TRANSMISSION AND PROGRESSION OF BOVINE LEUKEMIA VIRUS

By

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ABSTRACT

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Bovine leukemia virus (BLV) is a deltaretrovirus which infects more than 40% of the United States cattle population and more than 85% of U.S dairy herds. Upon infection of a susceptible host, BLV reverse transcribes its viral RNA genome into a DNA provirus that integrates into the cellular DNA of the host, resulting in a lifelong infection. Clinical outcomes of BLV infection are the development of lymphocytosis in more than 30% of infected cattle and the development of lymphoma in less than 10%. Furthermore, BLV infected cattle have been shown to be immunocompromised, to produce less milk, and to have shortened lifespans when compared to their uninfected herd mates. Collectively, these impacts warrant the consideration of disease eradication from the U.S. cattle population. In fact, eradication has already been achieved in more than 21 countries, predominately within the European Union.

One objective of our BLV research team has been to develop BLV management solutions that are both practical and economically feasible. The goal of this dissertation was to contribute to these efforts by providing a greater understanding of the routes through which BLV can be transmitted, by describing how BLV progresses over time to disease states associated with increased infection potential, and by understanding how newly available diagnostics can best be utilized to detect BLV infections.

To contribute to the identification of BLV transmission routes, a statistical model was built to examine the association between herd management practices and the BLV incidence rate among Michigan dairy herds. This analysis estimated the marginal incidence rate in Michigan

dairy herds to be 2.11 infections per 100 cow-months at risk. Herd management practices positively associated with herd-level rate of infection were herd prevalence, the frequency of needle reuse, housing post-parturient cows separately, and increased milking frequency. The use of sand bedding appeared to have protective effects and was negatively associated with the incidence rate.

To describe how BLV progresses over time, longitudinal observations on proviral load, lymphocyte counts, and ELISA test results collected during an intervention field trial were analyzed. The results from this analysis indicated that negligible increases in lymphocytes and small increases of approximately 3,000 proviral copies per 100,000 cells occurred over a six-month interval. Additionally, infected cattle with low proviral loads and normal lymphocyte counts were the most likely to experience changes in ELISA status that may result in ELISA false-negatives.

To determine our ability to detect BLV infections and to examine the role early infection plays in long-term disease progression, fifteen steers were experimentally infected with BLV and followed for 147 days. This study found that new infections are detected by PCR on average 24 days post infection prior to detection by ELISA at 36 days post infection. Furthermore, the observations on early viral kinetics suggest BLV proviral load and lymphocyte count levels may be established soon after initial infection.

Overall, the results of this dissertation contribute to ongoing BLV control efforts by identifying new potential routes of transmission that can be explored in future intervention trials, finding that the development of PVL and LC may not be the result of gradual disease progression but may be established shortly after infection, and determining the relationships among the various BLV diagnostic tests.

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KEY TO ABBREVIATIONS

AIC:	Akaike Information Criterion
BIC:	Bayesian Information Criteria
BLV:	bovine leukemia virus
CoCoMo:	Coordination of Common Motif
CI:	confidence interval
DIM:	days in milk
DNA:	deoxyribonucleic acid
DPI:	days post inoculation
EBL:	enzootic bovine leukosis
ELISA:	enzyme-linked immunosorbent assay
ELISA-SP:	ELISA-suspect or ELISA-positive
GLMM:	generalized linear mixed model
IN:	infected
IPNC:	in-pen negative control
K_2EDTA:	dipotassium ethylenediaminetetraacetic acid
LC:	lymphocyte count
LMM:	linear mixed model
IRR:	incidence rate ratio
NB:	negative binomial
NC:	negative control
OD:	optical density

OR:	odds ratio
PCR:	polymerase chain reaction
PRM:	Poisson regression model
PVL:	proviral load
RNA:	ribonucleic acid
qPCR:	real-time PCR
ZINB:	zero-inflated negative binomial
ZIP:	zero-inflated Poisson

Chapter 1 :

Introduction

Abstract

Bovine leukemia virus (BLV) is a prevalent retroviral infection in cattle in the United States and around the world. Clinical signs of infection include the development of lymphocytosis or lymphomas. Estimates indicate more than 40% of dairy cattle and 90% of dairy herds in the U.S. are infected with BLV. Furthermore, infection may increase susceptibility to infectious diseases and lead to economic losses accrued through treatment costs, decreased production and longevity, trade-restrictions, and carcass condemnation at slaughter. Given these effects, disease control efforts are warranted. To successfully control the disease, a greater understanding of the strengths and limitations of current efforts as well as of transmission routes and disease progression is needed.

Introduction

In 1878 Siedamgrotzky identified and reported the development of tumors in cattle. Later in the 1920s and 1930s, meat-inspection statistics began to report the incidence of leukotic tumors in Germany and the United States. Around the same time, it was also discovered that infected animals frequently had an increase in white-blood cells, creating a condition of lymphocytosis. The term enzootic bovine leukosis (EBL) was assigned to these outbreak clusters and early control programs relied on the identification and removal of cattle in these disease states. In 1969, Miller *et al* identified the causative agent of these lymphoma and lymphocytotic disease states to be the bovine leukemia virus (BLV), a c-type retrovirus (Miller et al., 1969). Sequenced in 1985 by Sagata, BLV was assigned to the family Retroviridae and grouped with the human T-lymphotropic virus in what is now the genus deltaretrovirus (Sagata et al., 1985). As a retrovirus, BLV is an RNA virus that reverse transcribes itself into a DNA provirus and integrates into the host genome resulting in a persistent, lifelong infection.

Identification of BLV as the causative agent of EBL allowed for the development of diagnostic assays to identify infected animals by detection of anti-BLV antibodies or the BLV provirus. The natural hosts for BLV are both cattle and water buffalo (EFSA Panel on Animal Health and Welfare, 2015). However, other ruminant species (e.g. sheep, goals, deer, alpacas), pigs (Mammerickx et al., 1981), chickens, rabbits (Wyatt et al., 1989), and rats were reported to be experimentally susceptible. Further elucidation of the infection distribution indicated that the early reports likely only identified a small subset of infected cattle. We now know a substantial portion of infected animals do not exhibit any outward clinical signs or manifestations of infection. These animals can remain in an aleukemic state in which the infection can only be identified through the use of diagnostic assays that identify either the virus or an immune

response to the virus. The development of persistent lymphocytosis occurs in 30 to 40% of BLV-infected animals with less than 10% developing BLV-associated tumors, independent of lymphocyte count.

Routine Diagnosis of BLV

Lymphoma:

While bovine leukosis was first identified because of the incidence of tumors, the development of lymphoma or lymphosarcoma is now known to be the final clinical outcome of infection. Lymphomas associated with BLV are B-cell tumors that develop within a peripheral lymph node. Lymphosarcomas occur in the tissue and have been frequently observed in the retrobulbar space, abomasum, uterus, spinal cord, and internal organs. The development of these tumors is the result of a monoclonal or oligoclonal expansion of a BLV-infected lymphocyte (EFSA Panel on Animal Health and Welfare, 2015). While persistent lymphocytosis is observed in two-thirds of cattle with lymphoma, it is not a prerequisite (Ferrer, 1980). Although occasionally detected through the palpation of peripheral lymph nodes, the detection of lymphoma or lymphosarcoma generally occurs post-mortem at slaughter where it results in carcass condemnation (Haredasht et al., 2018; White and Moore, 2009).

Cell Counts:

When the incidence of tumors was discovered, an association with an elevated lymphocyte count was observed (Marshak et al., 1962). The development of lymphocytosis is now known to occur in approximately 30% of infected cattle (EFSA Panel on Animal Health and Welfare, 2015; Kabeya et al., 2001) and is a hallmark of BLV infection.

Early diagnosis of BLV relied on the identification of cattle with either lymphocytosis or tumors. In 1959, Hans Jørgen Bendixen created “Bendixen’s Key”, which was a revised

leukosis-key created by Götze et al (1953). This key established the normal reference intervals for lymphocyte parameters by age for the Danish cattle population and categorized animals into one of three disease groups: normal, dubious, or leukemic (Bendixen, 1963). Inclusion of an age-associated factor was important given the natural decline in lymphocytes observed with age. Failure to adjust for age would result in a higher proportion of young animals being categorized as abnormal. While this key was effective in the Danish control programs efforts to eradicate EBL from their herds, the effects of factors such as breed and environmental conditions on normal hematological parameters should be taken into consideration. For these reasons, this key has been modified for implementation in various BLV control programs (Akagami et al., 2019; Mekata et al., 2018b). Potential exists for the U.S. to develop a screening key for the U.S. cattle populations. Given more than 90% of the U.S. dairy herd is Holstein, a start would be to establish normal reference intervals for BLV-free Holstein populations.

Detection of Immune Response:

Leukocyte keys allow for the detection of animals in clinical disease states. However, with the identification of the viral agent it became evident that animals can be infected and exist in a subclinical, aleukemic state. In fact, 50-60% of infected animals are thought to be aleukemic. Identification of these animals requires the use of diagnostic assays that can either detect the host's immune response to the virus or directly detect the virus itself. The World Organization for Animal Health recognizes antibody-based assays as the gold-standard for EBL/BLV diagnosis. Serological assays do not directly detect the virus, but rather an immune response to the virus. Therefore, a limitation of serological assays is they cannot distinguish between antibodies that are acquired through passive transfer and those which are produced by the animal.

The two most frequently detected antibodies are anti-gp51 and anti-p24. The anti-gp51 antibody targets an envelope glycoprotein while the anti-p24 antibody targets a viral capsid protein. The results of a meta-analysis indicated the median time until detection of antibodies post-infection was 57 days (Monti and Frankena, 2005). However, the detection of anti-gp51 antibodies generally was reported to occur prior to the detection of anti-p24 antibodies (Klintevall et al., 1994). Serological assays that can be used to detect anti-BLV antibodies include radioimmunoassay (RIA), agar-gel immunodiffusion (AGID), and enzyme-linked immunosorbent assays (ELISA).

The use of RIA has been phased out of use for the diagnosis of BLV because it requires the use of radioactive substances. Analysis by AGID is labor intensive as it requires the purification of BLV antigen and the molding of agarose gels. In addition, the process is time intensive as plates need to be incubated at room temperature and read for three consecutive days ("Chapter 2.4.10.-Enzootic Bovine Leukosis," 2018). The sensitivity and specificity of the AGID assay have been estimated to be 79.7% and 99.0%, respectively (Trono et al., 2001). A limitation of the AGID when compared to ELISA is the required use of serum; ELISA can be used to identify antibodies in a wider range of samples including serum, milk, and colostrum. Several commercial ELISA kits are available, many of which have been comparatively evaluated for both sensitivity and specificity (Kuczewski et al., 2018; Reichel et al., 1998; Simard et al., 2000). The sensitivity of ELISA based assays is greater than 97% while specificity varies from 78-100%.

Our BLV research team at Michigan State University has previously utilized both serum- and milk-based ELISAs. The BLV-ELISA utilized by our research team is performed by CentralStar Cooperative (Lansing, Michigan) and is a modified IDEXX gp51 antibody capture

ELISA (Erskine et al., 2012a). Comparison of a milk ELISA to a gold standard AGID assay resulted in a sensitivity of 97% and specificity of 99.6% (De Boer et al., 1989). While detection of anti-BLV antibodies by serum- and milk-based ELISAs is reported to be in 100% agreement (Klintevall et al., 1991), a small comparison by our research team indicates the sensitivity and specificity of the milk ELISA are 86% and 100%, respectively, when compared to the serum ELISA (Erskine et al., 2012a). This agreement and relatively high sensitivity has been harnessed through the inclusion of optional diagnostic testing in conjunction with routine milk components testing provided by the Dairy Herd Improvement Association, which is utilized by approximately 40% of U.S. dairy herds with about 50% of U.S cattle enrolled.

Detection of the Virus:

Although serological based assays, which detect an immune response to BLV, are recognized as the gold-standard for diagnosis, the virus itself can be identified through the use of polymerase chain reaction (PCR), which amplifies a portion of the viral genome. As a retrovirus, BLV can potentially be identified through the detection and amplification of either the viral RNA or the DNA provirus which has been reverse transcribed and integrated into the host genome. Because of the virus's tendency to integrate and become latent (Kettmann et al., 1982, 1980), the long-held belief was the viral RNA could not be detected *in vivo* (Gupta et al., 1984; Tajima and Aida, 2005). Therefore, viral detection has predominately been achieved through identification of the DNA provirus.

To detect the BLV provirus, a segment of the proviral genome is amplified. The proviral genome includes three major components: the long terminal repeats (LTR), the structural and enzymatic genes, and the regulatory genes (Sagata et al., 1985). Structural genes include the genome associated genes (GAG), the polymerase gene used for reverse transcription (POL), and

the envelope genes (ENV). A portion of the regulatory genes are the microRNA cassette and the oncogenic protein (TAX). Primers for PCR can be designed for any of these genes or regions. Commonly amplified regions are the LTR (Jimba et al., 2010; Komiyama et al., 2009; Rulka et al., 2003), POL (Heenemann et al., 2012; Rola-Łuszczak et al., 2013), ENV (Heinecke et al., 2017; Licursi et al., 2002; Rulka et al., 2003), and TAX (Okagawa et al., 2017) genes. Given the functional role of the POL gene in reverse transcription, this region is highly conserved (Jaworski et al., 2018; Sagata et al., 1985) and therefore, frequently targeted for viral identification. However, an advantage of targeting the LTR is the presence of two copies per viral genome, which has the potential to increase the analytical sensitivity of PCR assays.

Detection of the BLV provirus by PCR can be either qualitative or quantitative. Qualitive detection is typically performed using standard endpoint PCR which uses agarose gels to indicate the presence or absence of the BLV provirus in a sample. Quantitative PCR (qPCR) takes BLV detection one-step further by quantifying the number of proviral copies in respect to either an endogenous gene or quantity of DNA. The results of quantification are typically used to indicate the proviral load (PVL).

To date, research from our team has utilized two different qPCRs: the CoCoMo qPCR produced and manufactured in Japan (RIKEN Genesis, Tokyo, Japan) (Jimba et al., 2012, 2010) and a newer qPCR developed by CentralStar Cooperative (Lansing, Michigan) and referred to as the SS1 assay. Major differences between the two assays are the amplified BLV and endogenous reference genes and their capability to detect multiples genes simultaneously. The CoCoMo assay utilizes degenerate primers to amplify the LTR region and the endogenous *Bos Taurus* DRB3 genes; however, these regions have to be amplified in separate PCR wells requiring two individual PCR reactions per sample. Utilizing proprietary primers, the SS1 assay is multiplexed

and only requires one PCR reaction per sample to detect and quantify the BLV POL gene, the endogenous *Bos Taurus* Bos actin gene, and a spike-in control ultramer. Both the CoCoMo and SS1 assays quantify the relative copy numbers through the use of plasmid-derived standard curves.

An overall limitation of PCR is the potential for PCR false negatives, or failure in detecting the viral DNA. This can be attributed to the potential for the virus to persist at low levels, to sequester in organs (Klintevall et al., 1997), or the analytical sensitivities of the assays. To circumvent these problems, nested PCRs, which involve two sets of primers utilized in two consecutive PCR assays, have been used (Asfaw et al., 2005; Rola-Łuszczak et al., 2013; Wu et al., 2003). Nested PCRs, however, increase the analytical sensitivity of the assay at the expense of the specificity, which increases the likelihood of false positives (Belák and Ballagi-Pordány, 1993).

A limitation to qPCR that has become apparent is the ability to accurately quantify the number of proviral copies. In a ring-trial comparing six different qPCR assays, a wide variation was observed not only in the ability to detect the BLV provirus but also the quantification of PVL (Jaworski et al., 2018). The discrepancy in detection was most frequently observed when PVL was low, which may be reflective of variations in the analytical sensitivities of the compared assays. Primers for PCR are designed to bind and attach to a specific sequence of nucleic acids; therefore, single nucleotide polymorphisms and mutations associated with viral genotypes may partially explain variation in the detection and quantification (Marsolais et al., 1994; Mekata et al., 2015). Nonetheless, variation observed in reported PVLs emphasizes quantification is relative and is not absolute.

Viral RNA has long been considered to be undetectable *in vivo.* However, following sample collection and *ex vivo* culture, viral reactivation could be observed (Tajima and Aida, 2005). Therefore, the long-standing dogma was that the host immune response prevented active viral replication, leaving the virus to persist through the mitotic replication of infection cells (Florins et al., 2007; Tajima and Aida, 2005). Countering this belief, viral RNA has recently been reported to be transiently expressed *in vivo* (Alvarez et al., 2019; Chen et al., 2020; Jaworski et al., 2019). This was performed using a standard nested endpoint PCR (Alvarez et al., 2019) and quantitative reverse transcription PCR (qRT-PCR) (Chen et al., 2020; Jaworski et al., 2019). Inconsistency in the expression, however, hinders the use of the detection of viral RNA for BLV infection identification. Even so, viral RNA detection has interesting implications on the long-standing disease dogma and the understanding of disease progression.

Comparatively, there are various strengths and weaknesses to the detection of BLV by lymphocyte counts, serological based assays, and PCR. As indicated, detection using lymphocyte counts may only identify the 30 to 40% of infected animals which develop lymphocytosis. While performing better than lymphocyte counts alone, discrepancy has been observed between serological and PCR assay results. The percentage of ELISA positive samples testing PCR negative has been reported to be 1.1% (Ohno et al., 2015), 8.5% (17/201) (Heinecke et al., 2017), 17.9% (25/140) (Reichel et al., 1998), 45% (111/146) (Jimba et al., 2010) and 55.3% (242/437) (Lee et al., 2016). Meanwhile, PCR positive results were observed within 4% (2/59) (Jimba et al., 2010), 6.6% (6/93) (Ohno et al., 2015), 7% (31/445) (Monti et al., 2005), 8.5% (17/201) (Heinecke et al., 2017), and 13.5% (59/437) (Lee et al., 2016) of ELISA negative observations. The occurrence of ELISA false negatives is partially attributed to the occurrence of new infections (Monti et al., 2005; Nagy et al., 2007); however, several studies have reported the

observation of cattle which were persistently negative on serological tests and positive by PCR tests (Fechner et al., 1997; Monti et al., 2005; G. E. Monti et al., 2007). While Fechner *et al* (1997) speculated it was the result of different viral genotypes, sequencing of persistently negative cattle by Monti *et al* (2005) identified the cattle belonged to different genotypes.

Prevalence of BLV Worldwide

Following the identification of BLV in 1965, the push to eradicate the virus has largely been regionally specific. While BLV is a notifiable disease to the World Organization for Animal Health, many countries do not have national testing schemes in place. Therefore, the absence of a reported infection is not indicative of the absence of the virus. A majority of the reported international BLV prevalence estimates are from analytical studies and thus are not systematic samplings to accurately determine national BLV prevalence estimates. However, these analytical studies can provide insight to the distribution of BLV infection.

The European and Australasia continents have been the most proactive in identifying, controlling, and eradicating BLV. A 2016 report shows the European Union has granted disease free status, which requires at least 99.8% of herds to be BLV free, to 20 countries (European Commission, 2016). These countries are Austria, Belgium, Cyprus, Czech Republic, Denmark, Germany, Spain, Finland, France, Ireland, Latvia, Lithuania, Luxembourg, Netherlands, Norway, Slovakia, Slovenia, Sweden, Switzerland, and the United Kingdom. In addition, regions of Italy, Poland, and Portugal are considered BLV free. Although the EU has implemented control programs, BLV is still present in Greece, Bulgaria, Romania, Hungary, Croatia, and Estonia. In 2004, the prevalence in Bulgaria was estimated to be 22.3% (Sandev et al., 2006). In the Australasia continent, both Australia and New Zealand report BLV has been eradicated following the implementation of control programs (System, 2016; Voges, 2011).

Reports of national studies of BLV among in Asian countries are scarce and the implementation of control programs is country dependent. In Turkey, where BLV became a notifiable disease in 2011, it was reported that 11.8% (132/1116) of tested herds had at least one positive animal. However, the prevalence among cows was estimated to be 1.6% (460/28,982) (Şevik et al., 2015). Two different reports from Iran estimated the prevalence in three provinces were 22.1% (144/657), 29.8% (108/362), and 1.5% (1/66) indicating prevalence may be province dependent (Mousavi et al., 2014; Nekoei et al., 2015). The prevalence of BLV infection was reported to be 8% (31/400) in an Iraqi study aimed at establishing a screening program for Iraqi cattle (Khudhair et al., 2016). This indicates other middle eastern countries are impacted by BLV. The overall prevalence in the Philippines is estimated to be between 4.8-9.7% (Polat et al., 2015). A report from Mongolia found 3.9% (20/517) of cattle were infected (Ochirkhuu et al., 2016). Infection is reportable in Japan, but national control programs have yet to be implemented and BLV infection is endemic with 79.1% of dairy and 44.2% of beef herds infected. Twenty-nine per cent of all cows in Japan are infected, with variations between beef and dairy cows, 11.9% (173/1454) and 34.7% (1375/3966), respectively (Murakami et al., 2011). A study looking at milk production in China tested enrolled cows for BLV and found 49.1% of dairy cattle and 1.6% of beef cattle were infected (Yang et al., 2016). Prevalence in Thailand was reported to be regional specific with 58% (437/744) of cattle testing positive by at least ELISA or PCR (Lee et al., 2016).

National studies reporting BLV prevalence in South America are also limited. In a study testing cows for BLV in Peru, Paraguay, and Bolivia, 42.3% (139/328), 54.7% (76/139), and 30.8% (156/507) were infected, respectively (Polat et al., 2016). Two reports provide conflicting prevalence estimates for Chile reporting 29.1% (236/810) and 59% indicating prevalence may

vary by region (Felmer et al., 2009; Polat et al., 2016). In Argentina, a study sampling over more than 10,000 cows in 363 herds found 84% of herds and 32.9% of cows were infected (Trono et al., 2001). In Brazil, 37% (326/881) of tested cows were infected (Fernandes et al., 2009).

Individual reports from African countries are limited. A 2009 report from the OIE states that the BLV had not been reported in South Africa, Tunisia, and Egypt. However, without within-country, organized surveillance programs these reports, or lack of reports, should be taken cautiously as the lack of disease reports may not indicate the absence of BLV. For example, a 2014 study examining five dairy herds in Egypt found that 16% of cows (38/240) were infected with BLV, indicating BLV is present in Egypt (Zaher and Ahmed, 2014).

In North America, official control programs have not been implemented and infection with BLV is widespread. A nationwide study of 315 dairy farms in Canada from 1998-2003 found BLV on 78.3% (213/272) of herds (Nekouei et al., 2015). Studies from the Maritimes, and the Saskatchewan found the cow-level prevalence to be 20.8% (542/2604) and 37.4% (572/1530), respectively (Vanleeuwen et al., 2005, 2001). The BLV prevalence in Manitoba was reported to be 60.8% (732/1204) in dairy cattle and 10.3% (147/1425) of beef cattle (Vanleeuwen et al., 2006). In Mexico, 59% (118/201) of cattle sampled in the central region were positive for BLV (Heinecke et al., 2017).

In the last twenty years, research and awareness of BLV in the United States has increased, although no national program has been implemented to control the spread or eradicate the disease. The first report produced by the Animal and Plant Health Inspection Services in 1997 found 89% of dairy herds were positive with the within-herd prevalence estimated to be greater than 25% for 74.8% of herds (USDA, 1997). In 1999, a USDA estimate indicated almost 40% of beef herds and 10.3% of beef cattle were infected (USDA APHIS, 1999). A herd-level

survey in 2007 reported 83.9% (448/539) of dairy herds were infected (USDA, 2008). The prevalence among cattle presented for slaughter in 2017 was 38.6% (771/1996) with cow-level variations seen between predominately dairy, 47.6% (346/727), and predominately beef, 33.6% (308/918), slaughter facilities (Bauermann et al., 2017). A recently published report sampling 103 herds within 11 states found 94.2% (97/103) of herds were infected and the average within-herd prevalence at the cow level was 46.5%, indicating the U.S. BLV prevalence continues to increase (Ladronka et al., 2018).

Michigan is the only state that has conducted a statewide dairy prevalence estimate. This investigation sampled approximately 40 cows from 113 herds and found an average estimated within-herd prevalence to be 32.8% with 86.7% (98/113) of all herds and 34% (1309/3849) of all cows infected (Bartlett et al., 2013; Erskine et al., 2012a). In addition, members of our research team recently indicated 77.7% (21/27) of Midwest beef herds and 29.2% (918/3146) of beef cows were seropositive (Benitez *et al.*, manuscript in press). In a separate study, 45% (54/120) of beef bulls presented for breeding soundness exams at Michigan State University were infected (Benitez et al., 2019).

Factors that have been associated with increased herd prevalence in epidemiological studies include purchasing cattle (Kobayashi et al., 2014; Nekouei et al., 2015; Sargeant et al., 1997; Şevik et al., 2015) , sending heifers to common ranches (Kobayashi et al., 2014), the presence of flies (Kobayashi et al., 2014, 2010) or lack of fly control (Erskine et al., 2012b), reuse of needles (Erskine et al., 2012b) and palpation sleeves (Nekouei et al., 2015), gouge dehorning (Erskine et al., 2012b; Kobayashi et al., 2010), and several housing related variables including loose housing systems (Kobayashi et al., 2014, 2010), contact between calves and

adult cattle (Kobayashi et al., 2014; Sargeant et al., 1997), and calf housing (Sargeant et al., 1997).

Incidence of BLV

Limited studies have directly investigated the BLV incidence rate. Investigations of dairy heifers at the University of Florida reported the incidence rate ranged from 0 to 9.68 new infections per 100 cow-months at risk (Thurmond et al., 1982, 1983b). Significant differences in incidence rate by month or during periods of fly exposure, artificial insemination, or routine vaccination were not identified (Thurmond et al., 1982, 1983b). Exposure of heifers to the dry herd with a high BLV prevalence, however, corresponded to an increase in the incidence rate (Thurmond et al., 1982, 1983b). The incidence rate in four Argentinian dairy herds representing varying production stages was reported to range from 1.8 to 5.1 new infections per 100 cow-months at risk (Juliarena et al., 2016). In another report examining seven Argentinian herds, peaks in the BLV incidence rate were observed in the autumn and spring which corresponded to periods of parturition (G. E. Monti et al., 2007).

Transmission modeling has estimated the number of new cases attributed to an infected cow ranges from 1.9 to 3.7 per year, resulting in an R_0 equal to 8.9 new cases per lifetime (G E Monti et al., 2007). However, differences between the average cow longevity may impact this factor. For example, when Monti *et al* (2007) estimated the R_0 of 8.9, the median age at which cows first calved was 2.8 years and median age at culling was 9.1 years. This likely corresponds to more lactations than would be observed for the average U.S. dairy cow. Shorter longevity, or fewer lactations, would correspond to a reduced time for infected cows to transmit the virus to susceptible herd mates, and thus, a lower R_0. In a five-month period, Tsutsui *et al* (2010) estimated 0.6 new infections would occur if an infected animal was introduced into a completely

susceptible herd, corresponding to 1.2 new infections per year (Tsutsui et al., 2010). With this, they recommended infected animals should be removed within eight months of identification to prevent transmission. One limitation of these parameter estimates is the reliance on the assumption that the likelihood of transmission is constant. Radke *et al* (1992) indicated infected cattle may be the most infectious following the initial infection. In addition, the transient expression of viral RNA expression was recently reported. Collectively, this may invalidate the assumption of a constant infection potential.

Factors that have been associated with the incidence of BLV in epidemiological studies include increased herd prevalence (Lassauzet et al., 1991b) and being tied next to infected cattle (Kobayashi et al., 2015). Extensive research has been conducted to investigate potential routes of BLV transmission and factors associated with increased BLV incidence.

Modes of Transmission

Bovine leukemia virus has the potential to be transmitted through any bodily fluid or tissue that may contain infected lymphocytes. New infections have the potential to occur through the transmission of blood, milk and colostrum, saliva and nasal secretions, smegma and potentially semen. Transmission can either occur horizontally between herd mates or vertically from dam to offspring. Several herd-management practices that may facilitate the transmission of these infected tissues have been thoroughly investigated.

Horizontal Transmission:

Needle Reuse:

Early studies showing the ability of whole blood to induce new infections implicated the reuse of needles as a potential risk-factor for BLV transmission (Mammerickx et al., 1987; Ungar-Waron et al., 1999). Furthermore, needle reuse has been associated with increased herd

prevalence in analytical studies (Erskine et al., 2012b; Kobayashi et al., 2010). The greatest risk of needle-borne transmission may occur when a BLV-negative animal is sampled following a BLV-positive animal. Wilesmith (1979) reported that among animals sampled using a common needle, seronegative animals that were tied next to seropositive animals were more likely to have seroconverted at the subsequent test (Wilesmith, 1979). In a follow-up test conducted in a loose housing system, the risk of seroconverting was eight times greater when an animal was sampled following a positive animal, compared to following a negative animal (Wilesmith, 1979). On the contrary, Weber *et al.* (1988) failed to show viral transmission following the reuse of needles following intramuscular vaccine injections (Weber et al., 1988). Furthermore, BLV was not successfully transmitted following the reuse of bovine and avian tuberculin needles unless the needle was intentionally inoculated with blood (Roberts et al., 1981). In a recent intervention field trial, implementation of single-use injection needles and rectal palpation sleeves did not result in a reduction of either herd BLV prevalence or incidence (Ruggiero and Bartlett, 2019).

The possibility for BLV to be transmitted by the reuse of needles may be dependent on how the needle is being utilized. In the studies that resulted in successful transmission, the needle was used for blood collection, and therefore, was in direct contact with fresh blood. In the studies in which transmission did not occur, injections were given into tissue. In these cases, the needle may have come in contact with small blood vesicles, but not to the extent that occurs during blood collection.

Rectal Palpation:

Given the invasive nature and potential for hemorrhaging within the rectum associated with rectal palpation, the reuse of palpation sleeves has been considered a potential mode of transmission. Early inoculation studies using rectal palpation and blood from known positive

cows demonstrated that cattle could become infected via the rectal epithelium (Henry et al., 1987; Hopkins et al., 1988). The likelihood of transmission, however, may be dependent on the frequency and the skill of the personnel performing the palpation. Two studies reported the occurrence of transmission following palpation which resulted in intentional hemorrhaging of the rectal epithelium (Kohara et al., 2006; Wentink et al., 1993). On a university dairy farm where students learned rectal palpation technique, a controlled experiment comparing the reuse of sleeves compared to new sleeves demonstrated that negative cattle palpated after a positive cow with a common sleeve were 8.3 times more likely to seroconvert than negative cattle palpated with a new sleeve (Hopkins et al., 1991). However, in a commercial farm where palpation was performed by a skilled herdsman, negative cattle palpated with a common sleeve following a positive cow were not more likely to seroconvert then negative herd mates palpated with a washed sleeve (Hopkins et al., 1991).

In further support of transmission via palpation, Divers *et al* (1995) demonstrated that negative cattle intentionally palpated after positive cattle were 2.8 times more like to become infected than those which were not (Divers et al., 1995). In opposition, Lassauzet et al (1989) examined the incidence following palpation as a factor of group prevalence and indicated infection was not more likely to occur in groups with higher prevalence. In addition, an intervention field trial by our research time failed to demonstrate a significant reduction in BLV prevalence or incidence in a two-year period between groups of cows palpated with a common sleeve or a new sleeve (Ruggiero and Bartlett, 2019).

Differences seen between different farms within single studies and conflicting results of different studies indicate that rectal palpation has the potential to be a risk factors when common sleeves are used. These differences may depend on the skill or technique of the individual

performing the palpations, the extent of hemorrhaging in both donor and recipient animals, and the amount of blood contamination of the palpation sleeve.

Dehorning:

Gouge dehorning which results in blood and exposed tissue, is a potential mode of BLV transmission. In a Washington herd, calves that were gouge dehorned with no sanitary intervention were significantly more likely to BLV seroconvert than both calves that were gouge dehorned following tool sterilization and calves that were not dehorned at all (Digiacomo et al., 1985). In a follow-up intervention study, herd prevalence was observed to decrease over the course of a three-year period when a switch was made from gouge to electric dehorning (DiGiacomo et al., 1987). In a California herd, gouge dehorning was also strongly associated with transmission, especially for cattle dehorned following a known BLV-positive herd mate (Lassauzet et al., 1990). Subsequent risk factors analyses have associated the use of gouge dehorning with increased herd prevalence (Erskine et al., 2012b; Kobayashi et al., 2010). The role gouge dehorning plays in potential transmission is widely accepted and can be prevented through the use of alternative dehorning techniques or sanitation of dehorning equipment.

Presence of Flies:

Hematophagous insects may play a role in BLV transmission. The incidence of BLV infections has been correlated with the density of tabanids (Manet et al., 1989). Generally associated with the presence or incidence of tabanids is season. However, mixed associations between BLV incidence and seasons were reported. The BLV incidence in seronegative cattle exposed to groups of seropositive cattle was reported to be higher in the summer when the population density of tabanids was greater than in the winter (Bech-Nielsen et al., 1978). On the contrary, the cumulative BLV incidence rates were not higher during the fly season (defined as

June to September) than any other season (Thurmond et al., 1982). Additionally, one study reported the incidence rate was higher when cows were housed indoors during the winter months compared to the summer months when housed on pasture and exposed to tabanids (Wilesmith et al., 1980).

In experimental studies, BLV transmission was reported when feeding insects were translocated from BLV-infected to BLV-negative animals by investigators (Hasselschwert et al., 1993; Ohshima et al., 1981). Contradicting these results, Buxton *et al* (1985) showed calves receiving bites from 75 stable flies interrupted during feeding did not become infected. In addition, seronegative cattle maintained in an enclosure with two seropositive cattle in the presence of flies did not become infected over a range of one to four months (Buxton et al., 1985).

Direct Contact Transmission:

The greatest risk for direct contact transmission is likely through infected saliva and nasal secretions (Ressang et al., 1982; Yuan et al., 2015). Although new infections have been induced following inoculation with saliva or nasal secretions (Roberts et al., 1982), several inoculation studies using volumes of saliva ranging from 1 mL to 30 mL failed to demonstrate transmission (Dimmock et al., 1991; Gatei et al., 1989; Hoss and Olson, 1974; Miller and Van Der Maaten, 1979). The ability to transmit BLV via saliva or nasal secretions may be dependent on the infection status of the donor. The detection of BLV in nasal or saliva secretions was reported to occur only in cattle with proviral loads greater than 14,000 copies per 100,000 cells (Yuan et al., 2015); however, the proviral load was much lower in the nasal and saliva secretions when compared to the blood. In general, the number of lymphocytes in saliva and nasal secretions is substantially lower than blood; Gatei *et al* (1989) only found 2-4 and 3-7 lymphocytes per

milliliter of saliva and nasal secretions (Gatei et al., 1989). Therefore, the limiting factor for direct contact transmission may be the limited lymphocytes and proviral load in the nasal secretions.

Breeding:

An early study determining routes for BLV infection indicated that female cattle may become infected with BLV through their reproductive tract (Van Der Maaten and Miller, 1977). Shortly after, studies ensued with the aim of evaluating the potential for semen from seropositive bulls to transmit BLV to cows. In an early analysis of sire progeny, the prevalence of BLV infection was not significantly different between the progeny of seropositive and seronegative bulls conceived through artificial insemination (Baumgartener et al., 1978). In addition, artificial insemination with semen from positive bulls in BLV-seronegative herds over a five-year period did not result in new infections (Monke, 1986). Infection studies inoculating sheep with semen from infected bulls also failed to induce new infections (Kaja and Olson, 1982; Miller and Van Der Maaten, 1979). In a more comprehensive study, none of the 40,000 bull ejaculates tested induced new BLV infections in serologically negative calves or sheep (Schultz et al., 1982). Only one study in which the semen was collected from an infected bull using electroejaculation resulted in the infection of inoculated sheep (Lucas et al., 1980).

Examining later studies utilizing PCR to detect the BLV provirus, one study reported the presence of BLV in the semen of six bulls while two independent studies failed to identify BLV in semen (Benitez et al., 2019; Choi et al., 2002). For the first time, however, BLV was detected in the smegma of four bulls (Benitez et al., 2019). While the risk of transmission via semen or natural breeding likely is not high, it cannot be ruled out. In an epidemiological analysis, the use of a breeding bull for heifers was associated with a significantly higher herd seroprevalence

(Erskine et al., 2012b). However, a natural breeding study comparing the BLV incidence in heifers when bred by either a BLV-positive or BLV-negative bull did not identify transmission (Benitez-Rojas et al., 2018).

Vertical Transmission:

When examining the potential for vertical BLV transmission, differentiating between infections that occur in utero and those which occurs during parturition or shortly after infection is difficult. Collectively, the percentage of calves reported to be vertically infected at birth ranges from 0% (Meas et al., 2002; Van Der Maaten et al., 1981) to 26% (Kono et al., 1983), with the majority of studies reporting less than 10% (Gutiérrez et al., 2011; Jacobsen et al., 1983; Lassauzet et al., 1991a; Mekata et al., 2014; Ohshima et al., 1984; Thurmond et al., 1983a). The likelihood of *in utero* infection could not be predicted by infection of previous offspring (Piper et al., 1979) and was not associated with the dam's age, parity, or breed (Thurmond et al., 1983a). New infection of the dam during pregnancy also was not associated with the likelihood of *in utero* infection (Thurmond et al., 1983a; Van Der Maaten et al., 1981). Interestingly, the frequency was more likely to occur in calves born to cows with elevated lymphocyte counts (Lassauzet et al., 1991a) or co-infected with bovine immunodeficiency virus (Meas et al., 2002). *In utero* infection has also been positively correlated with the maternal proviral load (Mekata et al., 2014; Sajiki et al., 2017).

Milk/Colostrum:

Early studies aimed to find the causative agent of enzootic bovine leucosis reported virus-like particles among cells recovered from harvested lymph nodes of cattle with lymphosarcoma. Knowing the potential for disease transmission via milk secretions from other disease models, electron microscopy was used to identify virus-like particles believed to be associated with

bovine leucosis in milk and colostrum samples (Dutcher et al., 1964). Subsequent studies using sheep bioassays further identified milk's infectious potential and possible role in BLV transmission. Sheep inoculation studies using mixtures of virus culture and milk (Baumgartener et al., 1976), unaltered milk and colostrum from infected cows (Ferrer et al., 1981; Miller and Van Der Maaten, 1979), or cells isolated from infected cows and resuspended (Chung et al., 1986; Ferrer et al., 1981; Kanno et al., 2014) all showed milk and colostrum may transmit BLV to uninfected animals.

Discovery of colostrum and milk's infectious potential led to investigations of its role in BLV transmission and spread from dams to calves. Studies have shown the role of colostrum is complex as colostrum from infected dams can contain both antibodies that may prevent BLV infection and lymphocytes that may induce infection. Extensive research has shown calves and sheep fed colostrum from infected dams acquire transient antibodies against BLV that typically disappear between 3 and 6 months of age (Ferrer and Piper, 1981; Gillet et al., 2016; Gutiérrez et al., 2011; Mammerickx et al., 1980; Miller and Van Der Maaten, 1979; Nagy et al., 2006; Piper et al., 1979; Romero et al., 1983). These passively acquired antibodies may provide protection from early infection. In a BLV inoculation challenge, sheep which acquired antibodies from colostrum did not become infected (Mammerickx et al., 1980). The protective effect of passive transfer is further supported by studies showing calves that did not acquire colostral antibodies were more susceptible to BLV infection than calves who receives passive transfer (Nagy et al., 2006; Romero et al., 1983). In a statistical analysis, calves without detectable antibodies in the first week of life were 2-2.75 times more likely to become infected than calves with antibodies (Lassauzet et al., 1989). To prevent transmission of BLV to calves and still provide protective

antibodies, both pasteurization (Chung et al., 1986; Rubino and Donham, 1984) and freezing of colostrum (Kanno et al., 2014) have been shown to effectively inactivate infected cells.

Impacts of BLV

Infection with BLV has been associated with impacts on animal health and welfare, production and producer profitability, and potentially human health.

Animal Health and Welfare:

As a chronic lymphoproliferative disease, infection with BLV is causes immune dysregulation (Frie and Coussens, 2015). Cattle infected with BLV were demonstrated to have an impaired response to vaccination with reduced IgM production (Erskine et al., 2011; Frie et al., 2017, 2016). This immune disruption may leave infected animals more susceptible to infectious diseases (Emanuelson et al., 1992). Cattle infected with BLV were also reported to have delayed clearance of ringworm infections (Trainin et al., 1996). Furthermore, cattle infected with BLV and presenting with abnormal leukocyte differentials were more likely to experience subclinical mastitis compared to both BLV negative and BLV positive cattle with normal leukocyte differentials (Sandev et al., 2004). Additionally, the severity of mastitis was reported to be greater in BLV-infected cattle with high proviral loads (Watanabe et al., 2019). Infected cattle which progress to the development of lymphoma are reported to experience lethargy, loss of appetite, weight loss, and fever.

Production & Producer Profitability:

The increased risk of infectious disease and impaired immune response may result increased veterinary costs associated with the treatment of BLV infected cattle. In addition to the impact on animal health, BLV infected cattle may fail to reach their full production potential and producers may accrue economic losses through reduced milk production, shortened cow

longevity, and carcass condemnation at slaughter (Haredasht et al., 2018; White and Moore, 2009).

The implications of BLV infection on milk production are mixed with reports of milk production being greater in BLV infected cattle (Pollari et al., 1992), equivalent to non-infected cattle (Brenner et al., 1989; Huber et al., 1981; Sorge et al., 2011), or lower than BLV non-infected cattle (Erskine et al., 2012c; Nekouei et al., 2016; Ott et al., 2003). Positive herds were estimated to produce 218 kg less per cow which was estimated to cost the U.S. dairy industry more than $525 million per year (Ott et al., 2003). In addition, for every 10% increase in BLV prevalence, a loss of 115kg in rolling herd average was observed (Erskine et al., 2012c).

Differences in the impacts of infection on milk production may be the result of a failure to control confounders since many factors may influence milk production. Interestingly, an analysis of genetic potentials by Wu *et al* (1989) indicated seropositive cattle with both normal and abnormal lymphocyte counts had a higher genetic potential for milk production than seronegative herd mates. Although infected cattle had higher genetic potential for fat production, a non-significant difference was observed suggesting infected cattle may not reach their full potential.

In addition to impacts on milk production, BLV-infected cattle were reported to have shortened cow longevity when compared to their BLV negative herd mates (Bartlett et al., 2013; Emanuelson et al., 1992; Pollari et al., 1992; Rhodes et al., 2003). In one analysis, BLV infected dairy cattle were 23% more likely to be culled than their negative herd mates (Bartlett et al., 2013). When survival analysis was conducted in beef cattle, however, a non-significant difference overall was observed. When infected beef cattle were stratified by proviral load, cattle with high proviral loads had an 84% greater hazed of leaving the herd (Benitez et al, manuscript

in press). Interestingly, Rhodes *et al* (2003) reported cattle which seroconverted in the follow-up period had greater longevity than both their seropositive and seronegative herd mates.

Human Health:

In recent years, there is a rising concern for the zoonotic potential of BLV (Cuesta et al., 2018). Reports have indicated that approximately 70% of humans have anti-BLV antibodies (Buehring et al., 2019, 2003) and 25% have detectable BLV provirus in their blood (Buehring et al., 2019; Khalilian et al., 2019). These studies are in contrast to studies conducted in the 1970s which failed to detect the presence of antibodies in high risk populations (e.g. farmers, veterinarians, meat inspectors, creamery workers) (Burridge, 1981). In addition, whole genome sequencing of 51 breast cancer tumors failed to identify BLV (Gillet and Willems, 2016). In further opposition, query of the Cancer Genome atlas failed to identify BLV viral DNA among 750 breast carcinomas (Khoury et al., 2013) or BLV transcriptomic sequences among 810 breast cancer samples (Tang et al., 2013).

While there is controversy regarding the potential human infection with BLV, several case-control studies have reported a positive association between BLV and breast cancer with the odds of BLV detection in cancerous breast tissue being 2.73 to 4.72 the odds of detection in benign, non-cancerous tissue (Baltzell et al., 2017; Buehring et al., 2017; Schwingel et al., 2019). Studies investigating the relationship between BLV and breast cancer have suggested the attributable risk for BLV and breast cancer is 37-52% (Baltzell et al., 2017; Buehring et al., 2015).

While studies have reported the presence of BLV in humans, little is known about how the provirus is being transmitted. Identification of anti-BLV antibodies only indicates exposure to the virus or antigen, which could occur through the consumption of food products containing

BLV antigens. The detection of the DNA provirus, however, may be indicative of infection. Reports identifying BLV in humans have provided limited demographic information about study participants, which has been attributed to the utilization of self-selected populations and available tissue banks. Potential routes of transmission include direct contact with infected animals and the consumption of unpasteurized or undercooked dairy and beef products, which have been shown to contain the BLV provirus (Olaya-Galan et al., 2017).

BLV Control Programs

Similar to other infectious diseases, controlling BLV could be accomplished through vaccination, removal of infected animals, or interventions to prevent transmission. To date, the development of a traditional BLV vaccine has not been successful because of the failure to provide long-term antibody protection (Gutiérrez et al., 2014b; Rodríguez et al., 2011). A promising vaccination currently being investigated is the use of competitive, attenuated proviruses. In this approach, the DNA provirus is mutated to contain genes important for immune stimulation and replication but not be capable of inducing new infections (Gutiérrez et al., 2014b). This has been reported to successfully protect sheep from infection and studies in cattle are ongoing (Gutiérrez et al., 2014b). Unfortunately, the use of gene modification for viral attenuation would face regulatory restrictions that may prevent the use in United States cattle populations. Therefore, a search for natural mutants that have the same benefits of attenuated proviruses is underway (L. Willems, personal communication).

In the absence of a viable vaccine, control efforts have focused on removing infected animals or preventing transmission. Classical BLV control programs have predominately been grouped into three main categories: test and cull, test and segregate, test and manage (Rodríguez et al., 2011). While all programs require whole herd testing, they vary in actions taken after the

identification of infected animals. In the test and cull strategy, once an infected animal is identified it is sent to slaughter to prevent viral transmission. The advantages to this program are BLV eradication can be quickly achieved and additional measures to change housing or management facilities are not required. However, this solution is not generally feasible at high prevalences, requires frequent surveillance to identify reactors, and requires the replacement of culled cattle to maintain herd numbers.

In the test and segregate method, instead of being slaughtered, all infected animals are separated from their BLV-negative animals. This can be done by either maintaining separate BLV-negative and BLV-positive herds or modifying on-farm housing structures and systems to keep infected animals segregated. While this plan may be more feasible than test and cull because it does not require the replacement of infected animals, structural constraints prevent many farms from achieving complete separation of negative and positive animals. In the third program, test and manage, positive animals do not need to be slaughtered or separated from negative animals. Instead, management practices are modified in attempt to prevent viral transmission from positive to negative animals. Management interventions generally target the potential routes of transmission. While positive animals do not need to be replaced and control is not constrained by farm facilities, this intervention is time-intensive and requires complete compliance by veterinarians and farm personnel. Both the test and segregate method and the test and mange method do not immediately eliminate BLV from the herd. Instead, these control strategies rely on breaking the cycle of transmission to prevent new infections until eventually all positive animals are naturally removed from the herd.

Reports of management programs have demonstrated mixed success (Gutiérrez et al., 2011; Ruppanner et al., 1983; Sprecher et al., 1991). In 1991, Sprecher *et al* reported the

implementation of single-use needles and palpation sleeves, disinfection of medical devices, and replacement or pasteurization of colostrum successfully reduced herd prevalence in a two-year period (Sprecher et al., 1991). An Argentinian dairy herd implementing similar interventions, however, did not find a significant reduction in the incidence or prevalence of BLV during a five-year intervention period (Gutiérrez et al., 2011). A field trial implementing single-use needles and palpation sleeves over a two-year period also did not find a significant reduction in BLV incidence (Ruggiero and Bartlett, 2019). Implementation of fly nets or pesticide control, however, has been reported to prevent transmission (Kohara et al., 2018; Ooshiro et al., 2013). The mixed success of management programs and the conflicting results of experimental studies investigating potential routes of transmission suggest that the main routes of BLV transmission may be farm dependent and that major routes of transmission have yet been identified.

Given the limitations associated with removing or segregating all infected animals or intervening on all routes of transmission, recent control efforts have focused on the selective removal of cattle thought to be the most infectious to their herd mates. Based on the theory that new infections occur following the transmission of infected lymphocytes, cattle with high proviral loads and high lymphocyte counts present the greatest risk for infecting their herd mates. In an early field trial, the selective removal of cattle which expressed viral antigen upon lymphocyte culture significantly reduced the BLV incidence in enrolled herds (Molloy et al., 1994). Serial dilutions of blood indicated antigen-positive cattle had more copies of the BLV provirus than antigen-negative cows. In a more recent field trial, the selective removal of cattle with high proviral loads or high lymphocyte counts resulted in significant reduction in the herd BLV prevalence and incidence (Ruggiero et al., 2019). While shown to be effective control strategies, these studies do not directly prove cattle with high proviral load or lymphocyte count

are the most infectious. A phylogenetic analysis, however, reported that five new infections in a BLV-negative herd were caused by a single BLV infected cow with persistent lymphocytosis even though seven BLV infected animals were introduced (Ooshiro et al., 2013).

In further support of the theory on infection potential, it was demonstrated that new infections did not occur following the introduction of BLV-positive cows with low proviral loads into BLV-negative herds (Juliarena et al., 2016; Mekata et al., 2018a). Furthermore, a phylogenetic analysis indicated cattle with less than 3,000 proviral copies per 100,000 cells did not spread the virus during a 30-month period (Mekata et al., 2015). Interestingly though, cattle with higher proviral loads, ranging 12,300 to 69,200 copies per 100,000 cells also did not transmit the virus in a follow-up period ranging from 10 to 19 months. To contribute to the success of control programs targeting the removal of cattle of with high proviral loads and lymphocyte counts, a greater understanding of disease progression is needed.

Disease Progression

Infection with BLV is thought to progress from an aleukemic state to a state of persistent lymphocytosis in approximately 30% of infected animals and result in the development of lymphoma or lymphosarcoma in less than 5% of animals. This is considered to occur through both infective and mitotic viral cycles.

The long-standing theory is infection is the result of the transmission of a lymphocyte infected with the BLV provirus from a BLV-positive cow to a BLV-negative herd mate. Upon this transference, viral reactivation occurs, and an infectious cycle is initiated in the new host. The infectious cycle is responsible for the active production and release of BLV viral particles containing the BLV-RNA virus. Upon entry into a susceptible host cell, the RNA virus is reverse transcribed into a DNA provirus which integrates into the host genome, resulting in lifelong

infection (EFSA Panel on Animal Health and Welfare, 2015; Gillet et al., 2013). After a period of time, the host immune response is suspected to suppress the active viral replication and production of virions. Due to this immune suppression, the infectious cycle ceases and the virus persists through the mitotic replication of infected cells (i.e. mitotic cycle). As BLV infection mitotically persists, animals may remain in an aleukemic state characterized by normal lymphocytes or they may develop a state of persistent lymphocytosis. Lymphocytosis occurs from the polyclonal expansion of infected lymphocytes (Kenyon and Piper, 1977). Independent of AL of PL status, lymphoma or lymphosarcoma may develop from the monoclonal or oligoclonal expansion of an infected cell (Ferrer et al., 1978). The development of lymphoma or lymphosarcoma results in imminent death or condemnation at slaughter.

In addition to the classic disease states of AL, PL, and LS, more recent research has further defined infected animals in terms of their proviral load (PVL), or number of BLV proviral copies per quantity of cells, genomic DNA, or volume of blood. The development of PVL levels requires further elucidation but has been linked to differences in host genetics (Juliarena et al., 2008; Mirsky et al., 1998; Miyasaka et al., 2013; Takeshima et al., 2019). Furthermore, high proviral load has been associated with lymphocytosis (Ohno et al., 2015) and the development of lymphoma or lymphosarcoma (Kobayashi et al., 2019; Somura et al., 2014).

While associations between proviral load and BLV disease states have been established, limited studies have examined the longitudinal progression and development of proviral load. While persistent lymphocytosis is thought to be the result of slow expansion of lymphocytes, it recently has been suggested that proviral load is established shortly after infection and is stable over time (Gillet et al., 2013; Lendez et al., 2015; Mekata et al., 2018a). The stability of proviral load may be supported by the observation that proviral load was not significantly different

between infected calves and adult cattle, which suggests proviral load may not be a result of an age associated increase (Merlini et al., 2016) In contrast, significantly higher proviral loads were reported for multiparous cows when compared to nulliparous cows; however, when the proviral load by parity was observed, apparent differences was not observed (Ohno et al., 2015). A limitation of these studies is that their cross-sectional design does not allow for the observation of individual cow progression. To date, limited studies have examined disease progression by longitudinally following BLV infected cattle and determining the relationship between proviral load and lymphocyte counts. The studies which have examined proviral load over time are constrained by they use experimental inoculation (Forletti et al., 2020; Gillet et al., 2013; Jimba et al., 2012), heifers(Gutiérrez et al., 2014a) or are limited to one lactation cycle (Konishi et al., 2018); these studies create a need for longitudinal observations on naturally infected cattle.

Conclusion

Bovine leukemia virus is an endemic disease in most parts of the world. Infection leads to negative impacts on animal health and welfare, producer profitability, and potentially human health. Given these impacts, control is warranted. Based on the prevalence of infection in most herds, control strategies that can prevent the occurrence of new infections until all infected cattle can feasibly be removed are necessary. Prior research on routes of transmission have demonstrated mixed success indicating that either major routes of transmission are farm dependent or all routes have not been identified. A new approach focused on the removal of cattle with high proviral loads shows promise for successful control and reduction; however, the natural development of this condition requires further elucidation.

Overall Objective and Research Aims

The overall objective of this dissertation was to determine herd-level management practices and cow-level factors associated with increased BLV transmission and progression. A greater understanding of disease transmission and progression will contribute to our long-term goal of creating effective management strategies that producers can implement to eliminate BLV and improve the sustainability of the dairy industry. The following specific aims were designed to contribute to this goal and objective:

Specific Aim 1: Determine herd management practices associated with BLV transmission in Michigan dairy herds.

Specific Aim 2: Determine factors associated with disease progression in naturally infected cattle.

Specific Aim 3: Determine early disease kinetics in experimentally infected steers.

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Chapter 2 :
Herd Management Practices Associated with Bovine Leukemia Virus Incidence Rate in Michigan Dairy Farms

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Abstract

The objective of this study was to identify associations between herd management practices and the incidence rate of bovine leukemia virus (BLV) infections in Michigan dairy herds. Previous management risk factor studies were of antibody prevalence rather than the rate of recent infections. Milk samples were collected from cohorts of cows on 112 Michigan dairy herds and tested for BLV using an antibody capture ELISA (n=3,849 cows). Cows were subsequently followed for an average of 21 months. Cows negative for anti-BLV antibodies and still present in their respective herds were retested by the same antibody capture ELISA to estimate within-herd incidence rates. The overall crude incidence rate was 1.46 infections per 100 cow-months at risk for the 1314 retested cows in 107 herds. The average within-herd incidence rate was 2.28 infections per 100 cow-months (range: 0 to 9.76 infections per 100 cow-months). A negative binomial regression model was used to identify herd management practices associated with the within-herd incidence rate. Results of the final multivariable model identified higher herd prevalence, milking frequency, needle reuse, as well as housing post-parturient cows separately, to be associated with increased incidence rate. Utilization of sand bedding for the lactating herd was found to be associated with decreased incidence rates. Results of this study suggest potential routes of BLV transmission which should be further investigated as disease control targets in ongoing control programs.

Keywords:

Cattle; Bovine leukemia virus; incidence rate; negative binomial regression model

Introduction

Bovine leukemia virus (BLV), the etiological agent of enzootic bovine leukosis, is an endemic retrovirus impacting the sustainability of the U.S. dairy industry. In the National Animal Health Monitoring Systems surveys, BLV was estimated to infect 85% of dairy herds and 40% of dairy cows in the U.S. (USDA, 2008, 1997). A more recent study indicated within-herd prevalence is approximately 47% with 94% of herds having at least one infected animal (Ladronka et al., 2018). BLV has been shown to negatively impact animal welfare and create economic losses through impaired host response to vaccination (Frie et al., 2017), reduced cow longevity (Nekouei et al., 2016; Rhodes et al., 2003), decreased milk production (Nekouei et al., 2016; Yang et al., 2016), restricted trade of embryos and live cattle (EFSA Panel on Animal Health and Welfare, 2015), condemnation of carcasses at slaughter (White and Moore, 2009) and suggested increased susceptibility to other diseases (Emanuelson et al., 1992). Furthermore, the dairy industry is threatened by a potential loss of consumer confidence due to rising public health concerns stemming from postulated links between BLV and human breast cancer (Buehring et al., 2014; Cuesta et al., 2018). The high prevalence and negative impacts of BLV warrant consideration of BLV control and possibly eradication from U.S. cattle herds.

Given the high prevalence of BLV infection in most U.S. dairy cattle herds, eradication in a herd through removal of all infected animals is usually not economically feasible. Hence, a primary goal of control programs should be to decrease herd prevalence by reducing the transmission of infected lymphocytes found in blood, milk, colostrum, and saliva (Hopkins and Digiacomo, 1997). Many control programs have attempted this goal through implementation of management interventions targeting risk factors previously identified in cross-sectional studies to be associated with BLV prevalence. Examples of interventions include the implementation of

single-use needles and palpation sleeves, disinfection of medical equipment, and pasteurization or freezing of colostrum. However, the results of these programs show variable success, with many not showing substantial reduction in prevalence (Gutiérrez et al., 2011; Ruggiero and Bartlett, 2019; Sprecher et al., 1991). For example, an Argentinian dairy herd implementing single use needles, single use rectal palpation sleeves, and disinfection of all other medical equipment (i.e. tattooing, ear-tagging, and dehorning equipment) did not observe a significant reduction in BLV prevalence or incidence in a three-year period (Gutiérrez et al., 2011). Additionally, a recent intervention field trial conducted by our research team in three dairy herds implementing single-use needles and sleeves did not find significant differences in disease incidence in an approximately two-year follow-up period (Ruggiero and Bartlett, 2019). These studies targeted risk factors associated with the prevalence of BLV, which reflects the proportion of cows infected and transmission that has occurred at an unspecified time in the past. A superior outcome of interest for disease control is BLV incidence, which reflects transmission that occurs in a prescribed period of time and is not affected by the duration of infection. It is possible that risk factors solely associated with the incidence of BLV have been overlooked, leaving primary risks of transmission unidentified. For these reasons, the objective of this analysis was to determine herd management practices associated with the BLV incidence rate in Michigan dairy herds.

Materials and Methods

Data Collection:

This analysis was performed on a previously described dataset (Erskine et al., 2012a). In 2010, all Michigan dairy herds participating in testing by the Dairy Herd Improvement Association and averaging $\geq$ 120 cows on-test during the previous 12 months were divided

equally into three size groups: small (120-174 cows), medium (175-295 cows), and large (296-6,492 cows). Herds within each size category were assigned random numbers to determine the order in which they were contacted and invited to participate. The goal was to enroll 40 herds in each herd size category. A total of 112 Michigan dairy herds were enrolled in the initial study (Erskine et al., 2012b). Herd management surveys and herd prevalence profiles (Erskine et al., 2012c) were performed at enrollment. Herd management surveys contained 118 management related questions and were performed by study personnel interviewing herd managers or owners. The included questions related to cow facilities and housing, vaccinations, reproductive management, and previously identified risk-factors for BLV.

Prevalence profiles were used to estimate apparent within-herd BLV prevalence by collecting milk from up to 40 (mean: 35; median: 36; range: 14 to 40) of the most recently calved cows to prevent selection bias when choosing cows for enrollment; up to 10 cows within each of the first, second, third and ≥fourth lactation group were sampled (Erskine et al., 2012c). Milk samples were submitted to a diagnostic lab (CentralStar Cooperative, East Lansing MI) for BLV antibody capture ELISA testing (IDEXX Laboratories Inc., Westbrook, ME). The serum ELISA test is reported by IDEXX to have "very high specificity" and "excellent sensitivity". An independent evaluation of a serum IDEXX ELISA reported diagnostic sensitivity to be greater than 99.8% and specificity to be 100% when compared to agar-gel immunodiffusion (Simard et al., 2000). The milk-based ELISA has been shown to have a sensitivity of 86.0% and specificity of 100% when compared to the serum ELISA (Erskine et al., 2012c). Milk ELISAs with a corrected optical density ≥ 0.1 were considered BLV ELISA-positive. A total of 3,849 cows within 112 Michigan dairy herds were enrolled in the original study. The overall cow seroprevalence was 34.0% (1309/3849) across all herds; the average estimated within-herd-

prevalence was 32.8% (range: 0 to 80.6%) (Norby et al., 2016). Cows sampled for the herd prevalence profile created a farm cohort which was subsequently followed for an average of 21 months for milk production (Norby et al., 2016), cow longevity (Bartlett et al., 2013), and BLV incidence.

Inclusion/Exclusion Criteria:

Herds were excluded due to the inability to schedule initial herd visits or follow-up retests at the end of the study. For a cow to be included in the analysis, it had to be ELISA-negative at enrollment in 2010, present in its herd in 2012, and retested in 2012 by BLV milk-ELISA. Eighty-four cows negative in 2010 and present in 2012 were not retested; these cows were excluded from the analysis. Cows culled in the follow-up period were excluded from the analysis since their infection status was unknown at the time of removal. Since BLV is considered a lifelong infection, cows which were ELISA-positive at enrollment were not retested; these cows would not be eligible for inclusion in our incidence rate study.

Statistical Analysis:

Seroconversion was defined as a cow testing BLV ELISA-negative at enrollment and BLV ELISA-positive by milk at the follow-up test. Anti-BLV antibody levels in milk are known to closely reflect antibody levels in serum (De Boer et al., 1989; Erskine et al., 2012c). Therefore, cows testing positive for anti-BLV antibodies in milk were considered to have seroconverted. The rate of seroconversion in the follow-up period was the primary outcome of interest and was modeled as within-herd incidence rate, defined as the number of retested cows seroconverting divided by the total retested herd time at risk, measured in months. Because the time at which each new infection occurred was unknown, the average time of seroconversion was assumed to be halfway through the follow-up period (Dohoo et al., 2009). Therefore, cows

which seroconverted contributed one-half the time between their first and second ELISA test to the retested herd's time at risk. Cows that did not seroconvert contributed the full length of the monitoring period to the total retested herd time at risk:

Incidence Rate= (Number of cows seroconverted) /Σ (cow time $_{BLV\,(-)}$ + (cow time $_{BLV\,(+)}$/2))

Descriptive Statistics:

All statistical analyses were conducted in Stata 15; Stata commands utilized in the analyses are provided as [command]. Overall cow and within-herd incidence rates were reported and further sub-grouped by lactation number and herd-size for descriptive and analytical statistics. Lactation number was based on the cow's lactation at enrollment and initial testing. Crude incidence rates were based on the observed data. For analytical purposes, cow-level and herd-level incidence rates were modeled and adjusted for random herd effects using mixed-effects logistic [melogit] and mixed-effects negative binomial [menbreg] regression models, respectively. Marginal incidence rates were calculated and statistically compared using the margins command [margins].

Identification of Herd Management Practices Associated with BLV Incidence Rate:

Analysis began with examination of the univariate distributions of herd-level factors and management practices. Categorical variables with less than 10 herds per category were condensed to fewer categories or were dichotomized. Calf and heifer-raising variables from the management survey were excluded since all retested cows were present in the milking herd at enrollment. The apparent point prevalence estimated by milk ELISA at herd enrollment and the proportion of retested cows in each lactation category (1st, 2nd, 3rd, ≥4th) were calculated.

Rate data can be analyzed using count distributions that model the number of events (i.e. the number of cows seroconverting) as the outcome and include the time at risk (i.e. the cow-

months at risk) as an exposure. Common count distributions include Poisson (PRM), negative binomial (NB), zero-inflated Poisson (ZIP), and zero-inflated negative binomial (ZINB). The appropriate count distribution was determined by building four separate multivariable regression models using identical model-building procedures [poisson, nbreg, zip, and zinb] which included the number of cows seroconverting as the outcome and herd cow-months at risk as an exposure (**Appendix B**). Model-building began with bivariable analysis to examine the relationship between herd-level management factors and the BLV incidence rate. All factors with a significance of $p \leq 0.15$ in the bivariable analyses were eligible for inclusion into the multivariable models. Spearman rank correlation [spearman] was used to calculate correlations between predictor variables eligible for inclusion into the model using a cutoff of rho=0.8 (Dohoo et al., 2009).

The multivariable models were subsequently created using manual forward selection. Herd size was included in the baseline multivariable model as a potential confounder. During each forward step, eligible variables were individually added to the model which was then compared to the nested model without the predictor using a likelihood ratio test [lrtest]. The variable with the smallest p-value was retained in the model at the end of each step. This process continued until all variables with $p \leq 0.05$ were included in the model. After all significant factors were included, all possible two-way interactions among main effects were examined. Variables that were eligible for inclusion, but were not significant in the final multivariable, were examined as potential confounders using a change of 10% or more in the incidence rate ratio as a cutoff. Robust standard errors were used for all final models to account for overdispersion and potential correlation (Hilbe, 2014); utilization of likelihood ratio tests for model building does not permit robust standard errors.

Information criteria and test statistics were used to compare the four individual count models. Final PRM and NB were evaluated using the deviance and Pearson $\chi 2$ goodness-of-fit tests to assess model-fit and overdispersion, respectively. Boundary likelihood-ratio tests were used to test the significance of the dispersion parameter, α, in both the NB and ZINB models. Because the Vuong statistic is no longer considered appropriate for comparing zero-inflated models against noninflated models (Wilson, 2015), the Akaike Information Criterion (AIC) and Bayesian Information Criteria (BIC) were used for comparison of all models [abic]. Deviance and Anscombe residuals of the selected model were calculated and tested for normality using the Shapiro-Wilks [swilk] test and by visual assessment.

Results

Descriptive Statistics:

In 2012, a total of 1,931 cows in 107 herds were still present, of which 1,398 (72.4%) were ELISA-negative in 2010. Retests were performed in 94.0% (1314/1398) of cows ELISA-negative on their first test in 2010 and still present in 2012 when herd files were collected. The average time between a cow's initial and follow-up milk ELISA tests was 627 days (range: 571-779). In the follow-up period, 26.6% (349/1314) of cows seroconverted, with an overall crude incidence rate of 1.46 cases per 100 cow-months at risk (**Table 2.1**). After accounting for random herd-level effects, the marginal cow-level incidence rate was 1.93 cases per 100 cow-months at risk. Non-significant differences were observed when cows were grouped by their respective herd size categories (p=0.105) or lactation number at enrollment (p=0.082).

Table 2.1
Cow-level incidence rate of bovine leukemia virus infection by lactation and herd size in 107 Michigan dairy herds (n=1,314 cows)

	N	# New Infections	Time at risk [a]	Crude Incidence Rate [b]	Marginal Incidence Rate [b, c]	Marginal Incidence 95% CI		p-value[d]
Overall	1,314	349	23,832	1.46	1.92	1.60	2.26	
Lactation[e]								0.082
1	528	144	9,570	1.50	1.78	1.43	2.14	
2	375	110	6,682	1.65	2.19	1.79	2.59	
3	247	59	4,547	1.30	1.90	1.47	2.33	
4+	164	36	3,033	1.19	1.91	1.43	2.39	
Herd Size								0.105
<174 cows	382	85	7,427	1.14	1.58	1.05	2.12	
175-295 cows	485	117	8,922	1.31	1.76	1.22	2.30	
>295 cows	431	147	7,483	1.96	2.43	1.82	3.04	

[a] Time in months. Cow-months at risk was calculated as the sum of the full time between first test and retest for BLV-negative cows and one-half the time between first test and retest for cows which became infected with BLV. [b] Rates expressed as cases per 100 cow-months at risk. [c] Marginal incidence determined using a mixed-effects logistic regression model accounting for a random effect for herds. [d] Statistical significance assessed using marginal contrast. [e] Lactation number is based on the lactation at enrollment.

Cases of seroconversion were identified within 83.2% (89/107) of retested herds. The descriptive distributions for the number of cows retested, time at risk, and the proportion of retested cows in each lactation category per herd are presented in **Table 2.2** below. An average of 12.3 cows were retested per herd (range: 2-25, median: 11); this number was dependent on both the number of cows originally ELISA-negative and the number still present within the herd. There was an average of 3.3 new infections per herd (range: 0 to 14; median: 3). The total herd-time at risk was non-normally distributed. The median herd time at risk was 188.3 cow-months and ranged from 39.7 to 516.4 cow-months (mean: 222.7 cow-months). On average, the highest proportion of retested cows per herd were in lactation 1 at enrollment and the lowest proportion of cows were in lactation 4 or greater.

Table 2.2
Herd-level descriptive distributions of bovine leukemia virus ELISA retests performed, herd time at risk, cows lost to follow-up, and proportion of retested cows among 4 lactation categories in 107 Michigan dairy herds (n=1,314 cows)

	Mean	**SD**	**Min.**	**P25**	**P50**	**P75**	**Max.**
# BLV-ELISA positive at retest	3.26	2.73	0	1	3	5	14
Number of cows retested per herd	12.28	5.35	2	8	11	17	25
Herd time at risk (in cow-months) [a]	223	119	40	125	189	341	516
Number of cows lost to follow-up [b]	10.25	5.54	0	6	9	14	25
Proportion of enrolled retested	0.36	0.15	0.13	0.23	0.33	0.47	0.68
Proportion of retested in Lactation 1	0.42	0.17	0	0.33	0.4	0.5	1
Proportion of retested in Lactation 2	0.29	0.15	0	0.2	0.29	0.36	0.8
Proportion of retested in Lactation 3	0.17	0.11	0	0.1	0.17	0.25	0.55
Proportion of retested in Lactation ≥4	0.12	0.1	0	0.05	0.11	0.18	0.5

[a] Cow-months at risk was calculated as the sum of the full time between first and retest for BLV-negative cows and one-half the time between first test and retest for cows which became infected with BLV [b] Cows lost to follow-up are cows that were seronegative for BLV at study enrollment and not retested at study conclusion due to being removed from the herd or missed at herd-sampling

The time at risk and the number of new infections in each herd were used to calculate a within-herd incidence rate, and when averaged among all herds, this resulted in a median within-herd incidence rate of 1.59 cases per 100 cow-months at risk. The distribution of within-herd incidence rate was right-skewed and ranged from 0 to 9.76 cases per 100 cow-months at risk (mean: 2.28 cases per 100 cow-months) (**Table 2.3**). Among the 107 herds retested, 18 herds had zero new infections among the retested cows in 2012. Eleven of these eighteen herds (61.1%) had zero BLV-positive cows among the cows tested at herd enrollment in 2010. After adjusting for random herd-level effects, the marginal herd-level incidence rate was 2.11 cases per 100 cow-months. Non-significant differences were observed for within-herd incidence rates when examined by herd size (p=0.287) or lactation number (p=0.301)

Table 2.3

Within-herd incidence rates of bovine leukemia virus infection overall, by lactation number, and by herd-size

	N[a]	Mean	Min.	P25	P50	P75	Max.	Marginal[c]	Marginal 95% CI		p-value
Overall	107	2.28	0	0.45	1.59	3.63	9.76	2.11	1.61	2.60	
Lactation[b]											0.301[d]
1	104	2.14	0	0	1.21	3.29	9.88	1.10	0.78	1.43	
2	101	2.88	0	0	1.4	4.65	10.2	1.44	1.00	1.88	
3	88	2.59	0	0	0	3.28	10.12	1.17	0.75	1.58	
4+	82	2.35	0	0	0	3.21	10.51	1.20	0.71	1.68	
Herd Size											0.287[e]
<174 cows	34	1.9	0	0	1.44	3.28	7.06	1.67	0.96	2.38	
175-295 cows	36	2.14	0	0.28	0.96	2.74	9.62	1.93	1.17	2.70	
>295 Cows	36	2.82	0	0.9	2.78	4.33	9.76	2.66	1.65	3.68	

Crude rates were calculated as the number of cows testing positive by BLV-ELISA divided by stratum retested cow-months at risk. Cow-months at risk was calculated as the sum of the full time between first test and retest for BLV-negative cows and one-half the time between first test and retest for cows which became infected with BLV. [a] "N" is equal to the number of herds contributing to the category rate distribution. [b] Lactation number and herd size are based on the status at enrollment. [c] Marginal rates, and the respective confidence intervals, were obtained from generalized linear mixed-effects models [d] Significance assessed using marginal contrasts following a multilevel mixed-effects negative binomial regression model which included a random intercept for herd. [e] Significance assessed using marginal contrasts following a negative binomial regression model.

Herd-Level Factors Associated with BLV Incidence Rate:

Results of the model-fit statistics for each of the four models can be found in **Table S2.6** . Examination of the Pearson $\chi 2$ statistic revealed both the PRM and NB models were over-dispersed; however, the Deviance statistic indicated the NB model provided a superior fit. Visual assessment of fit-distribution plots showed similar model fits with the largest model differences observed at predicted counts of 0 and 1; the PRM and NB models overpredicted counts of 1 while the ZIP and ZINB models overpredicted zero counts (Figure S2.1). Evaluation of AIC and BIC test statistics suggested the ZINB and NB models provided the best fit, respectively. Given the NB and ZINB models contain the same predictors (not shown), with the exception of the zero-inflated portion of the model which contained herd prevalence, the decision was made to report the more parsimonious NB model which also has a greater ease of interpretation than the zero-inflated model.

Bivariable Analysis:

Results for common risk factors and herd-level factors which were significant at $p \leq 0.15$ and eligible for inclusion in the multivariable NB model are presented in **Table 2.4**. Needle change and milking frequency were recategorized variables that were significant for inclusion in the multivariable model. Needle change was originally organized into five categories: after every injection, after 2-5 injections, after 6-10 injections, after 11-20 injections, and after 20 or more injections. After 2-5 injections and after 6-10 injections were not significantly different than the reference category and therefore, were aggregated to create a single category of <10 injections. Milking frequency was initially categorized into 2 times per day (2X), 3 times per day (3X), and a combination of 2-3 times per day frequency. The combination category contained 9 herds and was not significantly different than the 2X milking frequency (p=0.123) Therefore, the combined

2-3X frequency category was aggregated with the 2X category which was eligible for inclusion in the multivariable model (p=0.033).

Table 2.4

Results of negative binomial regression bivariable analysis of herd management practices associated with the incidence rate [a] of bovine leukemia virus infections

Continuous Variables	**N[b]**	**IRR[c]**	**IRR[c] 95% CI**		**p-value**
Herd Prevalence (%)	106	1.039	1.029	1.049	0.000
Proportion of Retested in Lactation 1	106	6.967	2.014	24.106	0.002
Proportion of Retested in Lactation 2	106	0.524	0.125	2.193	0.377
Proportion of Retested in Lactation 3	106	0.162	0.021	1.244	0.084
Proportion of Retested in Lactation 4	106	0.044	0.004	0.492	0.013
Number of Reproductive Palpations	106	1.233	0.997	1.524	0.055
Number of palpations to confirm pregnancy	106	1.333	1.049	1.693	0.020
Categorical Variables	**N[b]**	**IRR[c]**	**IRR[c] 95% CI**		**p-value**
Transition Cow (Post-Parturient) separate from others					0.090
No	52	--	--	--	
Yes	54	1.498	0.943	2.381	
Purchase Cows					0.003
No	58	--	--	--	
Yes	48	2.020	1.292	3.159	
Purchase some bulls, heifers, or cows					0.002
No	41	--	--	--	
Yes	65	2.167	1.366	3.439	
Calving/Sick Cows Separate from Lactating Cows					0.060
No	52	--	--	--	
Yes	54	0.641	0.405	1.015	
Milking Frequency					0.028
2x or Combination 2X-3X	62	--	--	--	
3X	43	1.694	1.064	2.700	
Reuse of Needles					0.373
No	18	--	--	--	
Yes	88	1.332	0.717	2.476	
Use only sand bedding for lactating cows					0.077
No	28	--	--	--	
Yes	82	0.617	0.359	1.061	
Frequency of Needle Change					0.022
After 10 or less injections	63	--	--	--	
After 11-20 injections	23	1.248	0.716	2.174	
After >20 injections	20	2.250	1.406	3.600	
Use of a breeding bull					0.124
No	81	--	--	--	
Yes	25	1.526	0.884	2.633	
Use Fly Control					0.694
No	22	--	--	--	
Yes	79	0.889	0.492	1.605	

[a] Incidence rate was modeled as the number of cows that tested BLV ELISA-positive as the outcome with an exposure term for the cow-months at risk. Cow-months at risk were calculated as the sum of the full time between first and retest for BLV-negative cows and one-half the time between first test and retest for cows which became infected with BLV. [b] N=Number of herds [c] IRR=Incidence Rate Ratio

Multivariable Model:

The results of the final multivariable negative binomial regression model, which included 105 retested herds, are presented in Table 5; two herds were excluded from the multivariable model due to the survey data missing from the dataset (n=1) and missing milking frequency (n=1). Using the Deviance $\chi 2$ goodness-of-fit test, the null hypothesis that the model was well-fit was not rejected (p=0.179). Deviance (p=0.570) and Anscombe (p= 0.359) residuals were normally distributed using the Shapiro-Wilks test. Increasing herd prevalence ($p < 0.001$), milking frequency (p=0.003), housing post-parturient cows (i.e. fresh cows) separate from other herd-mates (p=0.007), and needle reuse frequency (p=0.001) were significantly associated with increases in the incidence rate of BLV seroconversion. The use of sand bedding for the lactating herd resulted in a significantly lower incidence rate (p=0.005). The quadratic term for herd prevalence was significant ($p < 0.001$) and was included in the model. The relationship between herd prevalence and the predicted incidence rate was curvilinear with increasing predicted rates observed until herd prevalence reaches approximately 50% where the relationship changes to an observed decrease in predicted rates with increasing herd prevalence (**Figure S2.2**).

Table 2.5
Results of multivariable negative binomial regression model of herd management factors associated with the incidence rate[a] of bovine leukemia virus infection on 105 Michigan dairy herds

Risk Factor	IRR[b]	IRR[b] 95% CI		p-value
Herd Size				0.034
<175 cows	--	--	--	
175-295 cows	1.235	0.836	1.825	0.289
>295 Cows	0.569	0.347	0.934	0.026
Herd Prevalence (%)	1.130	1.098	1.163	<0.001
Herd Prevalence^2	0.999	0.998	0.999	<0.001
Transition Cows (Post-Parturient) separate from others				
No	--	--	--	
Yes	1.910	1.196	3.052	0.007
Sand bedding for lactating cows				
No	--	--	--	
Yes	0.650	0.482	0.875	0.005
Frequency of Needle Change				0.001
After 10 or less injections	--	--	--	
After 11-20 injections	1.641	1.170	2.303	0.004
After >20 injections	1.699	1.217	2.371	0.002
Milking Frequency				
2X or Combination 2X-3X	--	--	--	
3X	1.585	1.169	2.151	0.003
Constant	0.00135	0.00079	0.00232	<0.001
/lnalpha	-1.983	-2.904	-1.062	
alpha	0.138	0.055	0.346	

[a] Incidence rate was modeled as the number of cows testing BLV ELISA-positive as the outcome with an exposure term for the cow-months at risk. Cow-months at risk was calculated as the sum of the full time between first and retest for BLV-negative cows and one-half the time between first test and retest for cows which became infected with BLV. [b] IRR=Incidence Rate Ratio

Discussion

The marginal incidence rate among all cows retested was 1.92 cases per 100 cow-months at risk. This incidence rate is similar to studies reporting the incidence rate among dairy heifers in the United States, which ranged from 0.64 to 4.31 new infections per 100 cow-months at risk and was associated with age-related management interventions (Thurmond et al., 1983, 1982). The marginal within-herd incidence rate was 2.11 cases per 100 cow-months at risk, but the crude herd-level incidence rate ranged from 0 to 9.76 cases per 100 cow-months. Non-significant differences were observed in either the marginal cow-level or herd-level incidence rates when viewed by herd size or lactation category. Given all cows were already present in the milking herd at enrollment, we would expect an equal likelihood of exposure and infection regardless of lactation number. Previous studies have reported prevalence tends to increase concomitantly with lactation number (Ladronka et al., 2018). Non-significant differences in lactation-specific incidence rates suggest that these observed changes in prevalence are associated with increased lifetime exposure

The presence of 11 herds with an apparent herd prevalence of zero at enrollment and no new cases of BLV ELISA positive animals may represent true BLV-negative herds. We believe this apparent absence of infection provides hope for eradication programs as it indicates it is possible to maintain BLV-free herds once achieved. On the contrary, a few of the retested herds had high rates of new infections which suggests BLV transmission may occur rapidly. For example, in two herds, a large proportion, 87.5% (14/16) and 100% (12/12), of retested cows became infected.

Studies attempting to investigate transmission by specific risk factors have shown conflicting results (Buxton et al., 1985; Hasselschwert et al., 1993; Kohara et al., 2006;

Lassauzet et al., 1989; Roberts et al., 1981; Weber et al., 1988). This disunion of results suggests the mode of disease transmission varies among farms, major sources of transmission have yet to be identified, or study designs were varying in their abilities to identify the true significance and direction of associations.

Prior cross-sectional studies have identified several factors, such as the presence of insects, open herd status, and needle or sleeve reuse to be associated with BLV prevalence (Erskine et al., 2012a; Kobayashi et al., 2010; Kobayashi et al., 2014; Nekouei et al., 2015). However, cross-sectional studies cannot separate the effects of infection incidence from infection duration. The major aim of this study was to identify risk factors associated with BLV transmission, as best measured by the recent incidence rate. Identification of new infection risk factors may provide additional insight to disease transmission and may lead to a better understanding and control of BLV.

The final multivariable, negative binomial model, controlling for herd size and the possible confounding of other variables in the model, identified five factors to be associated with BLV incidence rate: herd prevalence, milking frequency, needle reuse frequency, housing post-parturient cows separately, and sand bedding for lactating cows. These factors were significant in all of the final multivariable models (PRM, NB, ZIP, ZINB; Appendix B).

Herd size was included in the baseline, multivariable risk factor model because of its role in initial herd enrollment and on the premise of being a theoretical confounder; herd size may influence herd management practices and may affect the rate of new infections. Additionally, inclusion of herd size may indirectly account for management practices that were not included in the herd survey but were influenced by herd size. When marginal incidence rates are examined, numerically higher rates are observed with increasing herd size. In the final multivariable model,

however, being a large herd (>295 cows) appears to be a protective factor with a lower incidence rate. This incongruity is likely because the coefficients, exponentiated to produce incidence rate ratios, are adjusted for other variables in the multivariable model and are additive. If the relationships between herd-size and the other variables in the final multivariable model are examined, all large herds housed post-parturient cows separately and were more likely to milk 3X per day. Both of these variables were associated with increased incidence rate. Removal of herd-size from the final multivariable model did not change the direction of the association between herd management risk-factors and the incidence rate but it did change the magnitude of several variables by more than 10%. Because herd size is related to these variables and to the incidence rate, the direct effects of herd size cannot be discerned from the multivariable model.

Increases in BLV incidence rate are intuitively associated with increased herd prevalence; a BLV-negative cow's exposure, and likelihood of infection, would be expected to concomitantly increase with prevalence in the herd (Lassauzet et al., 1991). As herd prevalence increases, the number of uninfected cattle in the herd decreases. This likely explains why the quadratic term for herd prevalence was significant and why an inflection point is observed when herd prevalence reaches approximately 50%.

Increasing needle reuse was significantly associated with increases in herd incidence rate, which aligns with our current understanding of proviral transmission (Hopkins and Digiacomo, 1997). BLV is known to be transmitted via infected lymphocytes present in blood. As needle reuse frequency increases, the chance of blood contamination on the needle likely increases. If a needle becomes contaminated with blood from an infected animal, the subsequent animal(s) may become infected, disseminating the virus (Wilesmith, 1979). However, a dichotomized version comparing no needle reuse with all frequencies of needle reuse was examined at the bivariable

level and was not eligible for inclusion (p= 0.373) suggesting that although needle reuse does not always result in new infection, as the frequency increases so does the likelihood of transmitting BLV.

Newly identified factors in the final multivariable model were milking frequency, housing post-parturient cows separately, and sand bedding for the lactating herd. Identification of an association between milking frequency and increased incidence suggests a potential mode of transmission which has largely been overlooked. It is known that BLV-infected lymphocytes are present in the milk of BLV- seropositive cows, which highlights the biological plausibility of the link between increased milking frequency and BLV incidence (Ferrer et al., 1981). Additionally, BLV has been reported to infect mammary epithelial cells, which can be sloughed off in the milking process (Boutinaud and Jammes, 2002). Although speculative, milking machines may facilitate the transmission of infected cells from BLV-positive to BLV-seronegative cows (Mammerickx et al., 1978). This potential mode of transmission has previously been discussed by an Argentinian study which reported a significantly higher prevalence between lactating and non-lactating animals tested at 30 months of age (Gutiérrez et al., 2011). Furthermore, a cross-sectional study conducted in Brazil found the odds of being seropositive were significantly higher in mechanically milked versus manually milked cows (Fernandes et al., 2009). Unintentionally, milking machines may result in damage to the external portion of the teat (Besier et al., 2016). If residual milk containing infected lymphocytes remains within the teat liner and a negative cow with minor teat damage is subsequently milked, it may be exposed to BLV-infected cells. Moreover, it is possible that residual milk could be refluxed into the mammary gland of the next cow during machine milking (Thompson and Miller, 1974). Under these hypothetical scenarios, increased milking frequency would be associated with increased

teat damage, increased exposure, and thus increased risk of infection. This interpretation, however, requires further investigation before true causal associations can be made. Potential infection during the milking process would further support the traditional BLV control method of test and segregate so BLV-negative cows can be milked prior to BLV-positive cows to prevent transmission (Bartlett et al., 2014).

Housing post-parturient cows separate from their herd-mates was significantly associated with increases in the incidence rate. While housing-related variables have previously been associated with BLV prevalence, to our knowledge this is the first-time housing during this specific stage in lactation has been identified as a risk factor. This significant association is biologically plausible. A decrease in anti-BLV antibodies has been reported in BLV-positive cows around parturition (Burridge et al., 1982). This wane in antibodies may allow for increased viral replication and lead to increased infection potential of BLV-positive cows. In fact, viral RNA was recently detected in blood of infected cows with an increased frequency around the time of parturition (Alvarez et al., 2019; Jaworski et al., 2019). Additionally, blood, tissues, and uterine fluids that are present following parturition have been recognized as a potential source of BLV transmission (Hopkins and Digiacomo, 1997). It is known that cows, independent of BLV-status, may become immune compromised around parturition (Sordillo et al., 2009). Thus, housing BLV-infected post-parturient cows, potentially experiencing an increase in viral replication, with BLV-negative post-parturient cows that are immuncompromised may create an environment in which increased viral transmission may occur.

The use of sand bedding for lactating cows was a newly identified factor associated with lower BLV incidence. Sand bedding for the lactating herd was used in 74.5% (82/110) of herds enrolled in 2010, which is representative of Michigan dairy herds. Alternative bedding identified

in the survey for lactating cows was shavings, pack, mattresses, or various combinations. Bedding during other stages of production were not significant at $p < 0.15$ at the bivariable level. A potential biological relationship could be the association between sand bedding and decreased presence of flies (Schmidtmann, 1991). It is also possible that the relationship could be representative of other herd management practices or be a spurious correlation. Future studies are needed to determine if there is indeed a relationship between sand bedding and decreased incidence rates of BLV.

Limitations:

The reported incidence rates are approximations, based on the sampling and testing occurring only twice over 2 years. The ability to calculate a more precise incidence rate estimate was limited by the uncertainty of the time at which new infections occurred in the two-year follow-up period, the possibility for BLV-infected cows to test ELISA-negative, and the exclusion of cows which were lost to follow-up, with no final BLV test when they left the herd. Since the exact time of seroconversion was unknown, it was assumed that given a constant incidence rate, the average time to infection would be halfway through the follow-up period (Dohoo et al., 2009). This assumption should not bias the overall seroconversion rate but may bias estimates at the herd-level, given the differing and sometimes limited number of cows resampled per herd.

A second possible limitation arises from our knowledge that BLV-infected animals may not test ELISA-positive for up to 2 months following infection (Monti and Frankena, 2005). Therefore, it is possible that cows newly infected with BLV may have tested ELISA-negative at study enrollment, affecting the incidence rate in the follow-up period. However, our assumption

is the false negatives at each of the two sampling points would likely cancel each other out, and therefore would not substantially change the observed incidence rates.

Thirdly, cows lost to follow-up introduce a potential selection bias as the BLV infection status of these animals were unknown. Our team and others have shown BLV-infected animals are more likely to be culled than their BLV-negative herd-mates, which could lead to underestimating the incidence rate (Nekouei et al., 2016; Bartlett et al., 2013). However, Rhodes et al. (2003) reported that cows which seroconverted in the follow-up period had a significantly lower cull rate than both their BLV-negative or BLV-positive herd-mates (Rhodes et al., 2003). Therefore, the true effects of the loss to follow-up on the incidence rate are unknown. The reason why 84 cows were still present when herd files were collected and were not retested is unknown; however, it can likely be attributed to cows being dry when milk samples were collected or being culled in the time between file and sample collection.

Another limitation for this study is the reliance on herd BLV prevalence estimates and management surveys from the time of the herd enrollment; estimates and surveys were not repeated at the conclusion of the study. Given that there is the potential for herd management practices to change in the follow-up period, our results are subject to a misclassification bias.

Conclusion

This analysis identified factors associated with BLV seroconversion incidence rate among cows on Michigan dairy farms. Herd prevalence, increased milking frequency, increased needle reuse, and separate housing of post-parturient cows were positively associated with higher herd-level incidence rates, while the use of sand bedding was negatively associated. Further studies are required to determine if these are causal associations and how interventions can be applied to diminish the spread of BLV.

Acknowledgements

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APPENDICES

Table S2.6
Model fit statistics for the multivariable models using four different count distributions

	Poisson	**Negative Binomial**	**Zero-Inflated Poisson**	**Zero-Inflated Negative Binomial**
Number of Herds[a]	103	105	105	105
Number of Parameters[b]	17	11	17	14
Log-PseudoLikelihood	-184.68	-205.13	-189.84	-198.93
AIC	4.21	4.21	4.19	4.15
AICn	433.36	442.25	439.69	435.86
BIC	5.74	4.75	5.56	4.85
BICn	517.68	484.72	519.31	486.28
Deviance	1.28	1.13	--[c]	--[c]
Pearson	1.29	1.11	--[c]	--[c]

[a] Number of herds in each model is due to variations in the number of herds that had complete survey results for variables significant and included in the model. [b] Number of parameters is the number of estimates for each model based on variables included and their respective number of categories. [c] Deviance and Pearson statistics cannot be calculated for zero-inflated models.

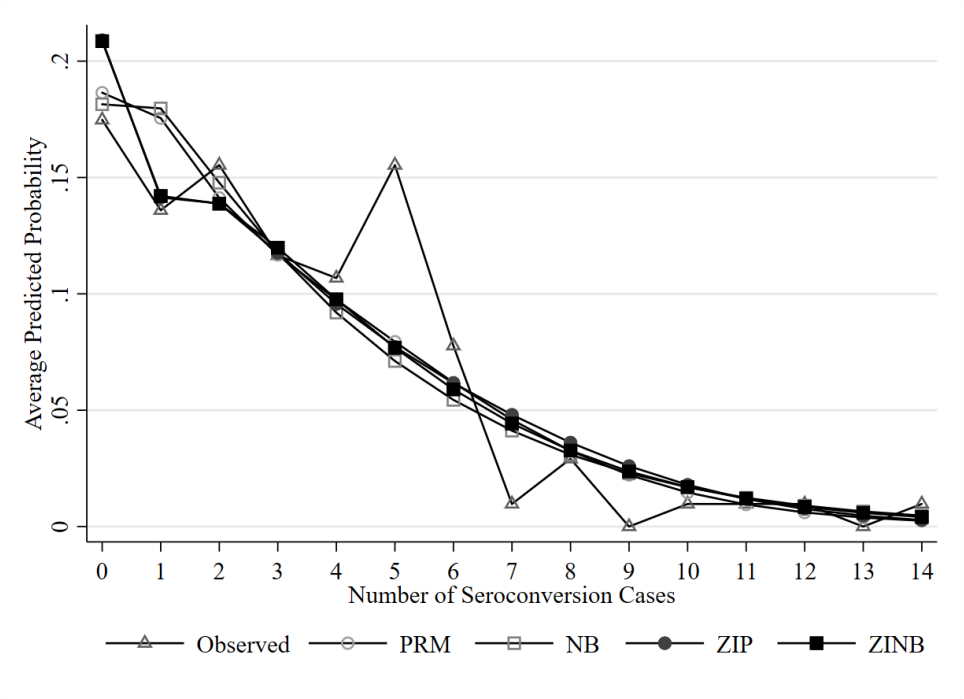

Figure S2.1 Fit of final multivariable models

Observed count frequencies overlaid with predicted count probabilities for the Poisson (PRM), Negative Binomial (NB), zero-inflated Poisson (ZIP), and zero-inflated negative binomial (ZINB) regression models

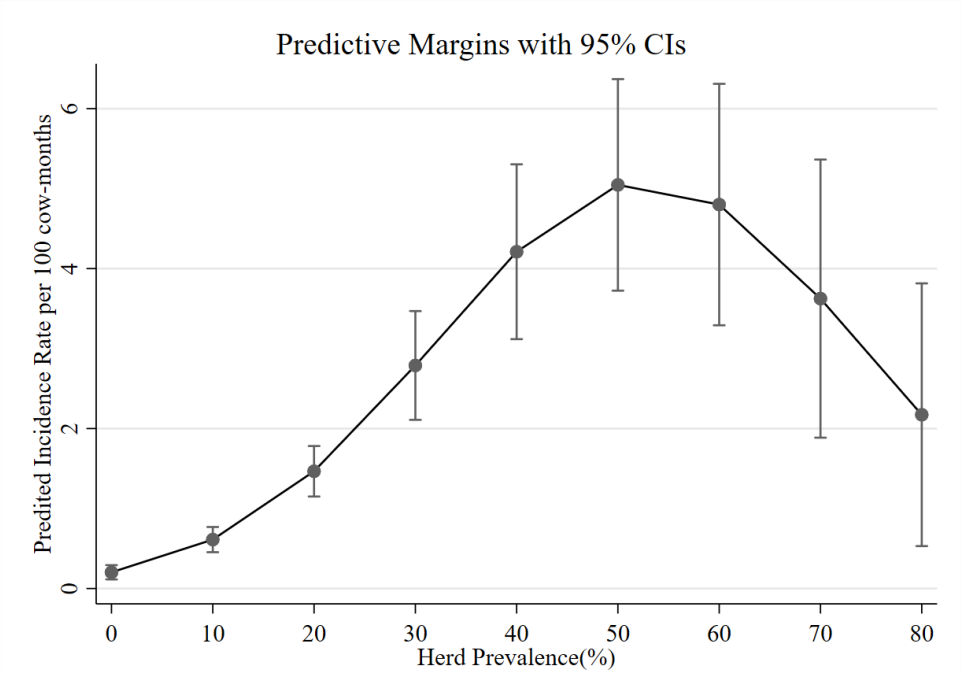

Figure S2.2 Marginal plot of the herd prevalence curvilinear effect in final, multivariable negative binomial model

Observed curvilinear relationship between herd prevalence and predicted rates of BLV seroconversion. Herd prevalence ranged from 0 to 76.3% (mean: 28.8%; median 26.7%) and was non-normally distributed

APPENDIX B

Results of Poisson, zero-inflated Poisson, and zero-inflated negative binomial Regression Models

Table S2.7

Results of Poisson bivariable analysis of herd management practices associated with the incidence rate[a] of bovine leukemia virus infections

Continuous Variables	**N[b]**	**IRR[c]**	**IRR[c] 95% CI**		**p-value**
Herd Prevalence (%)	106	1.034	1.030	1.039	<0.001
Proportion of Retested in Lactation 1	106	16.535	8.546	31.994	<0.001
Proportion of Retested in Lactation 2	106	0.444	0.174	1.131	0.089
Proportion of Retested in Lactation 3	106	0.145	0.052	0.405	<0.001
Proportion of Retested in Lactation 4	106	0.018	0.005	0.071	<0.001
Number of Reproductive Palpations	106	1.237	1.124	1.361	<0.001
Number of palpations to confirm pregnancy	106	1.319	1.187	1.465	<0.001
Number of Ovsynch Injections	105	1.055	0.988	1.125	0.108
Categorical Variables	**N[b]**	**IRR[c]**	**IRR[c] 95% CI**		**p-value**
Transition Cow (Post-Parturient) separate from others					
No	52	--	--	--	
Yes	54	1.492	1.202	1.851	<0.001
Purchase Cows					
No	58	--	--	--	
Yes	48	2.020	1.636	2.495	<0.001
Purchase some bulls, heifers, or cows					
No	41	--	--	--	
Yes	65	2.255	1.792	2.838	<0.001
Calving/Sick Cows Separate from Lactating Cows					
No	52	--	--	--	
Yes	54	0.648	0.524	0.801	<0.001
Milking Frequency					
2x or Combination 2X-3X	62	--	--	--	
3X	43	1.823	1.474	2.254	<0.001
Reuse of Needles					
No	18	--	--	--	
Yes	88	1.087	0.824	1.433	0.554
Use only sand bedding for lactating cows					
No	28	--	--	--	
Yes	82	0.645	0.509	0.819	<0.001
Frequency of Needle Change					
After 10 or less injections	63	--	--	--	
After 11-20 injections	23	1.355	1.048	1.752	0.02
After >20 injections	20	2.130	1.644	2.759	<0.001
Use of a breeding bull					
No	81	--	--	--	
Yes	25	1.475	1.149	1.894	0.002
Use Fly Control					
No	22	--	--	--	
Yes	79	1.185	0.898	1.564	0.229
Loose Housing for Dry Cows					
No	88	--	--	--	
Yes	18	1.673	1.291	2.169	<0.001

Table S2.7 (cont'd).

Dry Cows Fed TMR					
No	16	--	--	--	
Yes	90	1.554	1.122	2.153	0.008
First Lactation Housed Separate					
No	57	--	--	--	
Yes	48	1.207	0.976	1.493	0.082
Close-up Cows Housed Separate					
No	28	--	--	--	
Yes	78	0.806	0.633	1.025	0.079

Table S2.8

Results of multivariable Poisson regression model of herd management factors associated with the incidence rate[a] of bovine leukemia virus infection on 103 Michigan dairy herds

Risk Factor	IRR[b]	IRR[b] 95% CI		p-value
Herd Size				0.024
<175 cows	--	--	--	
175-295 cows	1.073	0.751	1.534	0.699
>295 Cows	0.582	0.387	0.876	0.009
Herd Prevalence (%)	1.156	1.115	1.198	<0.001
Herd Prevalence^2	0.999	0.998	0.999	<0.001
Transition Cows (Post-Parturient) separate from others				
No	--	--	--	
Yes	2.095	1.460	3.006	<0.001
Sand bedding for lactating cows				
No	--	--	--	
Yes	0.424	0.271	0.664	<0.001
Frequency of Needle Change				<0.001
After 10 or less injections	--	--	--	
After 11-20 injections	1.803	1.337	2.433	<0.001
After >20 injections	1.866	1.352	2.574	<0.001
Milking Frequency				
2X or Combination 2X-3X	--	--	--	
3X	2.100	1.500	2.942	<0.001
Additional Ovsynch Breedings	1.128	1.050	1.212	0.001
First Lactation Housed Separate				
No	--	--	--	
Yes	0.959	0.624	1.472	0.847
Dry Cows Fed TMR				
No	--	--	--	
Yes	1.688	1.215	2.345	0.002
Calving/Sick Cows Separate				
No	--	--	--	
Yes	0.914	0.372	2.245	0.844
Herd Prevalence x Calving/Sick Cows Separate				
Prevalence x No	--	--	--	--
Prevalence x Yes	0.979	0.963	0.995	0.011
Sand Only x Calving/Sick Cows Separate				
Yes x Yes	2.063	1.172	3.628	0.012
Milking Frequency x First Lactation Housed Separate				
3X x Yes	0.558	0.346	0.900	0.017
Constant	0.00065	0.00025	0.00170	<0.001

Table S2.9

Results of zero-inflated Poisson bivariable analysis of herd management practices associated with the incidence rate of bovine leukemia virus infections

Count Portion					
Continuous Variables	**N**	**IRR[a]**	**IRR[a] 95% CI**		**p-value**
Herd Prevalence (%)	106	1.032	1.026	1.038	<0.001
Proportion of Retested in Lactation 1	106	11.786	6.084	22.833	<0.001
Proportion of Retested in Lactation 2	106	0.311	0.124	0.781	0.013
Proportion of Retested in Lactation 3	106	0.156	0.056	0.436	<0.001
Proportion of Retested in Lactation 4	106	0.068	0.016	0.282	<0.001
Number of Reproductive Palpations	106	1.139	1.037	1.251	0.006
Number of palpations to confirm pregnancy	106	1.182	1.069	1.308	0.001
Categorical Variables	**N**	**IRR[a]**	**IRR[a] 95% CI**		**p-value**
Transition Cow (Post-Parturient) separate from others					
No	52	--	--	--	
Yes	54	1.541	1.237	1.920	<0.001
Purchase Cows					
No	58	--	--	--	
Yes	48	1.528	1.231	1.896	<0.001
Purchase some bulls, heifers, or cows					
No	41	--	--	--	
Yes	65	1.576	1.239	2.005	<0.001
Calving/Sick Cows Separate from Lactating Cows					
No	52	--	--	--	
Yes	54	0.637	0.513	0.790	<0.001
Milking Frequency					
2x or Combination 2X-3X	62	--	--	--	
3X	43	1.462	1.178	1.815	0.001
Reuse of Needles					
No	18	--	--	--	
Yes	88	1.473	1.114	1.947	0.007
Use only sand bedding for lactating cows					
No	28	--	--	--	
Yes	82	0.701	0.551	0.892	0.004
Frequency of Needle Change					
After 10 or less injections	63	--	--	--	
After 11-20 injections	23	1.473	1.135	1.913	0.004
After >20 injections	20	2.289	1.763	2.971	<0.001
Use of a breeding bull					
No	81	--	--	--	
Yes	25	1.289	1.002	1.658	0.049
Use Fly Control					
No	22	--	--	--	
Yes	79	0.829	0.626	1.097	0.190
Loose Housing for Dry Cows					
No	88	--	--	--	
Yes	18	1.271	0.978	1.653	0.073

Table **S2.9** (cont'd).

First Lactation Housed Separate					
No	57	--	--	--	
Yes	48	1.096	0.884	1.359	0.405
Close-up Cows Housed Separate					
No	28	--	--	--	
Yes	78	0.734	0.575	0.937	0.013
Only Sand Bedding for Dry Cows					
No	38	--	--	--	
Yes	66	1.265	1.007	1.589	0.043
Zero-Inflated Portion					
Continuous Variables	**N**	**OR[b]**	**OR[b] 95% CI**		**p-value**
Herd Prevalence	106	0.672	0.474	0.950	0.025
Number of Reproductive Palpations	106	0.685	0.404	1.161	0.16
Number of palpations to confirm pregnancy	106	0.630	0.339	1.172	0.145
Categorical Variables	**N**	**OR[b]**	**OR[b] 95% CI**		**p-value**
Purchase some bulls, heifers, or cows					
No	41	--	--	--	
Yes	65	0.109	0.026	0.455	0.002
Purchase Cows					
No	58	--	--	--	
Yes	48	0.129	0.023	0.742	0.022
Dry Cows Fed TMR					
No	16	--	--	--	
Yes	90	0.319	0.091	1.116	0.074
Milking Frequency					
2x or Combination 2X-3X	62	--	--	--	
3X	43	0.342	0.089	1.311	0.118

Table S2.10

Results of multivariable zero-inflated Poisson regression model of herd management factors associated with the incidence rate of bovine leukemia virus infection on 105 Michigan dairy herds

Count Portion				
Risk Factor	**IRR[a]**	**IRR[a] 95% CI**		**p-value**
Herd Size				
<175 cows	--	--	--	
175-295 cows	1.114	0.770	1.611	0.567
>295 Cows	0.751	0.518	1.089	0.131
Herd Prevalence (%)	1.077	1.052	1.103	<0.001
Herd Prevalence^2	0.999	0.999	1.000	<0.001
Transition Cows (Post-Parturient) separate from others				
No	--	--	--	
Yes	2.062	1.553	2.738	<0.001
Sand bedding for lactating cows				
No	--	--	--	
Yes	0.402	0.285	0.566	<0.001
Frequency of Needle Change				
After 10 or less injections	--	--	--	
After 11-20 injections	1.810	1.309	2.504	<0.001
After >20 injections	1.870	1.392	2.511	<0.001
Milking Frequency				
2X or Combination 2X-3X	--	--	--	
3X	3.165	2.132	4.700	<0.001
Calving/Sick Cows Separate				
No	--	--	--	
Yes	0.404	0.263	0.620	<0.001
Herd Size x Milking Frequency				
175-295 cows x 3X	0.584	0.326	1.044	0.07
>295 Cows x 3X	0.386	0.237	0.626	<0.001
Sand Only x Calving/Sick Cows Separate				
Yes x Yes	2.491	1.524	4.071	<0.001
Constant	0.00404	0.00247	0.00662	<0.001
Zero-Inflated Portion				
Risk Factor	OR[b]	OR[b] 95% CI		p-value
Herd Prevalence (%)	0.558	0.404	0.769	<0.001
Herd Prevalence^2	1.007	1.003	1.012	<0.001
Constant	1.873	0.535	6.560	0.327

Table S2.11

Results of zero-inflated negative binomial bivariable analysis of herd management practices associated with the incidence rate of bovine leukemia virus infections

Count Portion					
Continuous Variables	**N[b]**	**IRR[c]**	**IRR[c] 95% CI**		**p-value**
Herd Prevalence (%)	106	1.039	1.029	1.049	0.000
Proportion of Retested in Lactation 1	106	6.739	2.120	21.423	0.001
Proportion of Retested in Lactation 2	106	0.484	0.123	1.901	0.298
Proportion of Retested in Lactation 3	106	0.165	0.024	1.140	0.068
Proportion of Retested in Lactation 4	106	0.052	0.005	0.575	0.016
Number of Reproductive Palpations	106	1.214	0.989	1.490	0.064
Number of palpations to confirm pregnancy	106	1.303	1.033	1.644	0.025
Categorical Variables	**N[b]**	**IRR[c]**	**IRR[c] 95% CI**		**p-value**
Transition Cow (Post-Parturient) separate from others					
No	52	--	--	--	
Yes	54	1.556	1.007	2.404	0.046
Purchase Cows					
No	58	--	--	--	
Yes	48	1.968	1.226	3.158	0.005
Purchase some bulls, heifers, or cows					
No	41	--	--	--	
Yes	65	2.167	1.366	3.439	0.001
Calving/Sick Cows Separate from Lactating Cows					
No	52	--	--	--	
Yes	54	0.631	0.409	0.972	0.037
Milking Frequency					
2x or Combination 2X-3X	62	--	--	--	
3X	43	1.639	1.037	2.591	0.035
Reuse of Needles					
No	18	--	--	--	
Yes	88	1.457	0.827	2.570	0.193
Use only sand bedding for lactating cows					
No	28	--	--	--	
Yes	82	0.625	0.374	1.046	0.073
Frequency of Needle Change					
After 10 or less injections	63	--	--	--	
After 11-20 injections	23	1.322	0.782	2.232	0.297
After >20 injections	20	2.295	1.349	3.904	0.002
Use of a breeding bull					
No	81	--	--	--	
Yes	25	1.466	0.865	2.484	0.155
Use Fly Control					
No	22	--	--	--	
Yes	79	0.774	0.438	1.368	0.378
First Lactation Housed Separate					
No	57	--	--	--	
Yes	48	1.074	0.683	1.688	0.758
Close-up Cows Housed Separate					
No	28	--	--	--	
Yes	78	0.871	0.523	1.451	0.596

Table S2.11 **(**cont'd).

Zero-Inflated Portion					
Continuous Variables	**N[b]**	**OR[d]**	**OR[d] 95% CI**		**p-value**
Herd Prevalence	106	-0.425	-0.774	-0.076	0.017

Table S2.12

Results of multivariable zero-inflated negative binomial regression model of herd management factors associated with the incidence rate[a] of bovine leukemia virus infection on 105 Michigan dairy herds

Count Portion				
Risk Factor	**IRR[b]**	**IRR[b] 95% CI**		**p-value**
Herd Size				0.001
<175 cows	--	--	--	
175-295 cows	1.110	0.779	1.580	0.564
>295 Cows	0.466	0.307	0.709	<0.001
Herd Prevalence (%)	1.093	1.061	1.127	<0.001
Herd Prevalence^2	0.999	0.999	1.000	<0.001
Transition Cows (Post-Parturient) separate from others				
No	--	--	--	
Yes	2.299	1.514	3.490	<0.001
Sand bedding for lactating cows				
No	--	--	--	
Yes	0.697	0.530	0.917	0.01
Frequency of Needle Change				<0.001
After 10 or less injections	--	--	--	
After 11-20 injections	1.688	1.221	2.334	0.002
After >20 injections	1.797	1.320	2.446	<0.001
Milking Frequency				
2X or Combination 2X-3X	--	--	--	
3X	1.639	1.221	2.200	0.001
Constant	0.00213	0.00120	0.00378	<0.001
Zero-Inflated Portion				
Risk Factor	OR[b]	OR[b] 95% CI		p-value
Herd Prevalence (%)	0.570	0.416	0.782	<0.001
Herd Prevalence^2	1.007	1.003	1.011	<0.001
Constant	1.470	0.353	6.123	0.597
/lnalpha	-2.495	-3.694	-1.297	<0.001
alpha	0.082	0.025	0.273	

Table S2.13
Comparison of final multivariable models exponentiated coefficients

Risk Factor	Poisson	Negative Binomial	Zero-Inflated Poisson	Zero-Inflated Negative Binomial
Count Portion IRR				
Herd Size				
<175 cows	--	--	--	--
175-295 cows	1.073	1.235	1.114	1.110
>295 Cows	0.582	0.569	0.751	0.466
Herd Prevalence (%)	1.156	1.130	1.077	1.093
Herd Prevalence^2	0.999	0.999	0.999	0.999
Transition Cows (Post-Parturient) separate from others				
No	--	--	--	--
Yes	2.095	1.910	2.062	2.299
Sand bedding for lactating cows				
No	--	--	--	--
Yes	0.424	0.650	0.402	0.697
Frequency of Needle Change				
After 10 or less injections	--	--	--	--
After 11-20 injections	1.803	1.641	1.810	1.688
After >20 injections	1.866	1.699	1.870	1.797
Milking Frequency				
2X or Combination 2X-3X	--	--	--	--
3X	2.100	1.585	3.165	1.639
Use only sand bedding for lactating cows				
No	--	--	--	--
Yes	0.424	0.650	0.402	0.697
Calving/Sick Cows Separate from Lactating Cows				
No	--		--	
Yes	0.914		0.404	
Additional Ovsynch Breedings	1.128			
First Lactation Housed Separate				
No	--			
Yes	0.959			
Dry Cows Fed TMR				
No	--			
Yes	1.688			
Herd Prevalence x Calving/Sick Cows Separate				
Prevalence x No	--			
Prevalence x Yes	0.979			
Sand Only x Calving/Sick Cows Separate				
Yes x Yes	2.063		2.491	
Milking Frequency x First Lactation Housed Separate				
3X x Yes	0.558			
Herd Size x Milking Frequency				
175-295 cows x 3X			0.584	
>295 Cows x 3X			0.386	
Constant	0.00065	0.00135	0.00404	0.00213

Table S2.13 **(**cont'd).

Zero-Inflated Portion OR			
Herd Prevalence (%)		0.558	0.570
Herd Prevalence^2		1.007	1.007
Constant		1.873	1.470
/lnalpha	0.138		0.082

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Chapter 3 :

Natural progression of Bovine Leukemia Virus Infection in a longitudinal study

This chapter represents a manuscript in preparation for submission to a peer-reviewed journal

Authors who contributed to this study were: H. Hutchinson, B. Norby, V.J. Ruggiero, K.R.B., Sporer, P.C. Bartlett

Abstract

This study describes the longitudinal changes in bovine leukemia virus ELISA antibodies, proviral load (PVL), and blood lymphocyte counts observed over a 2.5-year period in naturally infected cattle. The dataset was from a BLV intervention field trial on three Midwestern dairy herds in which BLV diagnostic testing was used to inform culling and segregation decisions. The milking herd was BLV ELISA tested in the spring and fall. Proviral load quantification and blood lymphocyte counts were subsequently performed on cattle with ELISA-positive results. The results show ELISA false-negatives were more likely to occur in cattle with low PVL and normal lymphocyte counts. On average, negligible increases in lymphocytes counts were observed during six-month intervals and lymphocytes were almost as likely to increase as they were to decrease. Periods of lymphocytosis, defined as >10,000 lymphocytes per uL of blood, were observed in 31.5% (68/216) of test-positive cattle with two or more lymphocyte counts. An average increase of 2,900 to 3,100 proviral copies per 100,000 cells was observed during each subsequent six-month sampling interval. The difference between the minimum and maximum PVL observed for ELISA-positive cows with 3 or more observations ranged from 0 to 115,600 copies per 100,000 cells (median: 12,900; mean: 19,200). In mixed model analysis, proviral load was the greatest in ELISA-positive observations and minor changes in proviral load were associated with increases in lymphocytes. These results suggest that, following the identification of ELISA-positive cattle and the assessment of PVL and lymphocyte counts, subsequent tests to assess disease progression may not be needed. Further work is needed to determine how the available BLV diagnostic tests can be optimized to design cost-effective testing schemes for BLV control programs aimed at identifying and removing cattle with high proviral load and high lymphocyte counts.

Introduction

Bovine leukemia virus (BLV) is in the family *Retroviridae* and genus *Deltraretrovirus* and is the causative agent of enzootic bovine leukosis. As a retrovirus, the BLV RNA virus is reverse transcribed into a DNA provirus that integrates into the host genome leading to a persistent, lifelong infection. The primary target for BLV is host B-lymphocytes, although it has also been detected in other cells such as T-lymphocytes and mammary epithelium (Buehring et al., 1994; Panei et al., 2013; Yoshikawa et al., 1997). Endemic in the U.S. cattle population, BLV is estimated to infect approximately 40% of U.S. dairy cattle (Bauermann et al., 2017; Ladronka et al., 2018; USDA, 2008, 1997; USDA APHIS, 1999). A growing body of research shows that BLV negatively impacts animal welfare, the profitability of the dairy industry, and may affect human health. These impacts warrant the consideration of BLV control programs.

When the prevalence is low, BLV can be controlled through the removal or segregation of all infected cattle as was accomplished in many European countries that have achieved disease-free status (Acaite et al., 2007; Nuotio et al., 2003) For nations with a high prevalence, control through segregation or removal of all infected cattle is not economically feasible; the removal of infected cattle is prohibited by the need to replace those removed and segregation of infected cattle is constrained by farm facilities and logistics. Therefore, an alternative intervention is to reduce transmission until the prevalence is sufficiently low that the farm can survive the culling of all ELISA-positive cattle and thereby achieve eradication.

The transmission of BLV among cattle is thought to occur from the transference of BLV-infected cells which can be found in blood, milk, colostrum, saliva, and nasal secretions (Ferrer and Piper, 1981; Lucas et al., 1993; Miller and Van Der Maaten, 1979; Yuan et al., 2015). Potential modes for the transmission of infected cells include the reuse of medical equipment

(e.g. hypodermic needles, palpation sleeves), biting flies, and feeding of infected milk or colostrum (Hopkins and Digiacomo, 1997). Unfortunately, removal of all modes of transmission is costly and labor intensive. Furthermore, mixed success of management intervention control programs for disease reduction has been observed. For example, conflicting results were reported by two intervention field trials implementing both single-use needles and palpation sleeves and disinfection of medical equipment (Gutiérrez et al., 2011; Sprecher et al., 1991). Given the limitations of the aforementioned control strategies, a new approach has focused on the removal of cattle thought to be the most infectious (Gutiérrez et al., 2011; Molloy et al., 1994; Ruggiero et al., 2019). This approach requires an understanding of the epidemiology of BLV infection and progression with reference to the available diagnostic tests.

Infection with BLV has classically been thought to develop slowly across 3 progressive disease states defined by lymphocyte counts (LC) and tumor development: 1. aleukemic (normal lymphocyte count), 2. lymphocytosis (persistently elevated lymphocyte count), and 3. lymphoma or lymphosarcoma (development of tumors) (Schwartz and Lévy, 1994). At any given time, it has been estimated that 50-60% of infected cattle are aleukemic and 30-40% are persistently lymphocytic (EFSA Panel on Animal Health and Welfare, 2015). Independent of aleukemic or persistent lymphocytosis status less than 5% of infected cattle, can develop B-cell lymphoma or lymphosarcoma.

In addition to the three classic disease states, researchers have recently classified BLV-infected cattle by proviral load (PVL), expressed as proviral copies per quantity of cells or DNA (Juliarena et al., 2007; Nieto Farias et al., 2018). A current theory suggests that cows with a high PVL or high LC are at higher risk of infecting their herd mates (Rodríguez et al., 2011). This theory is supported by a recent field trial in which the targeted removal of cows with high PVL

and high LC was successful in reducing BLV prevalence and incidence (Ruggiero et al., 2019). In further support, cattle with low PVL do not appear to be at high risk of transmitting disease as BLV transmission did not occur when cattle with low PVL were introduced into negative herds (Juliarena et al., 2016; Mekata et al., 2018).

Factors which contribute to the development of high PVL and LC require further elucidation. The development of lymphocytosis is thought to result from the gradual, polyclonal expansion of BLV-infected cells (Bendixen, 1965; EFSA Panel on Animal Health and Welfare, 2015). The development of high PVL has been correlated with lymphocytosis (Alvarez et al., 2013; Ohno et al., 2015); however, Juliarena *et al* (2007) showed 40% of aleukemic cows had high PVL (Juliarena et al., 2007). Recently it has been suggested that PVL is established shortly after infection and is stable over time with minor fluctuations in PVL observed (Lendez et al., 2015; Mekata et al., 2018). In an experimental infection conducted by our research team, the average peak in PVL was observed 45 days post-inoculation followed by a decline or plateau to a relatively steady state until 147 days post-inoculation in a majority of the inoculated steers (Hutchinson *et al*, manuscript in preparation).

In another experimental infection study, the establishment of PVL was associated with the BoLA DRB3 haplotype, which has also been associated with high and low PVL disease states in several cross-sectional studies (Forletti et al., 2020; Juliarena et al., 2008; Lendez et al., 2015). Limited studies, however, have reported PVL and LC levels over time by longitudinally following naturally infected cattle. Among the studies which are published, many are limited by the use of experimental inoculation (Gillet et al., 2013; Jimba et al., 2012), heifers (Gutiérrez et al., 2014), or the follow-up duration (Konishi et al., 2018).

The objective of this study was to describe the longitudinal changes in BLV ELISA diagnostic results, PVL, and LC in cattle naturally infected with BLV and tested semi-annually over a 2.5-year period. More specifically, we wanted to examine the relationship between observed changes in ELISA test results and both the PVL and LC, to describe the observed fluctuations in the proviral load and lymphocyte count measurements over time, and to determine how changes in these measurements were interrelated.

Methods

Study Design:

The data used for this analysis were from a previously published field trial which aimed to reduce the prevalence and transmission of BLV using PVL and LC measurements as indicators of infection potential of cows that could be selected for culling or segregation until culling at a later date (Ruggiero et al., 2019). Three midwestern dairy herds were enrolled and sampled over the course of 2.5 years. This study was designed to semi-annually BLV test all cows that were lactating by antibody-capture milk ELISA. Subsequent on-farm sampling was conducted within one month to collect blood samples for PVL and LC testing from cattle that had ever had an ELISA-suspect or ELISA-positive result. In addition, blood samples were collected from cattle that were in the milking herd but were not lactating when semi-annual milk samples were collected for ELISA testing using either plasma or serum. (**Figure 3.1**). Herd managers utilized the PVL and LC measurements to inform culling decisions, prioritizing the removal or segregation of cattle with the highest PVL and LC. This study was approved by Michigan State University Institutional Animal Care and Use Committee (PROTO201900271; 08/16-143-00**).**

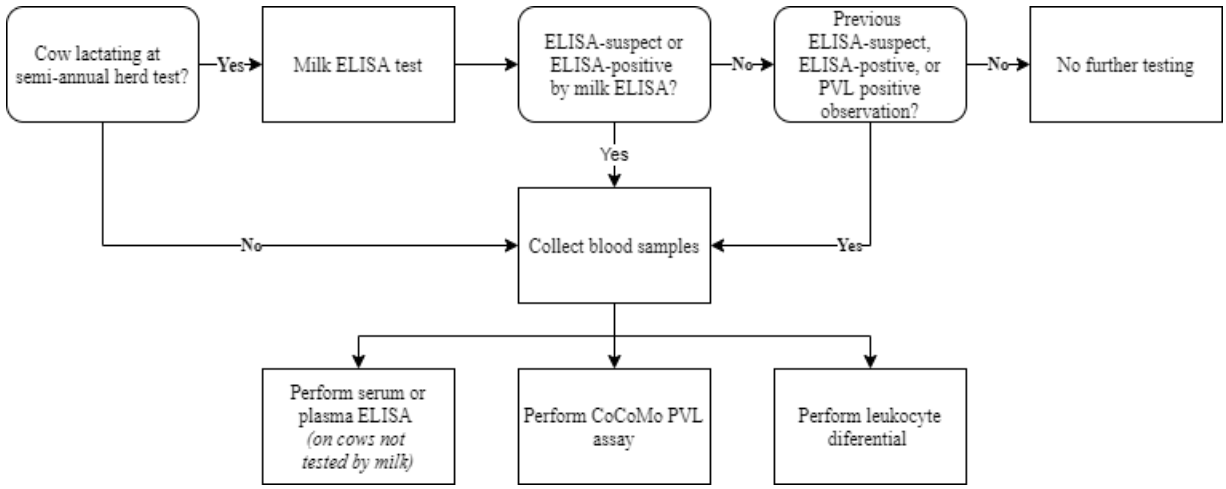

Figure 3.1 Semi-annual sampling scheme.

Anti-BLV antibody ELISA test:

Both the milk and serum antibody ELISA testing were performed by CentralStar Cooperative (Lansing, MI) using a modified, commercial gp51 antibody capture ELISA test (IDEXX Laboratories, Inc., Westbrook ME) as previously described (Erskine et al., 2012; Ruggiero et al., 2019). Briefly, sample aliquots were mixed with sample buffer using a 1:30 dilution and then added to precoated 96-well BLV ELISA plates. Following plate washing, an enzyme substrate was added to detect anti-BLV antibodies through a reaction with horseradish-peroxidase-labeled antibodies to bovine immunoglobulin. Reaction times were standardized by the color development of positive controls. The reaction was stopped by adding 0.5 N H_2SO_4. Results of all ELISA tests were reported as corrected 450 nm optical density (OD) measurements (raw sample OD-negative control OD) and were categorized as negative, suspect, or positive using established cut-off values. For milk ELISAs these cut-off values were negative <0.1 OD, suspect 0.1-0.3 OD, positive >0.3 OD. For serum or plasma, ELISA cut-off values were negative <0.5 OD, suspect 0.5-1.0 OD, positive >1.0 OD.

Leukocyte Differentials:

Blood leukocyte differentials were performed using an early version of the QScout blood leukocyte differential machine (Advanced Animal Diagnostics, Morrisville, NC). Briefly, 80 µL of blood collected in an EDTA tube was mixed with 20 µL of K-stain solution in a 2 mL microcentrifuge tube. Ten microliters of the mixture was pipetted onto a QScout slide which was then placed into the analyzer. The QScout captured and evaluated images using an analytical algorithm to calculate the leukocyte differential. The differential included the total leukocytes, lymphocytes, neutrophils, and their relative percentages. Leukocyte differentials were not performed at the first cross-sectional sampling.

Quantification of Proviral Load:

Using Wizard Genomic DNA Purification kits (Promega Corporation, Madison, WI), DNA was extracted from 300 µL aliquots of EDTA anti-coagulated blood. In short, blood samples went through a series of cell lysis, nuclei lysis, and protein precipitation solutions. Thereafter, the cellular proteins were pelleted and the DNA was precipitated from the supernatant using pure isopropanol followed by a 70% ethanol solution. DNA pellets were subsequently resuspended using a DNA rehydration solution. Eluted DNA was then normalized to 30 ng/µL.

Proviral load was quantified using the CoCoMo qPCR method (RIKEN Genesis, Tokyo, Japan) (Jimba et al., 2010), 150 ng of DNA, and TaqMan Gene Expression Master Mix (Life Technologies, Carson, CA). The assay used degenerate primers to amplify a portion of the BLV proviral long terminal repeat. Proviral copies were standardized to the number of nucleated cells through quantification of the BoLA-DRA gene and were reported as proviral copies per 100,000 cells.

Data Analysis

All descriptive and analytical statistics were performed using Stata 15 (StataCorp LLC, College Station, TX)

Descriptive Analysis:

We considered cows that tested either ELISA-suspect or ELISA-positive to be test-positive (Erskine et al., 2012; Ruggiero et al., 2019). However, these ELISA results were kept separate so that potential factors associated with this difference between ELISA-suspect and ELISA-positive results could be investigated.

A change in ELISA status was defined as a cow having different ELISA test results (i.e. negative, suspect, positive) reported at consecutive semi-annual sample times; an ELISA change could be from negative to positive, negative to suspect, suspect to positive, or vice-versa. Changes in ELISA status were attributed to ELISA new infections, ELISA false-negatives, and ELISA false-positives based on the criteria presented in **Table 3.1**.

Table 3.1
Required criteria for changes in ELISA to be defined as new infections, false-negatives, and false-positives

ELISA New Infection
▪ ELISA-suspect or ELISA-positive result ▪ Concurrent or subsequent PVL-positive result(s) ▪ Prior ELISA-negative result(s) ▪ No Prior PVL-positive result(s)
ELISA False-Negative
▪ ELISA-negative result ▪ Concurrent, or prior and subsequent, PVL-positive test(s) ▪ Prior ELISA-suspect or ELISA-positive result(s)
ELISA False-Positive
▪ ELISA-suspect or ELISA-positive result ▪ Concurrent PVL-negative result ▪ Prior or subsequent ELISA-negative result(s) ▪ Prior or subsequent PVL-negative result(s)

Observations from cattle that were determined to be BLV infected by combined ELISA and PCR results were considered to be BLV "test-positive". Only the observations from test-positive cattle contributed to the descriptive distributions of both PVL and LC. In evaluating the LC of BLV test-positive cows, persistent lymphocytosis was defined by all observed LC being greater than the specified cutoff; transient lymphocytosis was defined by the observance of both normal and lymphocytic results in a BLV test-positive cow. Cut-offs of both 7,500 and 10,000 lymphocytes per μL of blood were examined and used to define lymphocytosis.

Analytical Analysis:

A generalized linear mixed model (GLMM) approach was used to examine how PVL, LC, and DIM affected changes in ELISA status in BLV-test positive cattle across semi-annual sampling points. Using a binomial distribution and a logit link, two separate outcomes were examined: any change in ELISA status and a change associated with an ELISA false-negative. Linearity of continuous variables in the logit was assessed using the Box-Tidwell statistic. The low frequency of false negatives resulted in categorical variables with zero counts that could not be included in bivariable logistic regression models because of perfect prediction. Therefore, the associations between categorical predictors and ELISA false-negative were assessed using Fisher's exact tests.

Changes in LC and PVL over time in BLV test-positive cattle were evaluated using linear mixed models (LMMs). Models of PVL included random effects at both the herd- and cow-level. The PCR assay number was included as a random crossed effect in PVL models to account for systematic variation in proviral load that may have been the result of inter-plate variability. Potential explanatory variables in PVL models were LC, ELISA status, and DIM. Models of LC included a random effect for each cow. The standard errors of herd-level random

effects could not be estimated in the unconditional lymphocyte model and, therefore, herd was not included as a random effect. Explanatory variables examined in LC models included ELISA status and DIM. In addition, linear growth models were created by including the semi-annual test number as both a fixed and random effect slope to estimate both the average and individual change over time. Only cows with three or more outcome measurements were included in the linear growth models. All LMMs were built using restricted maximum likelihood estimation using Kenward-Rodger adjustment for degrees of freedom. The normality of residuals was visually assessed by histograms and Q-Q plots. Levene's test was used to evaluate the homogeneity of variance across categorical predictors. Wald's test was used to assess the statistical significance of independent variables. The statistical significance of marginal comparisons was adjusted using the Bonferroni correction for multiple comparisons.

Results

A total of 779 cows were tested and contributed 2,058 sampling observations. The average number of observations per cow was 2.6 (range: 1 to 5). The relative frequencies of total observations, ELISA, PVL, LC tests as well the frequency of positive outcomes are presented in **Table 3.2**. Information on the total days-in-milk (DIM) at ELISA testing was available for a subset of cows from two of the dairy herds (n=166 cows; 650 observations; median= 4 observations per cow). Total DIM was divided into five categories: dry, 0 to 100 DIM, 101-200 DIM, 201-300 DIM, 301+ DIM.

Table 3.2
Cow-level observation, ELISA, proviral load, and leukocyte differential test frequencies (n=779 cows)

	Total Number of Observations					
	0	**1**	**2**	**3**	**4**	**5**
Observations Overall	--	29.8% (232/779)	21.1% (164/779)	18.0% (140/779)	17.6% (137/779)	13.6% (106/779)
ELISA Tests	0.4% (3/779)	30.7% (239/779)	20.6% (161/779)	18.2% (142/779)	17.2% (134/779)	12.8% (100/779)
ELISA-SP[a]	47.9% (373/779)	12.2% (95/779)	10.9% (85/779)	10.8% (84/779)	10.4% (81/779)	7.8% (61/779)
PVL Tests	47.1% (367/779)	16.6% (129/779)	14.3% (111/779)	11.4% (89/779)	9.0% (70/779)	1.7% (13/779)
PVL-Positive	51.2% (399/779)	16.8% (131/779)	13.0% (101/779)	10.4% (81/779)	7.1% (55/779)	1.5% (12/779)
Leukocyte Differentials	52.5% (409/779)	16.2% (126/779)	11.3% (88/779)	11.2% (87/779)	8.9% (69/779)	--[b]
Combined ELISA, PVL, LC	53.5% (417/779)	16.7% (130/779)	11.3% (88/779)	10.9% (85/779)	7.6% (59/779)	--[b]

[a] ELISA-SP= ELISA-suspect or ELISA-positive outcome
[b] Leukocyte differential were not performed at the first semi-annual test

ELISA:

Two or more ELISA tests were available for 68.9% (537/779) of cows. For descriptive purposes, the observed patterns in ELISA test results were grouped into seven categories as shown in **Table 3.3**; in addition, this table includes the respective counts for new infections, ELISA false-negatives, and ELSIA false-positives The ELISA pattern categories are not based on the time-order of the result, but rather on the presence of a particular ELISA result. For example, the "Negative/Suspect" category contains cows which experienced either a change from ELISA-negative to ELISA-suspect or a change from ELISA-suspect to ELISA-negative. In some instances, cows contained within the pattern category had multiple changes between the respective ELISA diagnostic outcomes. The respective ELISA patterns and their relative frequencies are provided in the supplemental data (**Table S3.5**). Consistent ELISA results were observed in 76.0% (408/537) of cows with multiple observations.

Table 3.3
Categorization of observed ELISA tests results for all cows with two or more semi-annual ELISA tests (n=537 cows)

ELISA Pattern Categories [a]	N [b]	Mean # ELISA test	SD	New Infection [c]	False Negative [c]	False Positive [c]
Always Negative	236	3.28	1.12	0	--	0
Always Suspect	5	2.8	1.3	0	0	0
Always Positive	167	3.14	0.98	0	0	0
Negative or Suspect	32	3.69	1.15	8	16	9
Negative or Positive	28	3.5	1.14	22	2	4
Negative, Suspect or Positive	11	4.18	0.6	6	7	0
Suspect or Positive	58	3.62	1.11	0	0	0

[a] Cows with two or more ELISA results were assigned ELISA pattern categories based on observed results. Patterns are not based on the time-order of results but rather the presence of a particular ELISA result at any of the semi-annual tests [b] N= total number of cows [c] The occurrence of ELISA new infections, false-negatives, and false positives were inferred from previous, concurrent, and subsequent PCR tests when available. These occurrences are not mutually exclusive. For example, it is possible for a cow to have a new infection and an ELISA false-negative.

At least one change in ELISA status was observed for 129 cows with two or more observations. Multiple changes (2+) in ELISA status were observed for 20.1% (47/234) of cows with three or more ELISA tests. In fact, 12 cows experienced three changes and four cows experienced four changes in ELISA status.

In separate GLMM models, increases in LC and PVL were negatively associated with the odds of experiencing a change in ELISA status. The LC for observations with a change in ELISA status ranged from 1,800 to 16,600 with 75% of lymphocyte counts being less than 6,700 lymphocytes per uL. The odds of a cow experiencing a change in ELISA status were reduced by a factor of 0.764 for each increase of 1,000 lymphocyte per µL ($p<0.001$) (**Figure 3.2**).

The PVL associated with a change in ELISA status ranged from 0 to 106,800 copies per 100,000 cells. However, 75% of observations had a PVL less than 5,400 copies per 100,000 cells. The quadratic term for proviral load was significant; the marginal probability for a change

in ELISA status is depicted in **Figure 3.3**. Categorical DIM was not associated with the likelihood of experiencing a change in ELISA status (p=0.989).

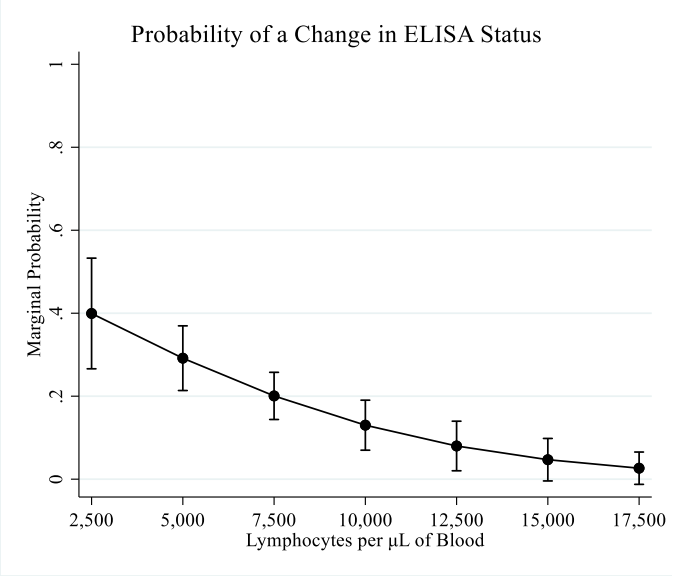

Figure 3.2 Marginal probability of experiencing a change in ELISA status as lymphocytes increases

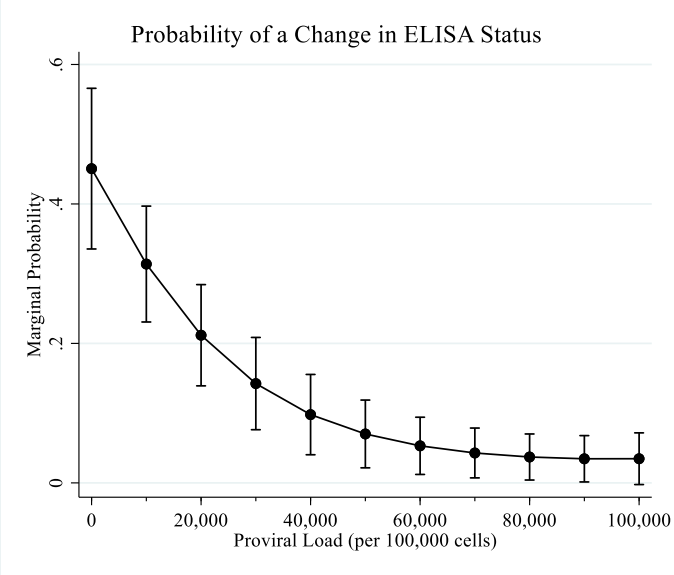

Figure 3.3 Marginal probability of experiencing a change in ELISA status as proviral load increases

Changes in ELISA status indicated new infections for 36 cows. Interestingly, additional changes in ELISA status were observed in 45.5% (10/22) of cows that had new infections and

were subsequently retested. This change was an ELISA false-negative for six cows that tested ELISA negative and PCR positive at a subsequent observation. The remaining four cows with new infections experienced changes from ELISA positive to ELISA suspect (n=3) or vice-versa (n=1).

Aside from those which occurred following new infections, ELISA false-negatives were observed in an additional 13 cows. Three cows were observed to have two ELISA false-negatives and one cow was observed to have three ELISA false-negatives.

Collectively, a total of 25 ELISA false-negative results, from 19 cows, were observed among 609 observations from 254 cows assumed to be BLV test-positive based on combined ELISA and PCR data. The occurrence of ELISA false-negatives was not associated with herd (Fisher's Exact p=0.342) or the semi-annual test (Fisher's Exact p=0.486). A significant difference in the likelihood of an ELISA false-negative was observed for lactation stage (Figure 3.4). False negatives by ELISA were not observed among samples collected during the dry period (n=7) or after 300 DIM (n=88). An increased frequency, however, was observed between 101 to 200 DIM. When the analysis was performed on the herds individually, the increased frequency was only significant in one of two herds. In this herd, the ELISA false-negative results were evenly dispersed among semi-annual sampling timepoints and cows with multiple ELISA false-negatives were not observed to have DIM categories with more than false negative observed. This suggest the occurrence of ELISA false-negatives is not an artifact of testing.

Table 3.4

Frequency of ELISA false-negatives by DIM in a subset of cows in two herds (n=166 cows; 391 observations).

		DIM					
		Dry	**0 to 100**	**101 to 200**	**201 to 300**	**301+**	**p-value[a]**
Overall	# ELISA False-Negative	0	8	9	6	0	0.017
	Total Observations	7	106	84	106	88	
Herd J	# ELISA False-Negative	--	2	7	3	0	0.006
	Total Observations	--	30	37	40	49	
Herd KBS	# ELISA False-Negative	0	6	2	3	0	0.461
	Total Observations	7	76	47	66	39	

[a] Statistical significance assessed using a Fisher's Exact test. This test determines if there are nonrandom associations between categorical variables.

The median PVL associated with ELISA false-negative results was 110 proviral copies per 100,000 cells. One extreme value of 71,773 copies per 100,000 cells was associated with an ELISA false-negative; the remainder of the samples had PVLs less than 1,400 copies per 100,000 cells. Lymphocyte counts associated with ELISA false-negatives ranged from 3,300 to 10,200 (median: 4,900) per µL of blood. A significant association was identified between lymphocyte counts and the odds of testing ELISA negative; for each increase of 1,000 lymphocytes, the odds of an ELISA false-negative decreased by a factor of 0.71 (p=0.010) which corresponded with a decrease in the probability of ELSIA false-negative (**Figure 3.4)**.

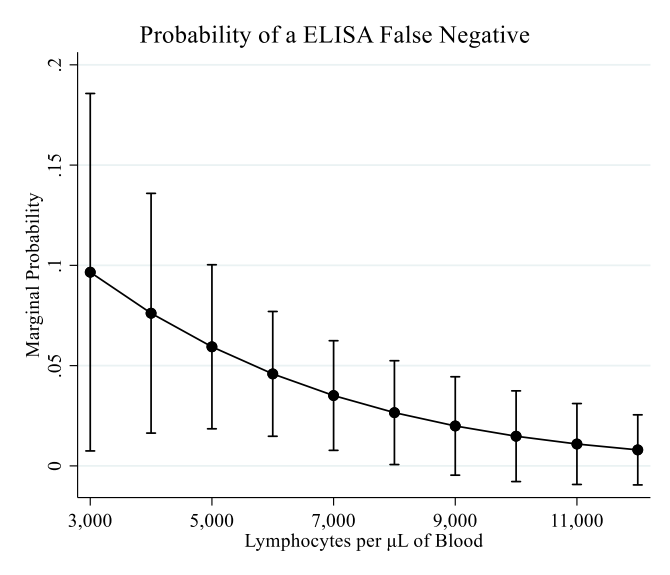

Figure 3.4 Marginal probability of experiencing a change in ELISA status as lymphocytes increase

Lymphocyte Count:

Lymphocyte counts were determined in 728 samples collected from 324 BLV test positive cows. The observed LC ranged from 1,800 to 23,600 lymphocytes per µL of blood (median: 6,600; mean: 7,700). Lymphocytosis (>7,500 lymphocytes per µL of blood) was observed for 40.7% (296/728) of observations with at least once incident of lymphocytosis

observed in 52% (167/324) of BLV test-positive cows. Among cows with 2 or more LC, 49.1% (106/216) were consistently aleukemic, 30.1% (65/216) were persistently lymphocytotic, 9.3% (20/216) progressed from aleukemic to lymphocytotic, and the remaining 11.6% (25/216) were transiently lymphocytotic. When the definition of lymphocytosis was increased to 10,000 lymphocytes per µL, 23% (170/728) of observations were lymphocytotic and 33% (106/324) of cows had at least one incident of lymphocytosis. Using the cutoff of 10,000 lymphocyte per µL, 68.5% (148/216) of cows with 2 or more observations were aleukemic, 13.9% (30/216) were persistently lymphocytotic, 9.3% (20/216) progressed from aleukemic to lymphocytotic, and 8.3% (18/216) were transiently lymphocytotic.

To compare LC across ELISA results categories, and DIM, the lymphocyte marginal means which are adjusted for repeated measures were used. Marginal LC were higher among observations with ELISA positive results (8,100 lymphocytes per µL) than ELISA suspect results (7,300 lymphocytes per µL) ($p=0.008$). Comparing lymphocytes across assigned ELISA pattern categories (**Table 3.3**), marginal LC in "Always Positive" cows (8,900 lymphocytes per µL) were significantly greater than cows with "Suspect or Positive" results (6,100 lymphocytes per µL; $p< 0.001$) and "Always Suspect" cows (5,900 lymphocytes per µL; $p=0.024$). The marginal LC in BLV test-positive cows were observed to be the lowest in dry cows (7,400 lymphocytes per µL) and to increase across DIM categories with the highest marginal lymphocytes observed for cows that were 301+ DIM (8,100 lymphocytes per µL) ($p=0.052$).

Observed changes in the LC of BLV test-positive cows that occurred over an approximately six-month long interval are presented in **Figure 3.5A.** While fluctuations upwards of 10,000 lymphocytes per µL were observed, the average change between two consecutive semi-annual tests was an increase of 200 lymphocyte per µL; 90% of all changes were between a

decrease of -2,900 lymphocytes per µL and an increase of 3,700 lymphocytes per µL. Looking at the difference between the first and last lymphocyte count for cows with 3 or more observations, while an average increase of 200 lymphocytes per µL was observed, the overall difference was evenly split between a increases and a decreases; 90% of cows experienced changes of -3,100 and 4,500 lymphocytes per µL between the first and last lymphocyte counts (**Figure 3.5B**). The median amplitude (i.e. difference between the minimum and maximum lymphocyte count) for BLV test-positive cows with three or more observations was 2,200 (range: 100 to 10,600) lymphocytes per µL. This amplitude, however, was less than 3,200 lymphocytes per µL in 75% of cows (**Figure 3.5C**).

In an unconditional mean model accounting for repeated measures, 81% of the variation in observed lymphocyte counts was attributed to between cow differences. In an unconditional growth model accounting for repeated measures, only 1% of within-cow variation was accounted for by the inclusion of time as a random slope. A non-significant (p=0.877) marginal increase of 11 (95% CI: -100 to 200) lymphocytes per µL was observed for each subsequent cross-sectional sampling for BLV test-positive cows with three or more leukocyte counts (n=135). Addition of categorical DIM (p=0.138) and ELISA status (p=0.085) to the model were non-significant.

Proviral Load:

Proviral load was measured in 850 samples from 359 BLV test-positive cows. The observed PVL ranged from 0 to almost 180,000 provirus copies per 100,000 cells and was right skewed. Not accounting for repeated measures, PVL was significantly higher in ELISA positive (median= 43,000 copies; mean=46,100 copies; range: 0 to 179,900 copies per 100,000 cells) samples than ELISA suspect samples (median=180 copies; mean 10,400 copies; range: 0 to 136,700 copies per 100,000 cells) (Kruskal-Wallis p<0.001). This was confirmed using a LMM

accounting for repeated measures. When viewed across the assigned ELISA pattern categories (**Table 3.3**), the marginal PVL for "Always Positive" cows (55,023 copies per 100,000 cells) was greater than both "Suspect or Positive" cows (18,823 copies per 100,000 cells; $p<0.001$) and " Always Suspect" cows (5,149 copies per 100,000 cells; $p<0.001$). Non-significant differences in PVL were observed across DIM categories ($p=0.275$).

The average change in proviral copies between consecutive, cross-sectional samplings was an increase of 2,900 copies per 100,000 cells (range: -72,000 to 115,600 copies per 100,000 cells) over the approximately 6-month period (**Figure 3.5B**). The median difference between the first and last PVL observation for cows with 3 or more observations was 800 proviral copies per 100,000 cells (mean: 7,800 copies per 100,000 cells); the interquartile range was -300 to 14,600 copies per 100,000 cells (**Figure 3.5D**). Because it has been suggested that proviral load undulates around a relatively steady state (Gillet et al., 2013; Lendez et al., 2015; Mekata et al., 2018), the PVL amplitude (i.e. the difference between the minimum and maximum observed proviral load) was calculated. The average maximum change in PVL was 19,200 copies per 100,000 cells (median: 12,900; range: 0 to 115,600 copies per 100,000 cells) (**Figure 3.5F**).

An unconditional mean model was utilized to partition the observed variation in PVL for cows with 3 or more observations; 7.6% of variation can be attributed to inter-herd differences while 79.5% is attributed to cow-level differences. The addition of semi-annual test number (i.e. time) as both a fixed- and random-effect accounted for 19.2% of the cow-level variation. A marginal increase of 3,072 proviral copies per 100,000 cells was observed with each subsequent 6-month sampling ($p=0.001$).

Independently, the model which included time as fixed and random effects was conditioned on ELISA status, DIM, and lymphocytes per μL of blood. Overall marginal

differences in proviral copies were observed when ELISA status was added to the growth model ($p=0.023$). Marginal differences in PVL were not statistically significant between ELISA-suspect and either ELISA-negative (5,754 copies per 100,000 cells; $p=0.361$) or ELISA-positive (4,599 copies per 100,000 cells; $p=0.120$) observations. Differences between ELISA-positive and ELISA-negative, however, were significant ($p= 0.033$) with a marginal increase of approximately 10,400 copies per 100,000 cells. An increase of 11 proviral copies was observed for every increase of 1,000 lymphocytes per μL of blood ($p<0.001$). Similar effects were observed in a multivariable model containing sampling number ($p=0.004$), ELISA status ($p=0.021$), and lymphocyte count ($p<0.001$). Non-significant differences in the marginal PVL were observed when the growth model was conditioned on categorical DIM (range: 387 to 4,047 copies per 100,000 cells; $p=0.403$).

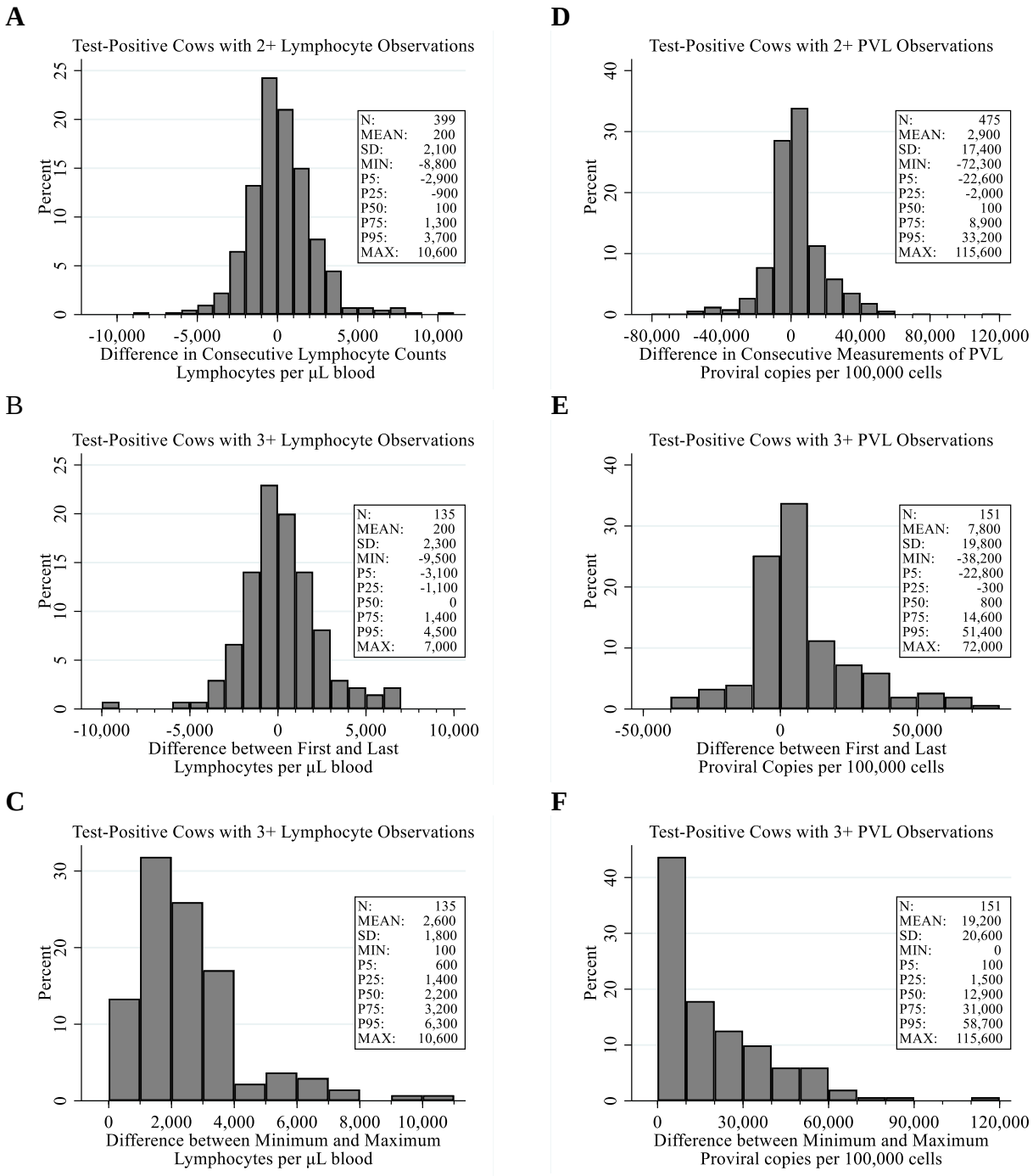

Figure 3.5 Distributions of consecutive differences, overall differences, and amplitude of lymphocytes in BLV test-positive cattle

The consecutive difference graphs show the relative difference in lymphocytes (A) and proviral load (D) between two consecutive observations in test-positive cows. The graphs of the difference between the first and last lymphocyte (B) and proviral load (E) observation show the general trend in proviral load over time. The graphs of lymphocyte (C) and proviral load amplitude (F) show the differences between the minimum and maximum observations and indicate the absolute fluctuations in BLV test-positive cows.

Discussion

The control and eradication of BLV are justified by the negative impact infection has on animal health and productivity. One of our research priorities is to design cost-effective control programs that cattle producers can successfully implement to reduce BLV incidence, and in turn reduce BLV prevalence. A control program which shows promise is the selective removal of infected cattle with high PVL (Ruggiero et al., 2019).

Currently, PVL testing is not broadly available, and is both time- and labor-intensive since the collection of blood samples is required. While PVL testing is now available through our commercial partner, PVL assays are more expensive, costing approximately $10 USD compared to $6 USD for ELISA tests (CentralStar Cooperative, Lansing, MI). Therefore, we typically rely on ELISA testing which can be performed on milk samples in conjunction with routine milk components analysis for initial BLV testing. Blood samples for the analysis of PVL are then subsequently collected from cattle which test ELISA-suspect or ELISA-positive. Through this sampling scheme, infected cattle need to be identified by ELISA for PVL assays to be performed; therefore, the occurrence of ELISA false-negatives can undermine control program success. In addition, once a cow is identified to be infected with BLV it may be tested multiple times over the course of the intervention program to quantify proviral load. A greater understanding is needed of the progression of BLV infection over-time and of how proviral load and lymphocyte counts change. This understanding may augment control programs by reducing the frequency, and therefore cost, of repeated samplings. For these reasons, the overall objective of this analysis was to describe the changes and the relationships between the ELISA tests, lymphocyte counts, and PVL measurements observed during the course of a 2.5-year intervention field trial.

With regard to ELISA tests, we were most interested in the cows which had changes in ELISA status, especially false-negatives, and the associated proviral load and lymphocyte counts. The ELISA assay provided an optical density that is then categorized as negative, suspect, or positive. The general purpose of the suspect category is to express caution in the test result since residual milk carryover (milk from the previous cow tested) has been shown to cause false positives (Nekouei et al., 2015). However, in this study, 85% (131/154) of ELISA suspect observations were PVL positive. While the OD is not the equivalent of antibody titer, it may be representative of the relative abundance of antibodies or may be associated with other BLV impacts or disease measurements. For example, a previous analysis conducted by our research team found significant differences in milk production when the BLV-ELISA optical density was categorized (Norby et al., 2016).

In this study, the true frequency of ELISA false-negatives (i.e. ELISA negative, PVL positive cows) was unknown given the sequential testing design used for data collection. However, PVL was evaluated for cattle that tested ELISA negative but had a previous test at which they were ELISA-suspect, ELISA-positive, or PVL positive. Through this, ELISA false-negatives were observed in 7.4% (19/258) of cows known to be BLV test-positive. This proportion is similar to another study which reported 6.8% (5/73) cows to be serologically negative and PCR positive (Jacobs et al., 1992). The observance of ELISA false-negatives in this study may partially be explained by the imperfect sensitivity of the milk ELISA, which was estimated to be 86% when compared to the serum-based ELISA. Interestingly, multiple ELISA false-negatives were observed in 26.3% (5/19) of these cows suggesting there may be more PVL positive, ELISA-negative cows among the 236 cows which consistently tested ELISA-negative and were never tested by PCR.

We observed that both the probability of having a change in ELISA and the probability of an ELISA false-negative was inversely associated with both the proviral load and lymphocyte count. While PVL was not examined, the identification of viral RNA was recently reported and was associated with elevated lymphocyte counts (Chen et al., 2020). Given that lymphocyte counts are correlated with proviral load (Alvarez et al., 2013), it is plausible that cows with low PVL and LC may experience less viral reactivation, and thus reduced immune stimulation and antibody production. This theory is further supported by our observation that PVL was significantly lower in ELISA suspect observations compared to ELISA positive observations.

The frequency of ELISA false-negatives was the greatest between 101 and 200 DIM. The spontaneous production of viral RNA was recently reported and was thought to be associated with periods of increased stress.(Alvarez et al., 2019; Jaworski et al., 2019) Two of the most physiologically stressful events in the production cycle of a dairy cow are the abrupt cessation of milking at dry-off and the events associated with calving. If these periods are associated with the increased likelihood of viral reactivation, and thus immune stimulation and antibody production, it is plausible antibodies levels have begun to wane by the time period of 101 to 200 DIM. However, this would not explain why cows 201 to 300 DIM do not have increased ELSIA false-negatives. Given samples collected in the dry period would have been serum samples, the absence of ELISA false-negatives in dry period may be reflective of differences in the sensitivity of the serum- and milk-based ELISAs or an artifact of small sample size. In a separate study conducted by members of our research team, seroconversion, based on ELISA tests performed on plasma samples, was observed to occur during the transition period (Wisnieski et al., *manuscript in press*). Taken together, the effect of production stage on both serum- and milk-based ELISA assays in BLV positive cows may be worthy of further investigation.

The cows with changes in ELISA status, specifically ELISA false-negatives, do not likely pose an immediate threat to control programs which focus on the removal of cattle with high PVL and LC. In the long-term, however, the small subset of cows with repeated ELISA false-negatives could prove problematic for BLV eradication programs that rely on serology to initially identify infected cattle.

Regarding both the LC and PVL, the main outcome of interest was the observed changes over time. We sought to answer the question of whether or not LC and PVL gradually increase over time in BLV-infected cows and, in essence, if high PVL and persistent lymphocytosis were the result of a gradual disease progression.

The results from our study suggest that on average, negligible increases in lymphocytes are observed during an approximately 6-month period. Addition of time into a growth model only accounted for a small (1%) portion of within-cow variable. Combined with the observed changes in LC, these results suggest that fluctuations in lymphocyte counts may be random and not the result of gradual disease progression. Given lymphocyte references intervals typically span an approximate range of 5,000 lymphocytes (2,500 to 7,500 lymphocytes per µL), the observed fluctuations may be normal. There was, however, a subset of cows that experienced absolute changes upward of 11,000 lymphocytes per µL during the observed study period. Whether these changes were associated with BLV-disease progression or are explained by other physiological processes or infections could not be determined.

Similar to the lymphocyte counts, both increases and decreases in PVL were observed between sequential six-month sampling intervals and between the first and last cross-sectional observation. In addition, absolute fluctuations averaged 19,200 proviral copies per 100,000 cells. In an experimental infection study, we observed fluctuations in proviral load to occur after the

initial peak (Hutchinson *et al.*, manuscript in preparation). Thus, it is plausible that the observed variation is just undulations around a relatively steady state. Unlike lymphocyte counts, however, a statistically significant increase of 3,072 proviral copies per 100,000 cells was observed per each six-month observation period in the LMM. Furthermore, addition of sampling number to represent time in the statistical model accounted for 19% of within-cow variability. The current thought is that BLV persists through the mitotic replication of infected cells. Our observation of a non-significant increase in lymphocytes coupled with small, yet significant, increases in PVL either support the idea that infected cells survive longer and accumulate more than non-infected cells (Florins et al., 2008) or suggest new cells are becoming infected, which may occur during periods of viral reactivation. While significant increases in PVL over time were observed, the differences between the first and last observation for cows with 3 or more observations was less than 15,000 proviral copies per 100,000 cells for over 75% of BLV test-positive cows. Given the sampling scheme, 3 or more observations corresponds to a 1.5-year time period. This indicates large increases in PVL do not occur for the majority of infected cattle.,

Collectively, our observations indicate that while variations are observed, the majority of BLV test-positive cows experience minor increases in lymphocyte counts and proviral load over time. This suggests a relatively steady infection state may exist. Once cattle are identified to be infected and the lymphocyte and proviral load are quantified, repeated testing may not be necessary. At a minimum, the frequency of testing could be reduced. For ongoing control programs, this may reduce the costs associated with the disease control programs and may incentivize more producers to undertake control efforts. While random intercepts and slopes were included in models of lymphocytes and proviral load, linear mixed models borrow analytical power collectively from all observations and therefore, do not perfectly fit the

observed measurements. Thus, it is possible that while on average cows experience negligible to minor increases in LC and PVL with time, there is a subset of cows which consistently increase. While reducing sampling frequency could fail to identify these cattle, a balance must be identified that allows for cost-effective, broad implementation of BLV control programs. Future studies should compare the effectiveness of intervention programs with varying sampling frequency and the ability to significantly reduce both the herd BLV prevalence and incidence.

Conclusions

In this analysis, we identified a small portion of cattle with changes in their ELISA test result status over 6-month sampling periods. These cattle tended to have low proviral loads and normal lymphocyte counts, and therefore do not likely pose a direct threat to current control programs aimed at removing those cattle with high infectious potential. Examination of the longitudinal changes in both LC and PVL identified the occurrence of variation over time but indicated that the majority of animals do not experience large changes indicative of gradual disease progression.

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APPENDIX

Appendix
Supplemental Figures

Table S3.5
ELISA pattern categories and the observed ELISA test patterns and their absolute frequencies

Negative	N
Neg-Neg	80
Neg-Neg-Neg	56
Neg-Neg-Neg-Neg	55
Neg-Neg-Neg-Neg-Neg	45
Total	**236**

Suspect	N
Susp-Susp	3
Susp-Susp-Susp	1
Susp-Susp-Susp-Susp-Susp	1
Total	**5**

Positive	N
Pos-Pos	53
Pos-Pos-Pos-Pos	43
Pos-Pos-Pos-Pos-Pos	17
Total	**167**

Suspect/Positive	N
Susp-Pos	9
Pos-Susp	2
Susp-Pos-Pos	8
Susp-Pos-Susp	3
Pos-Susp-Pos	1
Pos-Pos-Susp	2
Pos-Susp-Susp	3
Susp-Pos-Pos-Pos	3
Susp-Susp-Pos-Pos	1
Pos-Susp-Pos-Pos	3
Pos-Pos-Susp-Pos	2
Pos-Susp-Susp-Susp	1
Susp-Pos-Susp-Pos	3
Susp-Pos-Pos-Pos-Pos	7
Susp-Susp-Pos-Pos-Pos	1
Susp-Pos-Susp-Susp-Susp	1
Susp-Pos-Susp-Pos-Pos	1
Susp-Pos-Susp-Pos-Susp	1
Susp-Pos-Susp-Susp-Pos	1
Pos-Pos-Pos-Susp-Susp	1
Pos-Pos-Susp-Pos-Pos	1
Pos-Susp-Pos-Pos-Susp	1
Pos-Susp-Susp-Pos-Susp	1
Total	**58**

Negative/Suspect	N
Neg-Susp	3
Susp-Neg	4
Neg-Neg-Susp	1
Neg-Susp-Susp	1
Susp-Neg-Neg	2
Susp-Susp-Neg	2
Neg-Neg-Neg-Susp	1
Neg-Neg-Susp-Neg	2
Neg-Susp-Neg-Neg	1
Susp-Neg-Susp-Susp	1
Susp-Susp-Neg-Susp	1
Susp-Neg-Neg-Neg	2
Susp-Neg-Neg-Susp	1
Neg-Susp-Susp-Susp-Susp	1
Neg-Susp-Neg-Neg-Neg	2
Neg-Neg-Susp-Neg-Neg	1
Neg-Susp-Neg-Susp-Neg	1
Neg-Susp-Susp-Neg-Susp	1
Susp-Neg-Susp-Neg-Neg	1
Susp-Susp-Susp-Neg-Neg	2
Total	**32**

Negative/Positive	N
Neg-Pos	5
Pos-Neg	2
Neg-Neg-Pos	3
Neg-Pos-Pos	1
Neg-Pos-Neg	1
Pos-Neg-Neg	1
Pos-Pos-Neg	1
Neg-Neg-Neg-Pos	1
Neg-Neg-Pos-Pos	1
Neg-Pos-Pos-Pos	4
Neg-Neg-Pos-Neg	1
Neg-Neg-Neg-Neg-Pos	2
Neg-Neg-Neg-Pos-Pos	2
Neg-Neg-Pos-Pos-Pos	2
Neg-Pos-Neg-Neg-Neg	1
Total	**28**

Negative/Suspect/Positive	N
Neg-Susp-Pos	1
Neg-Susp-Pos-Pos	1
Neg-Neg-Pos-Susp	2
Neg-Pos-Susp-Susp	1
Susp-Neg-Neg-Pos	1
Susp-Neg-Pos-Susp	1
Pos-Neg-Susp-Susp	1
Neg-Pos-Neg-Neg-Susp	1
Neg-Susp-Neg-Susp-Pos	1
Pos-Susp-Neg-Susp-Susp	1
Total	**11**

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Chapter 4 :

Bovine Leukemia Virus detection and dynamics following experimental inoculation

This chapter represents a manuscript submitted for review by the journal *Research in Veterinary Science*

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Abstract

Bovine leukemia virus (BLV) infects more than 40% of the United States cattle population and impacts animal health and production. Control programs aiming to reduce disease prevalence and incidence depend on the ability to detect the BLV provirus, anti-BLV antibodies, and differences in blood lymphocyte counts following infection. These disease parameters also can be indicative of long-term disease progression. The objectives of this study were to determine the timing and to describe early fluctuations of BLV-detection by qPCR, ELISA, and lymphocyte counts. Fifteen Holstein steers were experimentally inoculated with 100 µL of a blood saline inoculum. Three steers served as in-pen negative controls and were housed with the experimentally infected steers to observe the potential for contract transmission. Five additional negative controls were housed separately. Steers were followed for 147 days post-inoculation (DPI). Infections were detected in experimentally infected steers by qPCR and ELISA an average of 24- and 36 DPI, respectively. Significant differences in lymphocyte counts between experimentally infected and control steers were observed from 30 to 45 DPI. Furthermore, a wide variation in peak proviral load and establishment was observed between experimentally infected steers. The results of this study can be used to inform control programs focused on the detection and removal of infectious cattle.

Keywords:

Bovine leukemia virus; epidemiology; disease detection; viral dynamics

Introduction

The United States cattle population is endemically infected with bovine leukemia virus (BLV), a deltaretrovirus that may lead to the development of lymphocytosis and lymphoma. The last NAHMS estimates indicated 84% of dairy operations and more than 40% of dairy cattle were infected with BLV (USDA, 2008, 1997). A more recent prevalence estimate from a nationwide cross-sectional convenience sample suggest the disease burden of BLV continues to increase as 95% of herds and 46% of cows were infected (Ladronka et al., 2018). Infection with BLV has negative impacts on animal health, herd profitability, and may impact human health. Infected cattle have dysregulated immune systems (Frie and Coussens, 2015) which manifests through impaired response to vaccination (Frie et al., 2016) and potentially increased susceptibility to infectious diseases (Emanuelson et al., 1992; Watanabe et al., 2019). Dairy producers endure negative economic consequences from decreased milk production (Norby et al., 2016; Yang et al., 2016), shortened cow-longevity (Bartlett et al., 2013; Nekouei et al., 2016), and carcass condemnation (Haredasht et al., 2018; White and Moore, 2009). Humans were reported to harbor the BLV provirus which was argued to be associated with the development of breast cancer (Buehring et al., 2019, 2014, 2003).

Bovine leukemia virus has been eradicated in more than 21 countries around the world, primarily by culling or segregating all ELISA-positive cattle (European Commission, 2016; System, 2016). In the United States, this approach is rarely economically feasible given the high within-herd BLV prevalence (Norby et al., 2016). Research from our team and others has focused on identifying BLV intervention programs that may be implemented by producers to reduce herd prevalence, incidence, and disease burden (Ruggiero et al., 2019, 2018). Initial disease control programs utilized lymphocyte counts to identify animals with persistent

lymphocytosis, a hallmark sign of BLV infection. However, it is now known that in current production systems only 30-40% of infected animals develop persistent lymphocytosis (EFSA Panel on Animal Health and Welfare, 2015). The remaining 60-70% of infected animals stay aleukemic and, hence, may go undetected without the use of either antibody-based assays, such as ELISA and AGID, or detection of the BLV provirus through PCR.

Discrepancies in ELISA- and qPCR-test results were observed in field trials. One potential explanation is the occurrence of new infections, as cows were reported to test positive by PCR prior to testing positive by ELISA (Nagy et al., 2007). The analytical sensitivities of PCR assays also may have hindered the ability to detect infections. For example, a recent publication that compared the performance of six different qPCR assays demonstrated a wide discordance in the ability to detect and quantify BLV infections (Jaworski et al., 2018). Additionally, ELISA assays were shown to vary in their sensitivity and specificity (Kuczewski et al., 2018). The first objective of this study was to determine the timing from exposure to the detection of new BLV infections and to determine the sequence of detection of BLV proviral DNA, anti-BLV antibodies, and changes in lymphocyte counts following new infections. A second objective was to examine the changes in proviral and lymphocyte dynamics following BLV infection to examine how early infection may play a role in disease establishment. Results of this study can be used to inform sampling timelines and protocols for BLV control programs.

Materials and Methods

Study Design:

Twenty-three Holstein dairy steers were purchased in December 2018 from a Michigan livestock auction at approximately nine months of age (weight range: 283 to 369 kg). The BLV infection status of the herd of origin was unknown. However, steers were determined to be BLV-

negative through testing for the BLV provirus and anti-BLV antibodies using qPCR and ELISA, respectively, three times prior to study onset on days -82, -53, -21. The experimental portion of the study began in March 2018 and continued until August 2018.

At the start of the trial (day zero), steers were randomly assigned to one of four study pens; three pens were designated as intervention pens and housed six steers each while the fourth pen served as a negative control (NC) pen and housed five steers. The animals in the NC pen were separated from the animals in the intervention pens by one empty pen. Within each of the three intervention pens, five of the six steers were intramuscularly injected with 100 μL of an infectious blood-saline inoculum. The sixth steer in each intervention pen served as a sentinel, in-pen negative control (IPNC) to examine the potential for direct contact transmission from newly infected to susceptible steers. The three IPNC and five NC steers were intramuscularly injected with 100 μL of a control blood-saline inoculum.

The infectious inoculum was an aggregate of pooled blood collected in K_2EDTA-tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) from three BLV-positive cows diluted with sterile, physiological saline to create a volume with a low concentration of white blood cells. The pooled blood had a total leukocyte count of 13,400 per μL and a PVL of 0.2809 copies per cell. The final infectious inoculum contained 8,300 leukocytes and 2,300 proviral copies, or the equivalent of 0.62 μL of blood per 100 μL of inoculum. The negative control inoculum was made by diluting blood from a known ELISA-negative cow with sterile, physiological saline. The negative-donor cow was enrolled in an ongoing BLV control trial and had two-years of biannual negative ELISA results. Furthermore, this cow was tested by qPCR and ELISA the week prior to and the day of study onset.

A sample size calculation was performed in Stata 15 (StataCorp LLC, College Station, TX) using a two-sample proportions likelihood ratio test assuming a power of 80%, significance of 5%, ratio of 3 to 1 for BLV exposed versus unexposed cattle, and infection rates of 80% among exposed and 10% among unexposed. The estimated sample size was 15 exposed and 5 unexposed. An additional three steers were added as IPNC as a means of examining the potential for direct contact transmission. This study was approved by the Michigan State University Institutional Animal Use and Care Committee (AUF: 09/17-152-00). All steers were housed at the Michigan State University, Beef Cattle Teaching & Research Center.

Sampling Scheme & Processing:

Blood samples were collected every three days for 45 days post infection (DPI), followed by every six days until 99 DPI, and then every twelve days until study completion at 147 DPI when the steers reached market weight (mean: 583 kg). Blood samples were collected using jugular or caudal tail venipuncture and K_2EDTA and serum vacutainer collection tubes (Becton, Dickinson and company, Franklin Lakes, NJ). New hypodermic needles were used for every steer at each sampling. Body weight was measured using the chute scale and rectal temperature was obtained using a digital thermometer. The NC steers were sampled prior to and kept out of contact with infection steers during sampling to prevent inadvertent virus transmission. Collected blood samples were placed on ice until they were transported to the university for sample processing. Plasma and serum were rendered from EDTA and serum tubes, respectively, by centrifugation at 1,500 x g for 15 min. Whole blood and serum samples were aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C until further analysis.

Sample Analysis:

Leukocyte differential blood counts were performed using Advanced Animal Diagnostics QScout® blood leukocyte differential machine (Morrisville, NC; Software version: 1.0.6.23009). Briefly, 10 μL of fresh blood collected in K2-EDTA tubes was aliquoted onto QScout® cassettes which were loaded into the machine for analysis. All leukocyte differentials were performed within four hours of sample collection.

DNA was extracted from frozen whole blood by using a Wizard Genomic DNA extraction kit (Promega Cooperation, Madison, WI) and following the manufacturer's protocol with a slight modification to repeat the cell lysis step. DNA samples were normalized to 30 ng/μL. Proviral load was quantified using the SS1 qPCR assay developed by CentralStar Cooperative (East Lansing, Michigan). Each qPCR reaction contained 3 μL of normalized DNA, 12.5 μL of 2X Primetime Gene Expression Master Mix (Integrated DNA Technologies, Coralville, Iowa), 1.25 μL of a 20X primer mix, 1 μL of an internal spike-in control developed by CentralStar, and 7.25 μL of DNA-free water. The primer mix contained proprietary primers and probes that target the BLV proviral polymerase gene, the endogenous *Bos Taurus* Beta actin gene (Bos Actin), and the spike-in internal amplification control ultramer. All qPCR was performed in duplicate using an Applied Biosystems 7500 Fast Real-Time PCR system (Foster City, CA) with qPCR conditions as follows: 95°C for 10 min, 40x (95°C for 15 sec, 60°C for 1 min), and then 60°C for 1 min. Total BLV and Bos Actin copy numbers were quantified via standard curve using linearized plasmid templates quantified and normalized by digital droplet PCR. Proviral load was calculated as the ratio between proviral BLV copies and Bos Actin copies and was expressed as copies per cell. To reduce the potential effect of inter-plate variability on describing intra-steer viral dynamics, an effort was made to analyze all of an

experimentally infected steer's samples on the same qPCR plate; this was accomplished for approximately 92% of samples.

Serum samples were submitted to CentralStar Cooperative for detection of anti-BLV antibodies using a modified IDEXX gp51 antibody capture ELISA (Westbrook, Maine). Serum BLV-ELISA results were reported back as corrected optical densities (OD; raw sample OD minus negative control OD) and interpreted as negative (OD: < 0.5), suspect (OD: 0.5 to 1.0), or positive (OD: >1.0). Serum samples collected prior to 21 DPI were not submitted for analysis because ELISA suspect or positive results were not observed until after 24 DPI.

Statistical Analysis:

SAS 9.4 software (SAS Institute Inc., Cary, NC) was used for data management and linear mixed model (LMM) analyses. Descriptive statistics and graphs were produced in Stata 15 (StataCorp LLC, College Station, TX).

Separate LMMs with repeated measures and random intercepts for individual steers were used to determine differences in lymphocyte counts and body temperature between experimentally infected and all negative control steers at each time point. A linear growth model that included time as a continuous variable with random intercepts for individual steers was created to examine the difference in average daily gain (ADG) between infected and control animals. Individual LMMs, which included only experimentally infected steers that tested positive for PVL, were created to model the association between changes in lymphocyte per μL (LC) and PVL overall and during the early (0 to 45 DPI) , middle (45 to 99 DPI), and late (99 to 147 DPI) sampling periods, respectively. All models contained infection status, DPI, and their interaction. Repeated measures and non-equidistant sampling intervals were accounted for using a spatial power covariance structure.

Results

Graphical representation of individual steers' LC, PVL, and ELISA measurements are presented in **Figure 4.1**and provided numerically in the supplemental data. Steer #542, an in-pen negative control, was removed from the study after 51 DPI.

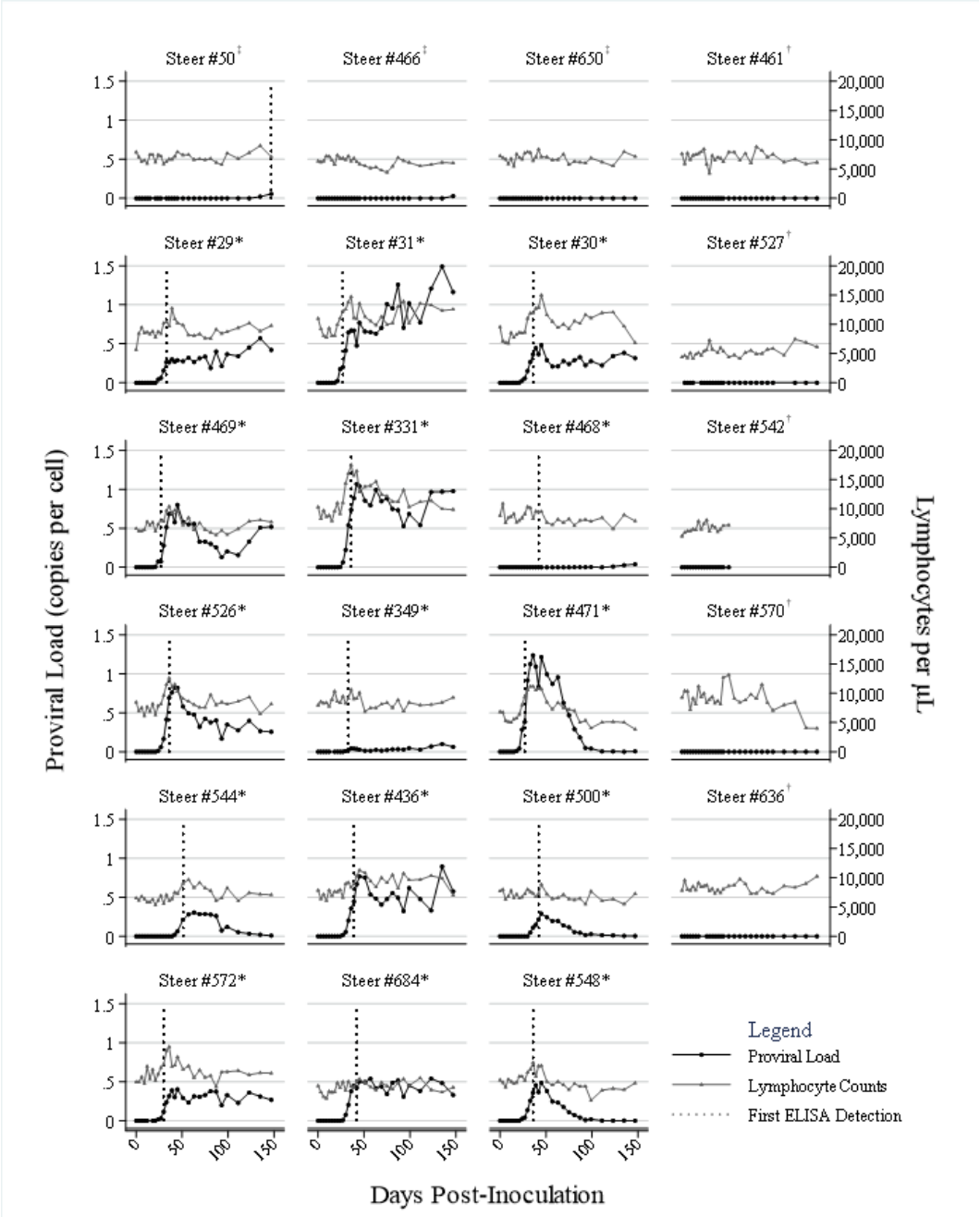

Figure 4.1

Proviral load, lymphocyte counts, and ELISA first detection overtime by individual study steers:

Each graph represents the viral dynamics for one steer. Each column represents a pen of steers. Pens one to three each housed five steers experimentally infected (*) with bovine leukemia virus and one in-pen negative control (‡). Pen four contained negative control steers (†) kept out of contact with experimentally infected steers. The black line represents proviral load (expressed as the ratio of proviral copies per copy of Bos Actin). The grey line represents lymphocyte counts. The vertical dashed line represents the first date at which anti-BLV antibodies were detected. Steer #542 was removed from the study at 51 DPI.

Lymphocytes per μL overtime ranged from 3,500 to 17,500 in experimentally infected steers and 4,000 to 13,100 in control steers. The average peak lymphocyte counts observed in infected and control steers were 11,700 per μL (range: 7,300 to 17,500) and 9,100 per μL (range: 7,400 to 13,100), respectively. The average lymphocyte counts per μL at each DPI were numerically higher among the experimentally infected steers than the control steers. Periods of lymphocytosis (>8,000 lymphocytes per μL) were observed in 93% (14/15) of infected steers. Lymphocyte counts greater than 10,000 lymphocytes per μL were observed in 73% (11/15) of steers. There were significantly higher LC marginal means from 30-45 DPI, at 93 DPI, and at 111 DPI among infected steers when compared to controls **(Figure 4.2)**. Extended periods of lymphocytosis, >8,000 lymphocytes per μL after 45 DPI, were observed in 80% (12/15) of steers. Persistent lymphocytosis, subjectively defined as more than 75% of lymphocyte observations after 45 DPI above 8,000 lymphocytes per μl, was observed for 40% (6/15) of infected steers (#29, #30, #31, #331, #436, #526). This definition was chosen based on the observation of non-significant marginal differences in lymphocyte counts after 45 DPI.

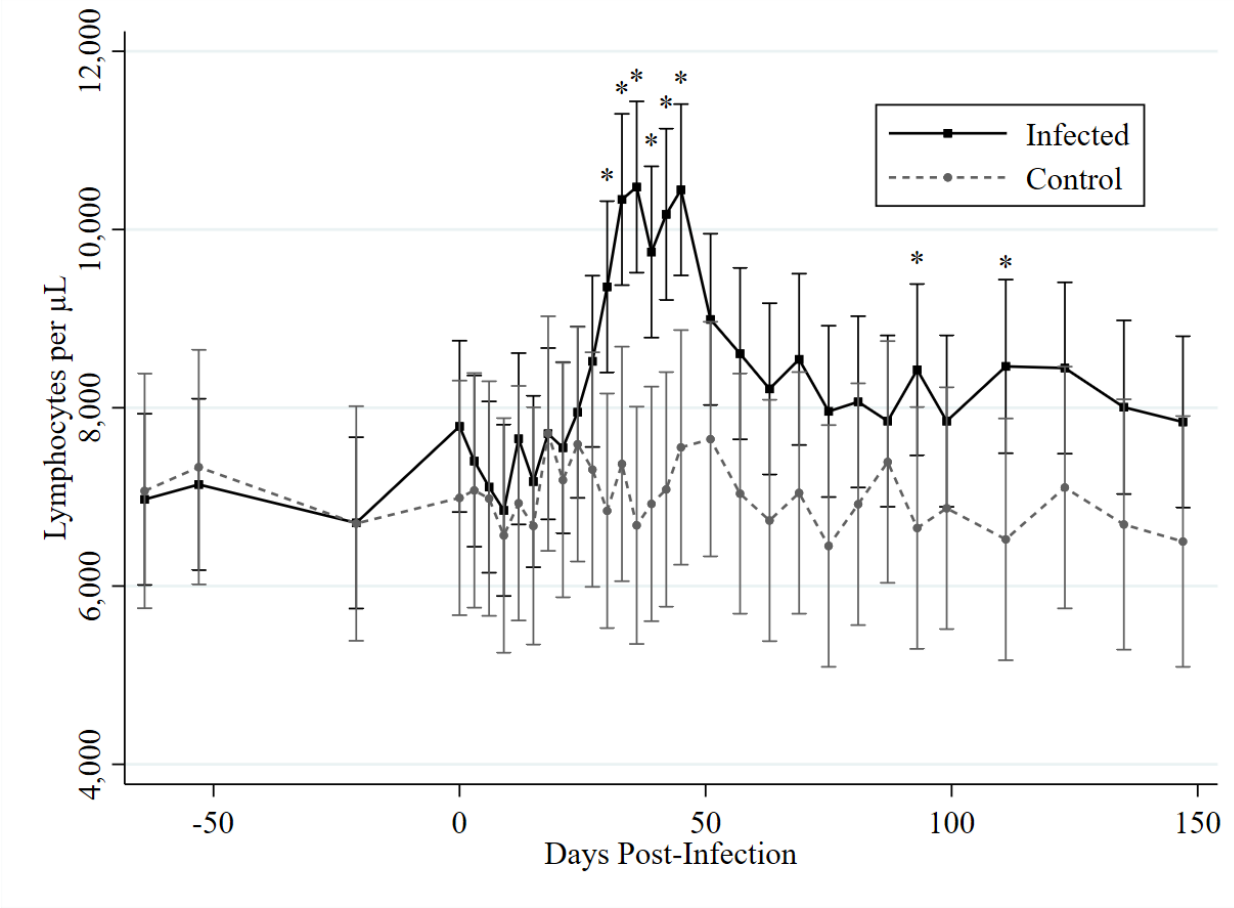

Figure 4.2
Marginal lymphocyte dynamics in both experimentally infected and control animals
Steers were experimentally inoculated with a blood-saline solution on day 0. The experimentally infected steers (n=15) received a solution containing blood from a BLV-infected donor while control steers (n=8) received a solution containing blood from BLV-negative donor. Lymphocytes were measured every three days for 45 days post-inoculation (DPI), followed by every six days until 99 DPI, and then every twelve days until study completion at 147 DPI. Lymphocyte counts were statistically compared using a linear mixed model accounting for repeated measures and including random intercepts for each steer. Results of marginal means ± 95% CI are depicted. Significant differences, indicated by *, were identified from 30-45 DPI, at 93 DPI, and at 111 DPI.

The median number of days until detection of BLV proviral DNA by qPCR in experimentally infected steers was 24 DPI (Range: 18 to 42 DPI). The PVL at first detection ranged from 0.0002 to 0.0475 copies per cell (median= 0.0029 copies per cell). The peak proviral load observed in each steer ranged from 0.0383 to 1.4910 copies per cell (median=0.5412 copies per cell). In 60% (9/15) of infected steers, the maximum PVL was

observed between 36 to 63 DPI (median= 45 DPI), shortly after (range: 12 to 24 d) first detection of proviral DNA, and was followed by a decline and/or plateau to a steady state at which proviral copies appear to undulate. In four of the steers (#471, #500, #544, #548) the decline resulted in a low PVL (< 0.1 copies per cell). In contrast, 40% of steers (6/15) had peak proviral loads between 123 and 147 DPI. In two of the steers (#29, #31) this peak was the result of a gradual increase in PVL observed after first detection. In three of the steers (#684, #349, #436), this peak appears to be relative to a plateau in PVL established shortly after detection. One experimentally infected steer (#468) maintained a low PVL which was first detected in one of the PCR duplicates at 42 DPI. This steer subsequently tested negative by qPCR until 81 DPI and later had a peak PVL of 0.0383 copies per cell observed at 147 DPI. Proviral DNA was detected by qPCR in two of three (#50, #466) IPNC steers at 135 and 147 DPI, respectively. The BLV provirus was not detected in any of the NC steers.

Changes in PVL were positively associated with changes in lymphocyte counts ($p<0.001$); every 1,000 increase in lymphocytes was associated with an increase of 28.02 proviral copies per 1,000 cells. When the relationship was examined by study period, the association between PVL and lymphocyte counts declined with increases in the length of infection. A 1,000 lymphocytes per µL increase was associated with an average increase of 45.45 proviral copies/1,000 cells ($p<0.001$) in the early period (0 to 45 DPI), increase of 12.37 proviral copies/1,000 cells ($p=0.156$) in the middle period (45 to 99 DPI), and an increase of 12.97 proviral copies/1,000 cells ($p=0.427$) in the late infection period (99 to 147 DPI).

Eighty percent (12/15) of the infected steers had one or more sampling points at which they tested ELISA suspect ($0.5<OD<1.0$) prior to testing positive by ELISA ($OD > 1.0$) for anti-BLV antibodies. The median DPI until BLV-suspect and BLV-positive results by antibody

capture ELISA were 36 DPI (range: 27 to 51 DPI) and 42 DPI (range: 27 to 57 DPI), respectively. One steer (#469) tested BLV-suspect at 27 DPI, negative until 36 DPI where it tested BLV-suspect, and then BLV-positive starting at 42 DPI. The median number of days from first detection of BLV provirus by qPCR to first detection of anti-BLV antibodies by ELISA (suspect or positive) was 12 d (range: 0 to 15 d). Once a steer tested BLV-ELISA positive it remained ELISA-positive at subsequent sampling points; exception: steer #349 tested BLV-ELISA suspect at 123 DPI. One IPNC steer (#50) tested BLV-suspect by ELISA at 147 DPI.

For experimentally infected steers in general, first detection by ELISA happened shortly after first detection of PVL and around the time of peak LC (Fig 1). The median number of days from ELISA first detection to peak PVL was 9 d (range: 3 to 18 d) in the nine steers that experienced max PVL peaks shortly after infection; in all infected steers the median was 15 d (range: 3 to 108 d). The median number of days from first detection by ELISA to individual peak lymphocytes counts was 6 d (range: 0 to 9 d) with two exceptions; one steer (#468) had a peak lymphocyte count 3 DPI and another steer (#684) had minor changes in lymphocyte count over the observed period with the highest LC observed at 111 DPI. This resulted in a difference of -39 d and 69 d between ELISA first detection and peak LC, respectively.

The median ADG for infected animals was 0.98 kg/day (range: 0.31 to 1.25 kg/day) and 0.85 kg/day (range: 0.31 to 1.36 kg/day) for control steers, respectively. The linear growth model for ADG, that treated time as a continuous variable and controlled for infection status and the interaction with time, identified a statistically significant ($p=0.020$) difference in the marginal ADG. Infected animals had a marginal gain of 1.02 kg per day while control animals had a marginal gain of 0.94 kg per day (**Figure S4.3**). The average body temperatures for infected steers was 38.7°C (range: 37.8 to 40.6 °C) and was 38.6°C (range: 37.1 to 40.4 °C) for control

steers. The average body temperature was significantly higher among infected steers when compared to control steers at 3, 21, 36 and 69 DPI.

Discussion

The first objective of this study was to determine the time from infection with BLV until the detection of the BLV provirus by qPCR, anti-BLV antibodies by ELISA, and deviations in lymphocyte counts. The BLV-provirus was first detected in experimentally infected steers between 18 and 42 DPI. These results are similar to, if not slightly later than, those reported by other studies (Forletti et al., 2020; Jimba et al., 2012; Klintevall et al., 1994; Nagy et al., 2007). The timing differences for first detection between this study and others may partially be attributed to the number of infected cells in the inoculum. Klintevall *et al*. (1994) reported that first detection by PCR occurred between 7 to 18 DPI in calves inoculated with 1 mL of blood containing 4 million lymphocytes but detection was delayed to 18, 21, and 56 DPI in three of six calves inoculated with approximately 40,000 lymphocytes. In this study, the inoculum contained the equivalent of 0.62 μL of blood with 8,300 leukocytes and 2,300 proviral copies Detection of BLV proviral load may be dependent on the analytical sensitivity of qPCR assays. The observed proviral load for steer #468 suggests the analytical sensitivity of the SS1 assay may not detect the presence of the provirus when PVL is low. While few samples with a lower detected PVL are observed, examination of the proviral load observations suggests the experiment-derived, analytical sensitivity of the SS1 assay is approximately 0.001 copies per cell, or 1 copy per 1,000 cells. This is based off the values associated with first PVL detection and the values observed in steers which maintained or developed low proviral loads. The lowest observed proviral load, however, was .0002 copies per cell, or 1 copy per 5,000 cells. Nonetheless, the presence of ELISA-positive and PCR negative cows have been observed in cross-sectional studies (Jimba et

al. 2012). Apart from analytical sensitivities, this may be a result of viral replication being restricted to an undetectable level by the host immune response or of the virus being sequestered in an organ and not circulating in the peripheral blood (Klintevall et al., 1994).

The first observed detection by ELISA was similar to previously reported experimental infection studies. Survival analysis on aggregated data from 36 studies found a median of 51 DPI to detection by ELISA (Monti 2005). Detection by ELISA previously was reported to follow first detection by PCR (Kelly et al., 1993; Klintevall et al., 1994) but to proceed peaks in PVL (Gillet et al., 2013). Indeed, these same observations were made in this study.

The observed rise in lymphocytes resulting in transient lymphocytosis post-infection previously was reported and attributed to expansion of the $CD5^+$ B-cell population (Klintevall et al., 1997; Ungar-Waron et al., 1999). Statistically significant differences in lymphocyte counts between the experimentally infected and the control animals were observed at eight timepoints. Inter-steer variations in lymphocyte counts resulted in wide standard errors, which likely contributed to the inability to detect significant difference in lymphocyte counts between experimentally infected and control animals at more timepoints. Regardless, the wide variation in lymphocyte counts observed between individual steers and the few statistically significant differences in lymphocyte counts between infection groups limits the ability to use lymphocyte counts as an indicator of infection status.

The algorithm version implemented by the QScout in this study (software version: 1.0.6.23009) groups the lymphocytes and monocytes into one sub-population. An analysis conducted by our team determined the QScout® tends to slightly overestimate this combined population by approximately 600 cells/μL (Hutchinson *et al*., manuscript in preparation). This difference does not impact the relative interpretation of lymphocyte counts within and between

steers over time enrolled in this study but should be taken into consideration when comparing results to other studies. The normal reference interval for cattle lymphocyte provided by the MSU diagnostic laboratory is 2,300-7,400 lymphocytes per μL. To account for the overestimation, lymphocytosis was defined as greater than 8,000 lymphocytes per μL. Forty percent of experimentally infected steers developed persistent lymphocytosis. This aligns with the current disease dogma that suggests approximately 30-40% of infected cattle will develop persistent lymphocytosis. While the follow-up time in this study was limited, these results suggest the development of persistent lymphocytosis may occur shortly after initial infection and is not necessarily the result of a slow, gradual increase following infection.

A 'flu-like' syndrome has been reported to occur following infection with BLV (EFSA Panel on Animal Health and Welfare, 2015). This was not reflected in the body temperature of steers following infection. Surprisingly, a significant difference was observed in the ADG with BLV infected steers having a higher average daily gain. Weight measurements were a secondary measure in this study and therefore, potential confounders were not controlled for in the study design. Plausibly, there could have been a difference in the amount of feed received as a result of either the housing density or structure. The experimental pens had one additional steer and were housed adjacently. The control pen was separated from the other pens. Additionally, one steer was removed from the control pen leaving four steers, compared to six steers housed in the other pens.

Recent reports have suggested lymphocyte count and proviral load are associated with a cow's infectious potential (i.e. the ability to transmit the virus to their negative herd mates) (Juliarena et al., 2016; Mekata et al., 2018; Ruggiero et al., 2019). Therefore, a second objective of our study was to examine the role early infection plays in the establishment of proviral load

and lymphocyte count. In this study, experimentally infected steers were given the same infectious inoculum and exhibited wide variations in lymphocyte and proviral dynamics. This indicates that individual animal responses to an infection are highly variable.

The establishment of PVL following infection was reported by three independent studies. In the first study (Jimba et al., 2012), proviral kinetics were observed for ten weeks and exhibited different patterns in the two inoculated cattle; one animal experienced a gradual increase in PVL while the other maintained a low, detectable PVL. The authors attributed the observed differences to the cattle's different BoLA-DRB3 haplotypes. The second study (Gillet et al., 2013) investigated the proviral integration sites in five experimentally inoculated cows which were followed for 250 DPI. The peak in PVL was reported to have occurred between 30 and 68 DPI followed by a decline or plateau. However, PVL was not reported between approximately 80 DPI and 252 DPI. While the same overall trend was observed in the majority of our steers, our results show fluctuations in the PVL still occurred in at least the time period of 80 to 147 DPI.

The most recent study (Forletti et al., 2020) investigated early viral dynamics in calves with known BLV-susceptible, resistant, or neutral DRB3 haplotypes for 180 days following experimental inoculation (Forletti et al., 2020). In this study, peak PVL occurred at 30 DPI followed by a decline in PVL for calves with either the resistant DRB3*0902 or neutral DRB3 allele. Calves with the susceptible DRB3*1201 allele maintained high PVL. While the haplotypes of the steers in our study were unknown, PVL dynamics can be grouped into four general patterns: peak and plateau, peak and decline, peak and rise, and remain low. Interesting differences between the Forletti *et al*. (2020) study and this study were the timing and magnitude of the proviral peak. In Forletti *et al*, non-significant differences in peak PVL were observed

between haplogroups at 30 DPI. In our study, the extent of the proviral peak was highly variable. Again, these differences may partially be attributed to the dose of infected cells provided to establish infections. While uncertain, it is assumed that when an infected cell finds its way into a susceptible host, the cell initiates an infectious cycle which leads to the infection of host immune cells followed by the clonal expansion of infected cells. The sheer quantity of cells used for inoculation in the Forletti *et al* study may have allowed for a robust viral replication and infection of cells that could not be contained by the innate immune system. The considerably smaller amount of infected cells utilized for inoculation in our study may have been better contained by the host immune response.

Insights from the studies of other retroviral diseases can further explain the observed proviral dynamics. The use of differential equations and mathematical modeling for HTLV, another delta retrovirus, suggests that proviral kinetics following infection are largely dependent on the immune responsiveness to the virus, which may be determined by the major histocompatibility complex and the cytotoxic T-cell response (Wodarz et al., 1999). These studies suggest that proviral load reaches a set point at which it may stabilize or exhibit a cycling dynamic, dependent on the relationship between the rate of infectious versus mitotic transmission, immune responsiveness of the host, and the rate of target cell death (Nowak and Bangham, 1996). While a cycling dynamic in proviral load was observed for several of the steers, a relative viral set-point can be discerned. This suggests that early disease establishment may influence long-term disease status. However, to conclusively determine this, newly infected animals would have to be followed for the remainder of their natural life.

Peak proviral load was observed around the time of first detection of anti-BLV antibodies. One might attribute this to the host immune response successfully controlling the

infection. However, mathematical models (Phillips, 1996) and experimental infection studies (Schwartz et al., 2018) of other retroviral infections have reported similar observations in the absence of an adaptive immune response. This suggests that the observed peak and subsequent decline or plateau in PVL occurs independently from the adaptive immune response and may be explained by depletion of susceptible target cells. In addition, peak proviral load does not appear to be associated with the relative equilibrium state observed following the peak. For example, steer #471 had one of the highest peaks in proviral load post-infection and exhibited a decline to low or undetectable levels. Future studies could attempt to utilize differential equation modelling to predict viral dynamics among BLV-infected cattle.

The time period in which peaks in proviral load and periods of lymphocytosis were observed in our study could be periods of increased infection potential. Research on simian immunodeficiency virus, a member of the retroviral genus lentivirus, found that infected donors were more infectious during the "ramp-up" period following a new infection than donors which had established a viral set-point (Vaidya et al., 2010). Whether this holds true for delta-retroviruses would require further investigation, but a similar observation could be associated with the transition from the infectious to mitotic replication cycles (Gillet et al., 2013)

Taken together, the results of the timing of disease detection and early disease progression can be utilized for disease control programs. The World Organisation for Animal Health (OIE) recommends the use of antibody-based assays for the detection of BLV-infected animals. A median difference of 12 d (range: 0 to 15 d) between first detection of BLV proviral DNA by qPCR and anti-BLV antibodies by ELISA was observed. During this brief period, disease control programs relying on antibody-based assays may fail to detect a small proportion of BLV-infected animals. However, the observation of a subset of BLV infected animals testing

negative for the BLV-provirus following disease establishment indicates the need for the continued use of antibody-based assays. When considering sampling frequency for the identification of infected animals and the determination of an animal's infection potential, a sampling interval of approximately 60 d may be the most appropriate. This frequency would allow for the detection of either the BLV-provirus or anti-BLV antibodies in the majority of newly infected animals. In addition, following the identification of infected animals, this frequency would provide relative insight to the disease incidence and viral dynamics. The majority of animals appear to establish a relative stability in their proviral load within 60 d of first detection which may be indicative of their infection potential.

New BLV infections were detected among the sentinel IPNC steers in this study. These steers were included to examine the potential for direct contact transmission. Efforts were made to prevent iatrogenic transmission during sampling. Toward the end of the study, flies were observed to be within the feedlot and resting on the steers. Thus, the IPNC steer may have become infected through fly transmission. Nonetheless, infection of IPNC steers illustrates the ease at which BLV can be transmitted from infected to susceptible herd mates.

Limitations:

The results of this study may not reflect what would be observed following a natural infection. However, an effort was made to use a volume of inoculum that would be small enough to mimic natural transmission yet large enough to ensure infection among inoculated animals. Previous experimental infection studies have shown the dose of blood from infected cows required to induce infections is donor dependent and varies by lymphocyte counts and antigen status (Mammerickx et al., 1987; Miller et al., 1985). Variation in infection potential can likely be attributed to differences in PVL; however, this has not been experimentally investigated.

Since the exact determinants of a donor's infection potential is unknown, blood was pooled from three known BLV-positive cows to create an inoculum thought to be sufficient for inducing infection. Additionally, detection following intramuscular injection maybe different than other possible routes of transmission (e. g. subcutaneous or intravenous).

For logistical, economic, and disease control reasons, we chose to use Holstein dairy steers housed on the university feedlot. Disease detection and progression may vary in dairy cows which experience a different set of physiological demands and environmental conditions. Lastly, our ability to follow disease progression in the steers was limited to the time they were housed at the university feedlot. While we successfully detected infections in all experimentally infected steers, it would have been preferable to follow the steers for a longer period of time given the observed fluctuating viral dynamics and the detection of infection in negative control steers.

Conclusion

In this study, the first detection of BLV proviral DNA by the SS1 qPCR and anti-BLV antibodies by ELISA were approximately 24- and 36-days post-infection, respectively. While all infections were detected by PCR prior to or concomitantly with the detection by ELISA, the observance of steers testing ELISA positive and PCR negative supports the continued use of antibody-based assays for the detection of BLV infections. In this study, all infections were detected by either PCR or ELISA by 33 DPI and solely ELISA by 51 DPI. Recent control programs have focused on removing cattle with high proviral load and high lymphocyte counts, as they are thought to be the most infectious (Ruggiero et al., 2019). However, viral dynamics among infected steers were highly variable. Our results suggest initial peak in proviral load and lymphocytes may not be indicative of long-term disease states. However, our observations

suggest high PVL and PL disease states might be achieved shortly after infection, independent of initial peaks. Taken together, the results of this study may inform necessary sampling intervals for herds attempting to eradicate BLV. Our data suggests sampling intervals of approximately 60 d may be sufficient to identify new infections and to identify the relative disease establishment which may be indicative of infection potential. Future studies should focus on elucidating the factors that determine whether an infected animal experiences continual increases, a plateau, or decline in measurements of viral dynamics following infection.

Acknowledgements

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APPENDIX

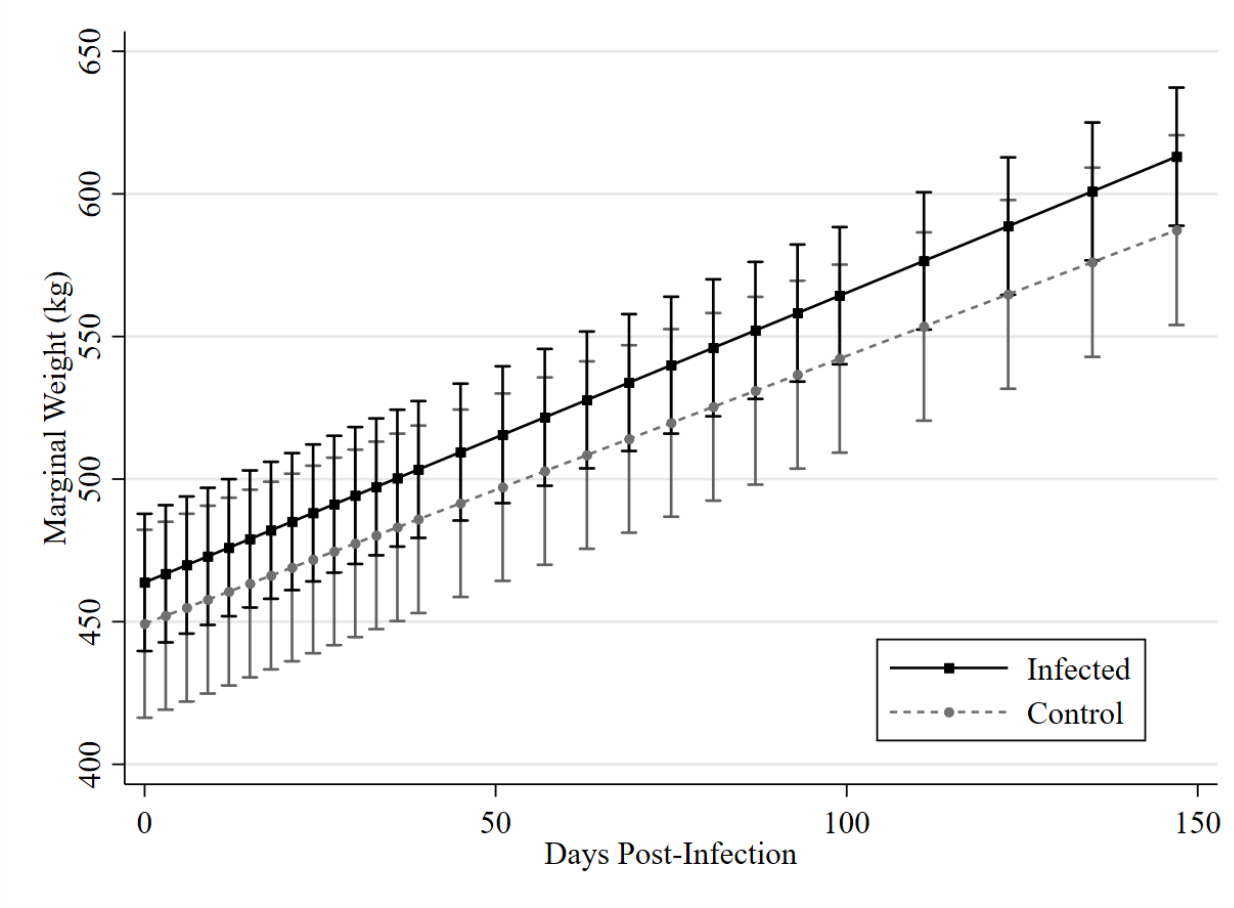

Figure S4.3

Marginal average daily gain in both experimentally infected and control animals

A linear growth model that included time as a continuous variable with random intercepts for individual steers was created to examine the difference in average daily gain (ADG) between infected and control animals. Infected animals had a marginal gain of 1.02 (95% CI: 0.98 to 1.05) kg per day while control animals had a marginal gain of 0.94 (95% CI: 0.89 to 0.99) kg per day (p=0.020)

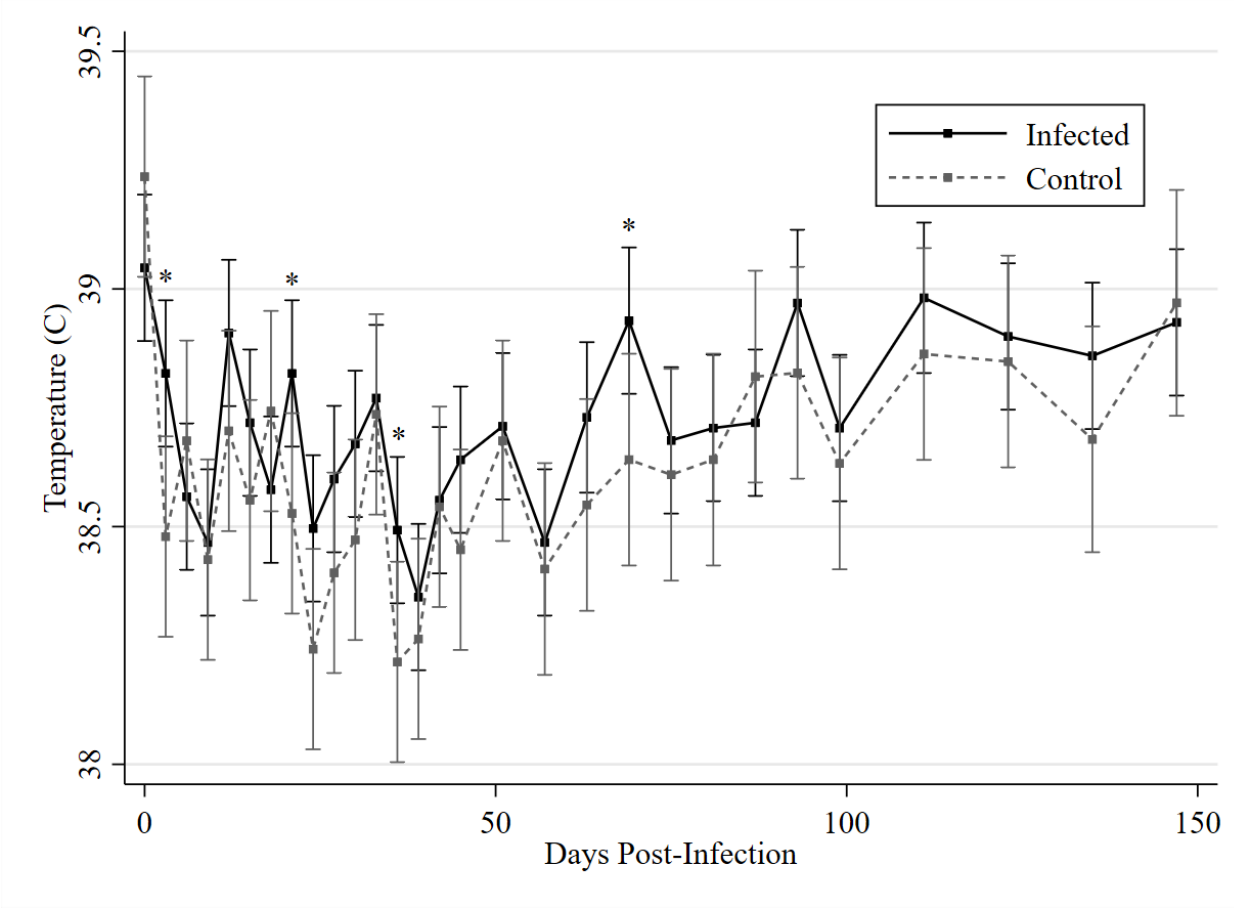

Figure S4.4
Marginal temperature in both experimentally infected and control animals

A linear mixed model with repeated measures and random intercepts for individual steers was used to examine differences in body temperature between experimentally infected and all negative control steers at each day post-inoculation. Significance at $p<0.05$ indicated by *

Table S4.1
Steer numbers and the associated infection status and pen number

Steer Number	Infection Status	Pen
50	IPNC	1
29	IN	1
469	IN	1
526	IN	1
544	IN	1
572	IN	1
466	IPNC	2
31	IN	2
331	IN	2
349	IN	2
436	IN	2
684	IN	2
650	IPNC	3
30	IN	3
468	IN	3
471	IN	3
500	IN	3
548	IN	3
461	NC	4
527	NC	4
542	NC	4
570	NC	4
636	NC	4

IPNC: In pen negative control; IN: Infected; NC: Negative Control

Table S4.2

Lymphocyte (per µL), proviral load (copies/1,000 cells), and ELISA results for experimentally infected steers by day

	Day	29	30	31	50	331	349	436	468	469	471	500	526	544	548	572	684
LC	0	5,659	9,541	10,988	7,892	10,313	7,966	7,853	8,833	6,614	6,833	7,733	8,470	6,531	6,958	6,618	5,965
PVL		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ELISA		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	3	8,412	7,112	9,240	7,038	8,300	8,569	6,406	10,867	6,190	6,742	7,977	6,982	6,153	6,542	6,645	4,896
		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	9,469	6,879	8,037	6,292	9,707	8,289	7,152	7,592	6,219	5,286	6,347	7,529	6,801	5,766	7,475	4,107
		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	9	8,531	6,754	7,782	6,465	8,635	8,377	6,072	8,462	6,419	5,137	6,801	6,115	6,391	7,056	6,379	3,829
		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	12	8,644	8,547	9,331	5,937	8,865	7,750	7,862	8,664	7,780	5,006	7,816	7,675	5,833	6,706	9,292	5,013
		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	15	8,380	7,748	8,003	7,442	7,929	8,932	6,918	9,133	7,214	5,584	6,554	6,893	5,872	6,405	7,166	4,855
		0	0	0	0	0		0	0	0	0	0	0	0	0		0
	18	8,789	8,264	8,082	7,425	9,610	10,351	7,622	7,701	7,668	5,702	7,167	8,178	6,273	6,532	8,734	4,969
		0	0	1.85		0	0	0	0	0	9.78	0	0	0	0	0	0
	21	7,840	8,599	9,957	6,174	11,002	8,624	7,619	7,974	6,118	6,401	6,514	6,301	5,365	7,573	6,911	6,475
		0	2.9	23.8	0.0	0.0	0.7	0.0	0.0	6.4	41.1	0.0	0.0	0.0	1.3	2.0	0.0
		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	24	8,724	8,484	11,031	7,415	9,117	8,391	7,954	8,445	7,354	7,982	6,661	7,856	6,131	7,573	7,957	5,597
		42.4	25.68	175.23	0	2.86	0	0.67	0	72.89	284.14	0	11.81	0	24.41	17.19	0
		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	27	8,386	8,624	12,228	7,193	11,050	9,466	6,615	9,111	8,021	9,411	7,248	8,135	6,917	6,900	9,074	6,635
		61.5	57.8	200.5	0.0	63.0	0.0	15.8	0.0	73.2	382.1	0.0	59.0	0.0	41.9	30.1	7.8
		N	N	S	N	N	N	N	N	S	P	N	N	N	N	N	
	30	10,190	11,007	12,550	5,879	14,369	8,293	8,964	10,337	7,876	10,561	8,019	9,607	5,537	8,233	9,616	5,190
		155.7	151.1	413.8		222.6	9.1	54.2	0.0	279.0	916.4	0.0	163.8	0.0	136.6	115.9	76.0
		N	N	P	N	N	N	N	N	N	P	N	N	N	N	S	N

Table S4.2 (cont'd)

	Day	29	30	31	50	331	349	436	468	469	471	500	526	544	548	572	684
LC PVL ELISA	33	10,407 265.5 S	11,911 265.9 N	13,815 645.4 P	6,279 0.0 N	16,015 538.4 N	9,144 24.4 S	9,172 201.2 N	10,151 0.0 N	9,663 513.8 N	11,167 1124.5 P	7,620 47.5 N	11,482 412.6 N	7,053 0.0 N	8,770 256.4 N	11,984 237.9 P	6,702 204.0 N
	36	9,681 262.3 P	11,979 364.2 S	14,712 671.0 P	6,687 0.0 N	17,501 736.3 P	10,626 41.6 P	8,360 357.0 N	8,281 0.0 N	10,396 682.9 S	11,164 1236.9 P	7,327 111.0 N	12,591 690.9 P	6,098 0.0 N	9,924 359.0 S	12,633 316.1 P	5,874 377.6 N
	39	12,718 299.7 P	12,701 444.9 S	11,059 670.0 P	6,645 0.0 N	15,671 886.1 P	9,053 38.9 P	8,926 444.7 S	9,569 N	9,210 690.6 S	10,515 1092.3 P	6,746 147.1 N	11,065 759.5 P	6,405 0.2 N	7,544 454.6 S	9,285 389.6 P	5,754 449.1 N
	42	10,912 271.4 P	12,885 365.3 P	10,989 476.5 P	7,171 0.0 N	16,461 1068.7 P	9,066 34.2 P	10,327 668.0 P	9,444 0.0 S	9,746 576.3 P	10,779 834.3 P	7,284 215.3 S	11,528 823.4 P	7,497 20.2 N	9,361 366.7 P	9,465 301.0 P	6,807 421.3 S
	45	10,247 289.4 P	14,988 483.4 P	13,547 768.9 P	7,922 0.0 N	12,897 1040.3 P	10,105 27.0 P	11,282 766.6 P	9,532 0.0 S	9,508 799.5 P	10,740 1215.3 P	8,842 290.0 S	10,322 820.4 P	7,270 61.9 N	9,396 486.1 P	10,915 402.9 P	7,099 500.8 S
	51	9,853 276.1 P	11,590 290.8 P	11,298 657.9 P	7,395 0.0 N	13,797 855.4 P	6,868 13.4 P	10,834 752.2 P	7,658 0.0 P	7,228 582.1 P	8,507 993.7 P	7,201 243.0 P	9,061 579.1 P	9,005 212.4 S	6,676 381.6 P	8,780 292.2 P	6,519 495.0 P
	57	8,178 320.7 P	10,478 209.0 P	10,554 650.0 P	7,452 0.0 N	13,966 793.1 P	7,498 12.3 P	9,443 535.1 P	7,258 0.0 P	8,501 550.4 P	7,260 869.6 P	6,413 195.3 P	8,624 493.0 P	9,675 279.9 P	6,106 246.1 P	9,368 232.9 P	5,787 537.8 P
	63	8,068 265.7 P	9,413 210.7 P	9,867 630.3 P	6,630 0.0 N	14,674 993.6 P	7,511 23.3 P	8,428 483.0 P	8,184 0.0 P	6,399 555.7 P	8,381 955.0 P	6,561 196.1 P	8,055 476.3 P	8,309 304.5 P	6,681 232.6 P	7,388 314.0 P	5,259 416.4 P
	69	8,345 314.6 P	10,005 278.4 P	11,297 703.9 P	6,710 0.0 N	12,477 846.4 P	8,118 14.3 P	10,015 404.9 P	7,656 0.0 P	7,566 327.0 P	7,538 627.6 P	7,121 138.5 P	7,574 320.2 P	9,129 284.0 P	6,144 176.0 P	8,653 305.9 P	6,517 439.2 P
	75	7,636 334.9 P	9,277 240.5 P	9,963 1009.6 P	6,584 0.0 N	12,153 880.7 P	8,386 21.6 P	9,146 476.9 P	8,268 0.0 P	6,453 329.0 P	7,235 468.3 P	6,376 114.8 P	7,584 424.5 P	8,255 285.6 P	5,331 110.0 P	7,393 330.0 P	5,941 342.5 P
	81	7,590 188.7 P	10,568 290.0 P	10,201 955.3 P	6,777 0.0 N	11,240 748.6 P	7,552 31.6 P	10,491 553.5 P	7,172 0.0 P	5,984 300.2 P	7,015 289.4 P	6,221 53.3 P	9,730 376.7 P	7,800 277.4 P	6,328 72.4 P	7,699 378.4 P	5,409 487.0 P

Table S4.2 (cont'd).

	Day	29	30	31	50	331	349	436	468	469	471	500	526	544	548	572	684
LC	87	9,119	10,132	13,023	6,124	11,214	8,860	8,170	7,995	5,542	4,979	6,449	8,029	6,105	5,874	5,809	6,462
PVL		400.8	329.2	1258.9	0.0	732.7	32.8	492.9	0.0	254.8	183.2	43.3	401.7	259.4	41.6	373.7	516.2
ELISA		P	P	P	N	P	P	P	P	P	P	P	P	P	P	P	P
	93	8,439	11,625	13,907	5,733	13,215	6,922	10,728	8,126	6,288	5,355	5,490	8,477	6,516	5,878	8,306	7,129
		213.5	224.6	705.9	0.0	527.8	27.8	322.3	0.0	129.7	49.0	15.5	167.1	74.1	9.9	198.2	309.1
		P	P	P	N	P	P	P	P	P	P	P	P	P	P	P	P
	99	8,727	11,081	10,199	7,696	10,320	8,417	9,616	7,862	5,586	4,044	7,676	8,125	8,252	3,497	8,369	5,981
		366.4	277.8	1018.9	0.0	683.5	46.5	618.2	0.0	204.1	40.4	26.3	348.0	119.5	19.3	328.8	451.8
		P	P	P	N	P	P	P	P	P	P	P	P	P	P	P	P
	111	9,397	11,968	13,554	6,788	11,172	7,981	9,670	8,485	6,575	5,036	6,066	8,600	6,061	5,209	8,557	7,299
		341.6	223.6	773.2	0.0	539.7	26.2	476.1	0.0	156.2	4.7	14.3	273.8	53.1	1.5	228.2	381.9
		P	P	P	N	P	P	P	P	P	P	P	P	P	P	P	P
	123	10,198	12,066	13,280	7,790	11,381	8,050	10,356	6,584	7,876	5,139	6,352	9,341	7,421	5,518	7,850	5,284
		452.8	342.5	1209.6	0.0	964.4	68.3	330.6	0.0	330.1	3.4	10.2	396.9	32.0	1.3	361.7	541.2
		P	P	P	N	P	S	P	P	P	P	P	P	P	P	P	P
	135	8,835	9,645	12,354	8,985	9,961	8,434	9,916	8,965	8,071	5,008	5,493	6,477	7,179	5,302	8,201	4,870
		570.3	383.7	1491.0	31.8	969.7	99.4	893.7	80.8	507.8	0.0	3.9	265.0	19.4	2.8	311.6	482.1
		P	P	P	N	P	P	P	P	P	P	P	P	P	P	P	P
	147	9,768	6,840	12,550	7,082	9,883	9,267	6,991	7,916	7,747	3,858	7,258	8,163	7,088	6,448	8,137	5,704
		421.2	316.1	1163.1	40.8	976.3	61.6	576.3	77.8	519.0	5.5	2.7	255.6	7.5	0.0	267.4	330.9
		P	P	P	S	P	P	P	P	P	P	P	P	P	P	P	P

Table S4.3

Lymphocyte (per µL), proviral load(copies/ 1,000 cells), and ELISA results for control steers by day

	Day	461	466	527	542	570	636	650
LC	0	7,598	6,359	4,400	5,319	9,264	7,813	7,269
PVL		0	0		0	0	0	0
ELISA		N	N	N	N	N	N	N
	3	5,903	6,189	4,638	5,939	10,410	9,572	6,899
		0	0	0	0	0	0	0
	6	7,733	6,371	4,257	6,184	10,359	7,979	6,676
		0	0	0	0	0	0	0
	9	6,622	7,225	5,112	6,196	7,157	7,857	5,905
		0	0	0	0	0	0	0
	12	7,340	7,162	4,121	6,492	9,129	8,485	6,769
		0	0	0	0	0	0	0
	15	7,472	6,538	4,845	6,392	8,131	7,878	5,469
		0	0		0	0	0	0
	18	7,649	5,756	5,118	7,885	11,179	9,121	7,552
		0	0		0	0	0	0
	21	7,956	7,360	4,706	6,510	9,414	8,439	6,954
		0	0	0	0	0		0
		N	N	N	N	N	N	N
	24	8,419	6,815	5,404	7,466	9,966	8,575	6,669
		0	0	0	0	0		0
		N	N	N	N	N	N	N
	27	5,827	6,868	5,621	8,072	8,394	8,732	7,747
		0	0	0	0	0	0	0
		N	N	N	N	N	N	N
	30	4,260	6,594	7,267	6,144	8,953	7,749	7,903
		0	0	0	0	0	0	0
		N	N	N	N	N	N	N
	33	7,307	7,211	5,725	7,129	9,396	8,108	7,805
		0	0	0	0	0	0	0
		N	N	N	N	N	N	N
	36	6,539	6,354		6,810	8,336	7,332	6,404
		0	0	0	0	0	0	0
		N	N	N	N	N	N	N
	39	6,973	6,835	5,173	6,091	8,688	7,917	7,051
		0	0	0	0	0	0	0
		N	N	N	N	N	N	N

Table S4.3 (cont'd).

	Day	461	466	527	542	570	636	650
LC PVL ELISA	42	6,858 0 N	6,233 0 N	5,982 0 N	6,519 0 N	8,114 0 N	7,417 0 N	8,376 0 N
	45	6,274 0 N	5,873 0 N	5,425 0 N	7,098 0 N	12,691 0 N	8,058 0 N	7,105 0 N
	51	7,888 0 N	5,528 0 N	4,394 0 N	7,202 0 N	13,144 0 N	8,585 0 N	7,050 0 N
	57	7,871 0 N	5,171 0 N	4,760 0 N		9,069 0 N	8,742 0 N	6,559 0 N
	63	6,517 0 N	5,325 0 N	4,142 0 N		8,462 0 N	9,781 0 N	6,625 0 N
	69	7,664 0 N	4,772 0 N	5,173 0 N		8,833 0 N	8,959 0 N	7,527 0 N
	75	6,024 0 N	4,458 0 N	5,525 0 N		9,801 0 N	7,299 0 N	5,774 0 N
	81	8,811 0 N	5,438 0 N	4,951 0 N		9,119 0 N	7,341 0 N	6,306 0 N
	87	8,158 0 N	6,933 0 N	5,008 0 N		11,500 0 N	8,178 0 N	6,160 0 N
	93	7,047 0 N	6,423 0 N	5,711 0 N		8,402 0 N	7,538 0 N	6,020 N
	99	7,524 0 N	6,114 0 N	5,860 0 N		7,065 0 N	7,284 0 N	6,879 0 N
	111	6,179 0 N	5,508 0 N	4,722 N		7,999 0 N	8,589 0 N	6,196 0 N
	123	6,676 0 N	5,758 0 N	7,453 0 N		8,495 0 N	8,350 0 N	5,530 0 N
	135	5,895 0 N	6,136 0 N	6,880 0 N		4,061 0 N	9,005 0 N	7,965 0 N
	147	6,147 0 N	6,024 564.87 N	6,134 0 N		3,993 0 N	10,251 0 N	7,145 0 N

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Chapter 5 :

Overall Conclusion and Future Directions

A purpose of our research team at Michigan State University is to design effective management strategies for cattle producers to reduce the burden of bovine leukemia virus (BLV) infections and improve the sustainability of the U.S. cattle industry. The overall objective of this dissertation was to determine factors associated with BLV transmission, to describe longitudinal changes in BLV disease parameters that may influence transmission, and to identify the ability to detect new infections. This was conceived from the previous work conducted by our research team which examined management practices associated with BLV prevalence (Erskine et al., 2012), investigated the negative impacts of BLV infection (Bartlett et al., 2013; Frie et al., 2017, 2016; Norby et al., 2016), and explored potential control programs (Ruggiero et al., 2019; Ruggiero and Bartlett, 2019). Unfortunately, limited success has been observed in control programs that have targeted management practices such as single-use hypodermic needs and palpation sleeves (Ruggiero and Bartlett, 2019). A control program focused on the removal of BLV-infected cattle with high proviral loads or high lymphocyte counts, and considered to be highly infectious, shows promise for the future success of BLV control programs (Ruggiero et al., 2019). Taken together, it is important to continue to investigate and identify which routes of transmission are the most important and how they can be controlled. Furthermore, understanding how BLV infections progress and lead to the development of "highly infectious" disease states will assist our efforts to control this disease.

When evaluating the success and limitations of control programs intervening on herd management practices, a limitation was the targeting of factors previously associated with BLV prevalence. While prevalence represents the proportion of cattle infected, it is a product of the

incidence rate and the duration of infection, which for BLV reflects a lifetime of exposure. Therefore, depending on the age range of cattle sampled, identification of risk factors associated with prevalence may be convoluted and may fail to identify critical routes of transmission. A superior outcome for examining disease transmission is the incidence rate which reflects transmission that has occurred at a recent and defined period of time.

To address the identified limitations of previous control programs, chapter 2 of this dissertation aimed to determine the herd management practices associated with the BLV incidence rate in Michigan dairy herds. This analysis was performed utilizing a database previously collected by our research team. While the precision of the calculated incidence rates would have been improved through increased testing frequency to reduce the assumptions made, to more accurately determine the timing of infection, and to reduce the proportion of negative cows lost to follow-up, this analysis created a foundation for future investigations of identified risk factors.

The risk factors identified were a combination of factors previously associated with BLV prevalence (i.e. frequency of needle reuse), housing-related practices (i.e. post-parturient cows housed separate, sand bedding), and milking frequency. Caution should be applied when evaluating these associations because correlation does not imply causation. Furthermore, the identified risk factors can plausibly be the result of farm facilities and, therefore, be surrogates representing or masking other routes of transmission not included in the conducted herd survey. Future work should further examine the factors identified to determine their potential for disease transmission.

A particular risk factor worthy of future investigation is increased milking frequency. Bovine leukemia virus has previously been found in the milk and colostrum of infected cattle

(Gutierrez et al., 2015; Jaworski et al., 2018). While alternative explanations could be conceived, a biologically plausible route for BLV transmission at milking was provided in the discussion of chapter 2. While the abundance of infected lymphocytes may be lower in milk when compared to blood and other bodily fluids (Jaworski et al., 2018; Watanuki et al., 2019; Yuan et al., 2015), dairy cows are milked multiple times a day in modern production systems. The frequency at which cows are milked far exceeds the frequency at which cattle are exposed to other commonly recognized modes of transmission which include breeding, injections, and dehorning. Even if the likelihood of transmission per event is lower, milking machines may serve as a silent, underappreciated route of transmission.

As indicated above, the limited success of management intervention control programs may partially be explained by the failure to target and identify critical routes of transmission. Another explanation is the limited convenience or practicality of specific management interventions in modern production systems. For example, implementation of single-use needles or palpation sleeves is costly and more time-intensive for farm personnel and veterinarians. Therefore, the success of control programs may be hindered by incomplete compliance to the intervention. Given these obstacles, alternative control programs that are both feasible and cost-effective are needed.

One alternative control program that has gained substantial traction in recent literature is intervention based on a cow's infection potential, or likelihood of transmitting the virus (Juliarena et al., 2016; Mekata et al., 2018; Ruggiero et al., 2019). To date, this has been associated with both the proviral load (PVL) and lymphocyte count of BLV-infected cattle. A field-trial conducted by our research team at Michigan State University successfully reduced both the prevalence and incidence of BLV through the selective removal of cattle with high PVL

and lymphocyte counts (Ruggiero et al., 2019). While the development of lymphocytosis is considered to be the result of a slow and gradual disease progression, the development of high PVL was linked through cross-sectional studies to genetic polymorphisms in the BoLA DRB3, BoLA DQA1, and TNF-α genes (Carignano et al., 2017; Forletti et al., 2013; Juliarena et al., 2008; Lendez et al., 2015; Takeshima et al., 2019). The successful identification and removal of cattle with high PVL or lymphocytosis can be enhanced by an understanding of longitudinal changes and progression of these measurements in naturally infected cattle.

To contribute to the understanding of the longitudinal progression of BLV infection, chapter 3 aimed to describe changes observed overtime in cattle naturally infected with BLV and to determine associated cow-level factors. To examine these changes, the database from the previously mentioned intervention field trial which conducted whole-herd antibody ELISA testing and PVL and lymphocyte testing of ELISA positive animals for selective removal was utilized. This database provided longitudinal observations on more than 150 naturally infected cows, which is substantially greater than cases described previously in the literature. The results of this analysis indicated that on average, a small increase in PVL and a non-significant increase in lymphocytes occur over time in naturally infected cattle. In respect to lymphocytes, this challenged the disease dogma that suggests the development of lymphocytosis is the result of a gradual increase in lymphocyte counts. The longitudinal observations on PVL in naturally infected cattle were among the first contributed to the literature. The only cow-level factor found to be associated with changes in PVL was ELISA status; categorical days in milk and changes in lymphocyte counts were not significantly associated.

A limitation of the analysis of longitudinal changes in naturally infected cows was not knowing how long the cattle had been infected. For this reason, chapter 4 aimed to examine early

viral kinetics in experimentally infected steers and to gain insight to the role early disease dynamics may play in long-term disease establishment. While limited to approximately 150 days post-inoculation, wide variation and distinct patterns in the establishment of PVL was observed in steers injected with the same volume of infectious inoculum. Four distinct subsets of steers, grouped by PVL dynamics, were observed in this study. One subset of steers was observed to maintain low PVL following infection, one subset experienced a gradual and consistent increase in PVL, one subset exhibited a peak at which PVL plateaued, and the last subset showed a peak followed by a decline and stabilization. These results indicated cattle have unique responses to infection which may influence long-term disease establishment.

Collectively, the results of chapter 3 and chapter 4 led to the formulation and proposition of a hypothesis which suggests the development of high PVL and of lymphocytosis in BLV infected cattle may occur soon after infection and may not be the result of a slow, gradual disease progression. Studies published in literature during the time in which the studies presented in this dissertation were performed further support this observation. In fact, while not providing supporting data, several studies (Lendez et al., 2015; Mekata et al., 2018) have proposed a similar hypothesis regarding the development of both low and high PVL set points. Furthermore, as discussed in chapter 4, comparative insights from research studies and statistical modelling of other retroviral diseases support the establishment of a PVL set-point following infection. More recently, greater insight to the role genetics and bovine leukocyte antigen haplotypes play in the development of disease states was published in the literature (Forletti et al., 2020). To fully support the notion that PVL and lymphocytes associated with BLV infection are stable over the course of disease, a study would need to be conducted following animals from the time they are infected until the end of their natural life. Nonetheless, if a steady state exists, control programs

focused on selective removal could become more practical and cost-effective by reducing the frequency at which known BLV-positive animals are repeatedly tested. To test the feasibility of this notion, ongoing and future control programs could look at the effects of varying the sampling frequency in which infected cattle are tested on the reduction in BLV prevalence and incidence.

The detection and identification of highly infectious cattle is important for current control programs; however, the detection of all infected animals is paramount for the long-term success of BLV control and eradication. Therefore, an additional objective of the presented studies was to determine our ability to detect BLV infections by qPCR, ELISA, and lymphocyte counts. This objective was addressed in chapter 4 using the experimental infection of Holstein steers. The results presented in chapter 4 indicated that the detection of infection generally first occurs by detection of the DNA provirus using PCR prior to the detection of anti-BLV antibodies by ELISA. However, the observance of ELISA positive, PCR negative cattle demonstrated the need for the continued use of serological based assays. Unfortunately, observations presented in chapter 3 indicated that a subset of cattle with low PVLs and low lymphocyte counts may test false negative by ELISA. While not an immediate threat to proposed control programs, the observation of infected cattle having discrepant test results, with the potential to be false negative by both ELISA and PCR, may be problematic for control programs aimed at BLV eradication; infected cattle potentially going undetected will allow BLV to lowly persist, as observed in countries in which BLV has been eradicated.

Future directions:

The future of BLV research is a target rich environment. Research efforts should continue to focus on the development of both feasible and cost-effective control programs. Furthermore, research should continue to investigate and target routes of transmission. As indicated above, the potential for transmission via milking machines warrants further investigation. In addition to classical control programs, further exploration of alternative control methods, such as the selective removal of high PVL and high lymphocyte count cows, should be conducted.

The results of the studies presented within this dissertation suggest these "highly infectious" disease states may be established shortly after infection. However, control programs focused on removal of highly infectious cattle should pursue deductive investigation to further identify what makes a BLV infected cow the most infectious. Research published in recent literature has reported the detection of viral RNA *in vivo* of infected animals, indicating viral reactivation from latency may occur (Alvarez et al., 2019; Chen et al., 2020; Jaworski et al., 2019). These observations conflict with the long-held belief that the virus is transcriptionally silent within infected animals.

Prior to the identification of viral RNA, viral transmission was thought to occur through the transmission of cells infected with the DNA provirus, and therefore, it was plausible that cattle with high PVL and high lymphocyte counts were responsible for the majority of transmission. The identification of viral RNA, which indicates viral reactivation, may challenge this paradigm as the transmission of active viral RNA may be the source of new infections. The events or factors that lead to viral reactivation still require elucidation but may be related to periods of stress (Alvarez et al., 2019; Jaworski et al., 2019). Nonetheless, the relationships

between PVL, lymphocyte counts, detection of viral RNA, and stage of production are worthy of investigation.

In addition to the further investigation of potential control programs, research should continue to investigate the negative implications of BLV on animal health and producer profitability. A greater understanding of the broad impacts of BLV infection will allow for a better estimation of the economic impact and will allow for researchers to better inform and provide incentive for more producers to prioritize the control of BLV. In addition, if studies can link the negative effects of BLV infection to high PVL and high lymphocyte count disease states, producers may be more likely to implement control programs focused on the selective removal of infected cattle.

Outside the scope of the studies presented within this dissertation, one last focus of future research should be to investigate the potential for BLV to be detected in human tissues and the associated implications. While the associations have been dismissed by many, the evidence for a relationship slowly continues to grow. As animal scientists we need to have a holistic understanding of the impacts of disease on the health and welfare of animals and the implications for producers as well as continue to ensure the products produced for human consumption are both safe and secure. Future BLV research needs to be proactive to prevent a forced reaction on behalf of the cattle industry by the general public if the reported associations were to ever gain traction.

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