reader at 450nm and the results obtained by comparing the sample optical density with the average optical density of the positive controls.

### **3.4.2 Reagents and materials**

The reagents that were used were stored at 2-8<sup>0</sup>C before use and they included; BLV antigen coated plates, positive controls, negative controls, conjugate concentrate (100X), dilution buffer N.1, dilution buffer N.2, TMB substrate N.13, stop solution N.3, wash concentrate (10X) N.2 and distilled or ionized water. The equipment, instruments and materials used were; precision pipettes for delivering 10 to 1000µl, disposable pipette tips, graduated cylinder for the wash solution, 96-well microplate reader (Halo LED 96 DYNAMIC ELISA machine), microplate washer, vortex, microplate covers (lid), centrifuge (2000 x g), incubator maintaining a temperature of 37<sup>0</sup>C (±3<sup>0</sup>C), microplate shaker and uncoated plates for sample preparation.

### **3.4.3 Reagents preparation**

#### ***Samples and controls***

The samples and controls were diluted in a ratio of 1:20 with the dilution buffer N.2 in pre-dilution plates for one hour at 37<sup>0</sup>C.

#### ***Wash solution***

The wash concentrate (10X) N.2 was diluted in a ratio of 1:10 with distilled water at 18-26<sup>0</sup>C and thus called the wash solution which was stored at 2-8<sup>0</sup>C. At these temperatures, the solution can be stable for up to 3 days.

### *Conjugate*

The conjugate concentrate (100X) was diluted in a ratio of 1:100 with the dilution buffer N.1 at 18-26<sup>0</sup>C. The solution can be stable at these temperatures for up to eight hours.

#### **3.4.4 Test procedure**

All the reagents were allowed to come to temperatures between 18<sup>0</sup>C and 26<sup>0</sup>C before use. The coated microplates were labelled and the position of each sample was identified. The precision pipettes were calibrated to measure 100µl of the reagents. About 100µl of the diluted negative control was dispensed into one well labelled negative control and 100µl of diluted positive control was dispensed in two adjacent wells labelled positive control. In the remaining 93 wells, 100µl of diluted samples were dispensed in the order they were organized in the test tube rack. The contents in the microplates were homogenized using a microplate shaker and the plates were then covered using a lid and incubated at 37<sup>0</sup>C for one hour. The solutions were removed and each well washed three times with approximately 300µl of wash solution then tapped onto an absorbent material to remove the residual wash fluids. 100µl of diluted conjugate (anti-bovine antibody enzyme) was dispensed into each well, covered and incubated at 37<sup>0</sup>C for 30 minutes. Thereafter the solution was removed and the wells washed with approximately 300µl of wash solution then tapped onto an absorbent material to remove any residual wash fluid. This was followed by dispensation of 100 µl of the TMB substrate N.13 into each well and incubated at 20<sup>0</sup>C for 20 minutes away from direct sunlight. About 100µl of stop solution was dispensed into each well to stop the reaction. The optical densities of samples and controls were measured at 450nm using the Halo LED 96 DYNAMIC ELISA machine. These measurements were recorded against the individual samples.

### 3.4.5 Validity criteria

#### *Controls*

$$\text{AVPC} = \{\text{PC}_1 (@450\text{nm}) + \text{PC}_2 (@450\text{nm})\} / 2$$

1. AVPC  $\geq$  0.350
2. AVPC: NC  $\geq$  3.00

Where: PC<sub>1</sub>- Positive control 1, PC<sub>2</sub> –Positive control 2, NC- Negative control and AVPC- average optical density of the positive controls.

### 3.4.6 Interpretation

Sample positivity (% S.P)

$$\% \text{ Sample positivity (S.P)} = \{(\text{SOD} - \text{NC}) / (\text{AVPC} - \text{NC})\} * 100$$

Where: S.P- sample positivity, SOD- sample optical density@450nm, NC- negative control, AVPC- average optical density of the positive controls.

For any given sample, if % S.P was  $\leq$  60, it was considered negative while if % S.P  $>$  60 the sample was considered positive.

### 3.5 Data analysis

The Microsoft® Excel 2013 spreadsheet was used to enter the raw data which was cleaned, coded and exported to Stata® 14 statistical package for analysis. The prevalence of bovine leukosis infection was established in the overall data; and in the specific categories of age, breed, sex, farming systems and Counties of origin. Univariate logistic regression models were used to determine the association between the risk factors and occurrence of bovine leukosis infection in Kenya using a 10% ( $p=0.1$ ) significance level. The statistically significant factors were fit into a fixed effects multivariate logistic model to assess how they were associated with having bovine leukosis infection in Kenya. With multi stage sampling, the hierarchical structure of the data

indicated that there was clustering at the County level. On accounting for this clustering the significant factors were fit into a multivariate mixed logistic regression model to determine their effects on the occurrence of EBL in Kenya. In this model, the variables age, breed, sex and farming system were fixed effects while County was modelled as a random effect. In addition to this, the mixed model was used to assess the difference in association of the risk factors before and after accounting for clustering of the data. The five percent ( $p=0.05$ ) significance level was used to assess the statistical associations between risk factors and bovine leukosis infection. The odds ratio of the predictors will be used to assess their strength of association to occurrence of bovine leukosis infection in the 14 Counties in Kenya.

## CHAPTER FOUR

### RESULTS

#### 4.1 Prevalence of Bovine Leukemia Virus antibodies

##### *Overall Prevalence*

The overall prevalence of bovine leukosis infection in the 14 Counties was 7.6% with 105 of the 1383 sera samples testing positive for antibodies against bovine leukemia virus.

##### *Prevalence by age*

Eighty six sera samples collected from cattle that were older than one year tested positive for anti-BLV antibodies out of 1,087 sera samples tested which indicated a prevalence of 7.9%. In comparison, cattle less than a year old had a slightly lower prevalence of 6.4 % where 19 cases were confirmed out of the 296 sera samples tested. The difference in the prevalence between the two age groups was not statistically significant at the five percent significance level ( $\chi^2=0.739$ ,  $p=0.390$ ).

##### *Prevalence by breed*

The prevalence of bovine leukosis infection in indigenous cattle in the 14 Counties was 10.8% where 101 out of the 939 tested were positive for antibodies against BLV. The exotic cattle (Holstein, Ayrshire, Guernsey and Jersey) had a prevalence of 0.9% with only 4 cases of bovine leukosis infection identified out of 440 samples tested. The difference in these prevalence was strongly significant at the 5% significance level ( $\chi^2=41.733$ ,  $p<0.0005$ ).

##### *Prevalence by sex*

Three hundred and nine sera samples from bulls were tested and 27 of these were positive for anti-BLV antibodies thus a prevalence of 8.7% while seventy eight samples taken from cows were positive for bovine leukemia virus of the 1,074 sera samples tested indicating a

seroprevalence of 7.3%. This slight difference in prevalence between the male and cows was not statistically significant ( $\chi^2=0.744$ ,  $p=0.388$ ).

#### ***Prevalence by farming system***

Cattle in pastoral systems had the highest prevalence of 18.3% where 90 cases were identified out of the 492 samples tested. Eleven cattle that were kept in ranches tested positive for antibodies against bovine leukemia virus out of the 253 cattle tested which indicated a prevalence of 4.4%. Zero grazed cattle had the lowest prevalence of 0.6% with only 4 cases confirmed out of 634 sera samples tested. This difference in prevalence was highly significant ( $\chi^2=128.210$ ,  $p<0.0005$ ).

#### ***Prevalence by County***

Positive cases of bovine leukosis infection were found in five of the fourteen Counties. Garissa County had the highest prevalence of 37.5% with 57 of the 152 sera samples collected there testing positive for antibodies against BLV. Marsabit County followed with a prevalence of 25.4% where 32 cases were identified out of the 126 sera samples tested. Twelve of the samples from cattle in Laikipia County were positive for anti-BLV antibodies out of the 350 samples that were tested thus a prevalence of 3.4%. Nandi County had a prevalence of 3.6% with 2 cases identified out of the 76 samples tested. Only 2 samples taken from cattle in Nakuru County tested positive for antibodies against BLV which indicated the lowest prevalence of 2.9%. Homabay, Kakamega, Kiambu, Muranga, Kwale, Machakos, Narok, Nyamira and Nyeri Counties had no samples testing positive for antibodies against bovine leukemia virus. These differences in prevalence in the counties were strongly significant ( $\chi^2=314.579$ ,  $p<0.0005$ ).

The seroprevalence of bovine leukosis infection in cattle in Kenya is summarised in the table below.

**Table 4.1 Variable definition and seroprevalence of EBL in animals in Kenya**

Variable	Definition	Levels & Frequency N=1383	Number of cases per level	EBL prevalence
BLV	Outcome	Negative (0)= 1278 Positive (1) = 105	Cases=105	Overall Prevalence=7.6%
Age	Predictor	Adult (>1yr)(0)= 1087 Young(=<1yr)(1)= 296	Adult=86 Young=19	0=7.9% 1=6.4%
Sex	Predictor	Female (0)= 1074 Male(1)= 309	Female=78 Male=27	0=7.3% 1=8.7%
Breed	Predictor	Indigenous(0)= 939 Exotic (1)= 440	Indigenous=101 Exotic=4	0=10.8% 1=0.9%
Farming system	Predictor	Zero- grazing(0)= 634 Pastoral (1)= 492 Ranching(2)= 253	Zero grazing=4 Pastoral=90 Ranching=11	0=0.6% 1=18.3% 2=4.4%
County	Predictor	Laikipia (0)=350 Machakos (1)=210 Garissa (2)=152 Marsabit (3)=126 Kakamega (4)=84 Narok (5)=84 Murang'a (6)=70 Nakuru (7)=70 Nandi (8)=56 Kiambu (9)=56 Nyeri (10)=55 Kwale (11)=42 Homabay (12)=14 Nyamira (13)=14	Laikipia =12 Machakos=0 Garissa =57 Marsabit=32 Kakamega=0 Narok =0 Murang'a=0 Nakuru =2 Nandi=2 Kiambu =0 Nyeri =0 Kwale =0 Homabay=0 Nyamira =0	Laikipia =3.4% Machakos=0 Garissa =37.5% Marsabit=25.4% Kakamega=0 Narok =0 Murang'a=0 Nakuru =2.9% Nandi=3.6% Kiambu =0 Nyeri =0 Kwale =0 Homabay=0 Nyamira =0



#### **4.2 Risk factors associated with Seropositivity from the mixed logistic regression model**

The variables that were considered as risk factors to the occurrence of bovine leukosis infection were; farming system, age, sex, breed and the County of origin of the cattle. Before accounting for clustering, Breed, farming system and County of origin were significantly associated with the occurrence of bovine leukosis infection in Kenya at a 5% significance level ( $p < 0.0005$ ). In addition to this, Sex of the cattle was not significantly associated with occurrence of bovine leukosis infection at a significance level of 5% ( $p = 0.444$ ). Age of the cattle was also not significantly associated with occurrence of bovine leukosis infection at the 5% significance level ( $p = 0.442$ ). On accounting for clustering at the County level, age was significantly associated with occurrence of bovine leukosis infection in Kenya ( $p = 0.01$ ) at the 5% significance level. Farming system was associated with occurrence of bovine leukosis infection in Kenya marginally at the 5% significance level ( $p = 0.063$ ).

The probability of bovine leukosis infection occurring in adult cattle that were zero grazed ranged from 0.01% to 2.42%. The odds of EBL occurring in the young cattle in a given county were 0.36 times less than in the adult cattle in the same county ( $OR = 0.36$ ) while the odds of EBL occurring in young cattle in any of the 14 Counties in Kenya were 0.56 times less than in adult cattle ( $OR = 0.56$ ). The probability of young cattle in a given county testing positive for bovine leukosis infection was 26.5 % and the probability of young cattle in any of the 14 Counties testing positive for bovine leukosis infection was 35.8%. The odds of EBL occurring in cattle in pastoral farms were 19 times higher than in zero grazed cattle in the same county ( $OR = 19$ ). These odds reduced to 5 times when comparing occurrence of EBL in cattle in pastoral farms and zero grazing farms in any of the 14 Counties in Kenya ( $OR = 5$ ). Cattle in pastoral systems in a given county have a 95% probability of having bovine leukosis infection. Cattle kept in a

pastoral farming system and randomly chosen from any of the 14 Counties has an 84.5% probability of testing positive for bovine leukosis infection. The odds of EBL occurring in cattle in ranches was 59 times higher than in zero grazed cattle in the same county (OR=59) while these odds reduced to 10 times higher when comparing ranches and zero grazed cattle in any of the 14 counties in Kenya (OR=10). The probability of cattle in a ranch in a given county having bovine leukosis infection is 98.3%. A randomly chosen animal in a ranch in any of the 14 counties in the study had a 91% probability of testing positive for bovine leukosis infection.

The occurrence of bovine leukosis infection varied across the 14 counties by a factor of log odds of 5.909. Given a cattle in a given county tested positive for EBL, the probability of a randomly selected cattle from the same county testing positive was 64.2% (ICC=0.642). If an animal was moved from a county with a low probability of bovine leukosis infection to a county with a higher probability of having EBL, the cattle's risk of getting EBL will increase 280 times in median parameters.

## CHAPTER FIVE

### DISCUSSION, CONCLUSION AND RECOMMENDATION

#### 5.1 Discussion

The overall prevalence (7.6%) was low compared to other African countries like South Africa, Namibia, Uganda and Tanzania that reported prevalence of 12.6%, 12.3%, 17% and 36% respectively (Ndou *et al.*, 2011; Kaura & Hbschule 1994; Adul & Olson 1981; Schoepf *et al.*, 1997; Azuba *et al.*, 1994).

Cattle older than one year had a slightly higher prevalence (7.9%) than young cattle (6.4%). This can be explained by the long latent period of the virus in the cattle before subclinical infection is manifested in the animals. In addition to this, dams with antibodies against bovine leukemia virus pass the maternal antibodies to the calves in colostrum subsequently protecting them against infection. The results were in agreement with previous studies that indicate occurrence of bovine leukosis infection being higher in adults due to the chronic nature of the disease (Nava *et al.*, 2011).

Indigenous breeds had a significantly higher prevalence of EBL (10.8%) compared to Exotic breeds (0.9%). This contradicts with a limited serological survey in South Africa that reported a 10% prevalence in Jersey herds and a much lower prevalence in South African indigenous breeds (Voster & Mapham 2008). It can be stipulated that most of the indigenous breeds are kept in pastoral systems in which many cattle are grazed together in communal lands. One of the vital methods of transmission of the bovine leukemia virus is through contact and these cattle had unrestricted contact with both infected and healthy cattle thus increasing the chances of spread

and infection with BLV. Other countries including; Columbia, Chile, Venezuela, Uruguay and Argentina have reported higher prevalence of bovine leukosis infection between 34% and 50% in the breeds of cattle specific to these countries (Rama *et al.*, 2011; Trono *et al.*, 2001). However, no specific reports have been made before for the prevalence in Kenyan local breeds.

Bulls had a higher prevalence of EBL (8.7%) than cows (7.3%). In Kenya, most bulls are kept in ranches and pastoral systems for beef and breeding with very few kept in zero grazing units. As indicated earlier, cattle in Pastoral systems and ranches have unlimited contact with other cattle which can be infected thus increasing the chances of infection. These results contradict with some studies that have reported higher prevalence in dairy herds than in beef herds in the U. S (Buehring *et al.*, 2014). This inconsistency can be explained by the different farming systems in Kenya and the U.SA, where dairy cattle are kept in large numbers in the Unites States and intra herd transmission of the bovine leukemia virus can increase the cases in the herds.

Cattle in pastoral farming systems had the highest prevalence (18.3%), followed by ranched cattle with 4.4% while zero-grazed cattle have the lowest prevalence (0.6%). This is consistent with the breed prevalence considering that indigenous breeds are predominant in the pastoral system while exotic breeds dominate the zero grazing systems. In ranches, indigenous breeds, exotic breeds and cross breeds are almost evenly distributed which explains the moderate prevalence in Laikipia County (3.4%) where most ranches are located. Counties with the highest prevalence, Garissa (37.5%) and Marsabit (25.4%) predominantly practice pastoral farming while Counties with the lowest prevalence; Nakuru (2.9%) and Nandi (3.6%) have high numbers

of zero grazing farms. It can be ascertained that the farming systems have a strong impact on occurrence of bovine leukosis infection in Kenya.

Age of the cattle was strongly associated with occurrence of bovine leukosis infection in the 14 Counties involved in the study ( $p=0.001$ ) at the 5% significance level. It can be stated that in Kenya younger cattle have a lower probability of having bovine leukosis infection compared to older cattle. This is could be due to the prolonged progression of the infection in cattle with lymphomas taking up to five to ten years to develop after the initial infection (Yoon *et al.*, 2005).

Cattle in pastoral systems had higher odds of bovine leukosis infection when compared to zero grazed cattle (OR=19.2). Pastoralists keep large herd of cattle and move from area to another in search of pasture and water. They graze their cattle together in communal grazing lands and the common disease control measure is mass vaccination of the livestock (IGAD 2013). Artificial transmission of BLV that occurs through blood contaminated needles, surgical equipment and rectal gloves (Mekata *et al.*, 2015) can be expected to be high in these cattle due to their close uninhibited interaction for prolonged time periods. Zero grazing farming system involves less than 10 cattle kept in one household and the cattle rarely get out of the units with vaccinations and treatment procedures carried out in every household. Transmission of BLV from cattle in one unit to other cattle in another unit is very unlikely due to the limited to no contact between the cattle. A herd in a zero grazing unit that is free of the BLV can progress without the disease for generations unless additions are made and the new cattle have the infection. The same applies for cattle in a zero grazing unit infected with BLV, the infection can persist in the unit for years but does not move to the neighbouring unit due to lack of contact. This could be one possible

explanation to the very low prevalence of EBL in zero grazed cattle. Trans-placental transmission accounts for 10-15% of the infections (EFSA 2015; OIE 2013). Cattle in pastoral systems calve in the fields and contact with body fluids and blood is unrestricted among the grazing cattle. This also may increase the number of infected animals and increase the spread of EBL among the cattle.

Ranches in Kenya keep a moderately large number of cattle enclosed in a given area. This is the major difference with pastoral systems and interaction with cattle from other ranches is very unlikely and rare. However, cattle in some ranches interact with cattle in pastoral systems as they move from one place to another. Transmission of BLV can occur naturally during parturition, artificially or trans-placental and contact with other animals can increase the transmission and probability of infection in this ranched cattle. This can explain the high odds of EBL occurring in ranched cattle compared to zero grazed cattle (OR=10.51). Age of the cattle and the farming system the cattle is in have a strong impact on the probability of that cattle having bovine leukosis infection in Kenya.

The strong variation between occurrence of EBL in cattle in the 14 Counties (log odds 5.909) and the large inter class correlation between cattle in the same County (64.2%) indicated that there was strong clustering at the County level. In Kenya, most Counties have one common farming system depending on the climate, availability of feed and demand for livestock products. Counties in the highlands where the annual rainfall is high commonly practice zero grazing farming system and keep dairy cattle. Counties in the arid and semi-arid region where the annual

rainfall is low most of the cattle are kept in pastoral systems and ranches. This distribution of farming systems in the Counties in Kenya explain the strong clustering and similarity among cattle randomly chosen from the same County. The large cluster-median odds ratio (MOR=280) indicates that the residual variation between Counties relevant in understanding the differences in the individual odds of a cattle having bovine leukosis infection in Kenya.

## **5.2 Conclusion**

1. Bovine leukosis infection was present in five of the selected Counties in Kenya
2. Adult cattle (> 1 year) had a higher chance of testing positive for bovine leukosis infection in the 14 Counties in Kenya in comparison to young cattle( $\leq$  1 year).
3. Indigenous cattle in Kenya had a higher chance of testing positive for bovine leukosis infection compared to cattle of exotic breeds.
4. Cattle kept in Pastoral farming systems had a higher probability of testing positive for bovine leukosis infection than cattle in ranches and zero grazing farming systems.
5. Counties in the Arid and semi-arid areas of Kenya e.g. Garissa and Marsabit had the highest number of cases and cattle in these areas have the highest probability of testing positive for bovine leukosis infection.

## **5.3 Recommendations**

1. Educate farmers on the spread, impact and control of Bovine leukosis infection in Kenya with emphasis to the Counties with the high number of cases
2. Additional research should be done to estimate the frequency of EBL clinical cases in slaughter houses in Kenya.
3. Estimate the economic losses attributed to Bovine leukosis infection in Kenya in a longitudinal study
4. County specific and national level control programs against bovine leukemia virus should be designed and implemented in Kenya.



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## APPENDICES

### Appendix 1. Univariate logistic regression models;

#### Appendix 1.1 BLV and Sex

. logit blv sex, nolog

```

Logistic regression              Number of obs   =    1,383
                                LR chi2(1)       =         0.57
                                Prob > chi2        =    0.4499
Log likelihood = -371.31897      Pseudo R2      =    0.0008
    
```

blv	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
sex	.1783604	.2331329	0.77	0.444	-.2785717	.6352925
_cons	-2.542006	.1175993	-21.62	0.000	-2.772496	-2.311515

#### Appendix 1.2 BLV and Age

. logit blv age, nolog

```

Logistic regression              Number of obs   =    1,383
                                LR chi2(1)       =         0.61
                                Prob > chi2        =    0.4334
Log likelihood = -371.29757      Pseudo R2      =    0.0008
    
```

blv	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
age	-.201973	.2625452	-0.77	0.442	-.7165522	.3126061
_cons	-2.45939	.1123474	-21.89	0.000	-2.679587	-2.239193

#### Appendix 1.3 BLV and Breed

. logit blv breed, nolog

```

Logistic regression              Number of obs   =    1,383
                                LR chi2(1)       =    56.45
                                Prob > chi2        =    0.0000
Log likelihood = -343.38144      Pseudo R2      =    0.0759
    
```

blv	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
breed	-2.584583	.513193	-5.04	0.000	-3.590423	-1.578743
_cons	-2.115898	.1053295	-20.09	0.000	-2.32234	-1.909456

## Appendix 1.4 BLV and Farming system

. logit blv i.fsystem, nolog

```

Logistic regression                               Number of obs   =       1,383
                                                    LR chi2(2)      =       135.97
                                                    Prob > chi2     =       0.0000
Log likelihood = -303.61829                       Pseudo R2      =       0.1830
  
```

blv	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
fsystem						
1	3.569112	.5149523	6.93	0.000	2.559824	4.5784
2	1.974712	.5887432	3.35	0.001	.8207963	3.128627
_cons	-5.065754	.5015747	-10.10	0.000	-6.048823	-4.082686

## Appendix 1.5 BLV and County

```

Logistic regression                               Number of obs   =       754
                                                    LR chi2(4)      =       124.78
                                                    Prob > chi2     =       0.0000
Log likelihood = -241.9342                       Pseudo R2      =       0.2050
  
```

blv	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
county						
1	0	(empty)				
2	2.827314	.3381746	8.36	0.000	2.164504	3.490124
3	2.26058	.3580226	6.31	0.000	1.558869	2.962292
4	0	(empty)				
5	0	(empty)				
6	0	(empty)				
7	-.1882213	.7752405	-0.24	0.808	-1.707665	1.331222
8	.0423024	.7776956	0.05	0.957	-1.481953	1.566558
9	0	(empty)				
10	0	(empty)				
11	0	(empty)				
12	0	(empty)				
13	0	(empty)				
_cons	-3.338139	.2937549	-11.36	0.000	-3.913888	-2.76239



### Appendix 3. Full mixed logistic regression model;

```

Mixed-effects logistic regression      Number of obs      =      1,383
Group variable:      county           Number of groups   =          14

Obs per group:
      min =          14
      avg =          98.8
      max =          350

Integration method: mvaghermite       Integration pts.   =          7

Log likelihood = -250.7971             Wald chi2(5)      =          15.95
                                         Prob > chi2       =          0.0070

```

blv	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
age	-1.05353	.3237696	-3.25	0.001	-1.688107	-.4189537
sex	.1414779	.2766271	0.51	0.609	-.4007012	.6836571
breed	.8381632	1.910047	0.44	0.661	-2.90546	4.581786
fsystem						
1	3.56135	2.114773	1.68	0.092	-.58353	7.706229
2	4.670518	2.293216	2.04	0.042	.1758968	9.165139
_cons	-7.112411	2.188019	-3.25	0.001	-11.40085	-2.823973
county						
var(_cons)	5.790018	4.492957			1.265207	26.49709

```

LR test vs. logistic model: chibar2(01) = 100.75      Prob >= chibar2 = 0.0000

```

.



#### Appendix 4. Final mixed logistic regression model

```
. melogit blv age i.fsystem|| county:, nolog
```

```
Mixed-effects logistic regression      Number of obs   =    1,383
Group variable:      county           Number of groups =     14

Obs per group:
    min =     14
    avg =    98.8
    max =    350

Integration method: mvaghermite      Integration pts. =     7

Wald chi2(3)      =    15.58
Prob > chi2      =    0.0014

Log likelihood = -251.02022
```

blv	Coef.	Std. Err.	z	P> z	[95% Conf. Interval]	
age	-1.01949	.3159524	-3.23	0.001	-1.638746	-.400235
fsystem						
1	2.954783	1.527947	1.93	0.053	-.0399375	5.949503
2	4.082703	1.735645	2.35	0.019	.6809022	7.484504
_cons	-6.432808	1.396259	-4.61	0.000	-9.169426	-3.69619
county						
var(_cons)	5.909116	4.61297			1.279468	27.29076

```
LR test vs. logistic model: chibar2(01) = 102.63      Prob >= chibar2 = 0.0000
```