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Exploring the Impact of Bovine Leukemia Virus Proviral Load on Production, and its Potential Use for Control

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Exploring the Impact of Bovine Leukemia Virus Proviral Load on Production, and its Potential Use for Control

by

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Abstract

The main aim of this dissertation was to evaluate the efficacy of a bovine leukemia virus (BLV) control program by selective removal of high proviral load (HPL) BLV-infected subsets. Six chapters are included. 1) To be acquainted with the current understanding on BLV infection, transmission routes, diagnosis, control, and most importantly, BLV proviral load, a literature review was conducted. This review explored the applicability of BLV proviral load in disease diagnosis, BLV transmission risk assessment, and BLV control. 2) We implemented a cross-sectional study to evaluate the impact of BLV proviral load on milk production of dairy cows. Data obtained from nine dairy herds in Alberta, Canada demonstrated a significant reduction in milk, fat, and protein production of HPL cows when compared with the BLV-negative counterparts. 3) The effectiveness of HPL-cow focused BLV control program in reducing BLV prevalence and seroconversions within the herd was evaluated by conducting a 3-year study among ten dairy herds. The BLV prevalence decreased in four herds whereas the BLV incidence was reduced in nine herds, which supported the notion that removal of HPL cows can offer a feasible and economical option for BLV control. 4) A 1.5-year longitudinal study was designed by enrolling subset of cows from BLV-seropositive (further classified into various proviral load categories) and BLV-seronegative group to monitor the dynamics of various parameters such as BLV proviral load, lymphocyte, white blood cell (WBC) count, antibody titer, CD3+, CD4+, CD8+, CD21+, and WC1+ cell proportions. A relatively stable pattern of BLV proviral load, WBC, CD3+, and CD4+ cell proportion was observed, indicating frequent testing might not be required for these parameters in monitoring BLV infection. 5) A cross-sectional study was

conducted to investigate the hematological and immunological impact of BLV infection which suggested a simpler categorization of HPL and LPL as an appropriate approach. Additionally, a lower proviral load cut-off was identified as an accurate threshold for identifying HPL cows. 6) Lastly, all the results and findings were thoroughly discussed, and future directions for using HPL-focused strategies as a potential tool for BLV control and management were elaborated.

Key words: bovine leukemia virus, control, dynamics, impact, proviral load, threshold

Preface

This doctoral thesis consists of four manuscripts: One has been published, one has been submitted, and two will be sent for publication next year. Written permission from the publishing journal and all co-authors have been obtained to include these manuscripts in this thesis on its entirety.

The following manuscripts are included in this thesis:

Chapter 2 (published):

Shrestha, S., K. Orsel, H.W. Barkema, L. Martins, S. Shrestha, and F. van der Meer. 2023. Effects of bovine leukemia virus seropositivity and proviral load on milk, fat, and protein production of dairy cows. J. Dairy Sci. In press. https://doi.org/10.3168/jds.2023-23695.

Chapter 3 (submitted):

Shrestha, S., K. Orsel, C. Droscha, S. Mijar, and F. van der Meer. Removing bovine leukemia virus infected animals with high proviral load leads to lowering within-herd prevalence and new case reduction.

Chapter 4:

Shrestha, S., K. Orsel, D.B. Nobrega, and F. van der Meer. Dynamics of proviral load, antibody titer, and immune cells in bovine leukemia virus-infected and non-infected dairy cows.

Chapter 5:

Shrestha, S., K. Orsel, and F. van der Meer. The association between hematological and immunological parameters to determine bovine leukemia virus proviral load in BLV-infected bovine.

Statement of Contribution

The study in Chapter 2 was designed in collaboration of Sulav Shrestha, Karin Orsel, Herman Barkema, and Frank van der Meer. The study designs in Chapters 3, 4, and 5 were contributed by Frank van der Meer, Karin Orsel, and Sulav Shrestha. Karin Orsel, Herman Barkema, and Frank van der Meer supported the identification of potential dairy farms to participate in the study of Chapters 2, 3, and 5. The study farm for Chapter 4 was identified with the support of Frank van der Meer. Farm visits to collect milk and blood samples were performed by Sulav Shrestha (Chapters 2-5), Larissa Martins (Chapter 2), Samita Shrestha (Chapters 2, 3, and 5), Sanjaya Mijar (Chapter 3), Karin Orsel and Frank van der Meer (Chapter 2-5). All farm sample processing and laboratory analyses were conducted by Sulav Shrestha with support from Samita Shrestha, Hadjira Hamou, and Frank van der Meer (Chapters 2, 3, 4, and 5). Casey Droscha helped in obtaining and setting up the BLV SS1 qPCR assay for proviral load quantification. Herman Barkema helped in finding the contact person to acquire the data for Chapter 2. All data were collected, cleaned, and analyzed by Sulav Shrestha to produce interpretable results with the guidance and support of Herman Barkema, Karin Orsel, Samita Shrestha, and Frank van der Meer. Diego Nobrega and Karin Orsel helped with the data analysis in Chapters 3 and 4. Sulav Shrestha was responsible to analyze the data and produce the manuscript from the results with feedback from Frank van der Meer. All co-authors contributed in the critical review and finalizing the manuscript before submission. Permission has been obtained from all co-authors and the publishing journals to reproduce the manuscript in this thesis.

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Thank you all.

Namaste!

Dedication

To my dad Laxman Kumar Shrestha and mom Bal Kumari Shrestha

To my wife Samita Shrestha

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AB	Alberta
AT	Antibody titer
AUC	Area under the curve
BC	British Columbia
BIC	Bayesian information criterion
BLV	Bovine Leukemia Virus
BSA	Bovine serum albumin
CA	California
CD	Cluster of differentiation
CI	Confidence interval
CO	Colorado
DHI	Dairy Herd Improvement
DNA	Deoxyribonucleic acid
EBL	Enzootic bovine leukosis
EDTA	Ethylenediamine tetraacetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
F305	305-days milk fat production
FMO	Fluorescence Minus One
HBSS	Hanks' Balanced Salt Solution
HTLV-1	Human T-lymphotropic virus type 1

HPL	High Proviral Load
IA	Iowa
ICC	Intraclass Correlation Coefficient
ICTV	International Committee on Taxonomy of Viruses
kg	Kilogram
LPL	Low Proviral Load
LTR	Long terminal repeats
M	Master-mix group
M305	305-days kg milk production
MA	Massachusetts
ME	Maine
MI	Michigan
miRNA	micro RNA
mL	milliliter
MPL	Moderate proviral load
n	sample size
ND	Not-detected
NEG	BLV-seronegative
ng	nanogram
NY	New York
OD	Optical density
ON	Ontario
OR	Oregon

P305	305-days kg protein production
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PVL	Proviral load
QC	Quebec
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
ROC	Receiver Operating Curve
SCC	Somatic Cell Count
SD	Standard deviation
SS1	Super spreader 1
T	Time-point
TX	Texas
USA	United States of America
VA	Virginia
VSACC	Veterinary Sciences Animal Care Committee
WA	Washington
WBC	White blood cell
µL	microliter
ρ	Rho

CHAPTER 1: General Introduction

1.1 Background information

The first "leukosis" disease in cattle was described as a yellowish nodule in the enlarged spleen in Lithuania in 1871 (Gillet et al., 2007). The spleen disruption occurring as a consequence of tumor formation is regarded as the most remarkable end point of bovine leukemia virus (BLV) infection. Series of reports described similar infections and disease characteristics, insinuating BLV as the possible causative agent (Schwartz and Levy, 1994). In 1969, it was recognized that lymphocytes obtained from cows demonstrating persistent lymphocytosis (a condition characterized by abnormally high blood lymphocyte level), contained virus-like particles (Miller et al., 1969). On further exploration, these virus particles were found to contain RNA-reverse transcriptase complex and had molecular characteristics that distinguished them from other RNA-oncogenic retroviruses (Kettmann et al., 1976).

1.1.1 Genomic structure

Bovine leukemia virus is an oncogenic *Deltraretrovirus* (ICTV, 2022). The BLV genome consists of 8,714 nucleotides (Sagata et al., 1985). This comprises of structural and enzyme coding genes such as *gag, pro, pol*, and *env,* and a *pX* region flanked on both sides with two identical long terminal repeats (LTRs) (Supplementary material, Figure S1-1). The *Gag* transcript generates three mature proteins: matrix protein (p15), the capsid protein (p24), and nucleocapsid protein (p12), out of which, p24 acts as a major target of host immune response (Polat et al., 2017). *Pro* gene generates protease. One of the important functions of the BLV *pol*

gene is that it translates and produces reverse transcriptase, which facilitates generation of a DNA intermediate copy of the BLV RNA genome. The viral *env* gene consists of a transmembrane unit and a surface unit which interact with the host cellular membrane receptors to facilitate virus entry (Lairmore, 2014). The *pX* region encodes the regulatory protein *Tax* and *Rex*, and the accessory protein R3 and G4. *Tax* triggers the activation of transcription from the LTR of the integrated provirus by binding to specific sites in the U3 region of the LTR, it can also promote lymphocyte proliferation through activation of cytokine production, inhibits DNA repair promoting mutations in cellular DNA, and may play a role in the development of lymphoma and leukemia, similar to the mechanism observed in human T-lymphotropic virus type 1 (HTLV-1) (Lairmore, 2014). *Rex* facilitates in nuclear transport of viral RNAs to the cytoplasm (Polat et al., 2017). Maintenance of high proviral loads is maintained by the R3 and G4 accessory proteins (Willems et al., 1994). The BLV genome also inhabits RNA polymerase-III transcribed viral microRNAs (miRNAs) encoding region between the *env* and *pX* region, which impact BLV pathogenesis (Kincaid et al., 2012). A strong expression of BLV miRNAs is noticed in preleukemic and malignant cells with supressed structural and regulatory gene expression, potentially leading to tumor onset and progression (Rosewick et al., 2013). Experimental infection conducted with recombinant BLV with ablated miRNAs demonstrated lower proviral load profile in animals and lower ex-vivo transcription of viral mRNAs compared to wild type BLV infection, highlighting the importance of BLV miRNAs in viral replication (Gillet et al., 2016). Additionally, a strong viral antisense transcription activity is observed from the 3′ LTR region in both tumor and non-tumor clones, suggesting its important role in the life cycle of BLV and potentially in tumorigenesis (Durkin et al., 2016).

1.1.2 BLV prevalence

In North America, almost 90% of the dairy herds are infected with BLV with the within-herd prevalence of approximately 40% (LaDronka et al., 2018; Kuczewski et al., 2019). There are limited studies on beef herds, however, a recent study conducted in Kansas, USA using convenience samples indicated similar pattern, with a 95% herd-level BLV prevalence (Huser et al., 2023). In the Canadian context, a 2018 study conducted in Atlantic Canada using bulk tank milk samples revealed 89.3% of the dairy herds with at least one BLV-infected cow (John et al., 2020). In other provinces of Canada, the reported BLV herd-level prevalence in dairy herds varied, with 97.4% in Manitoba (VanLeeuwen et al., 2006), 89.1% in Saskatchewan (VanLeeuwen et al., 2005), 69.6% in Ontario (Sargeant et al., 1997), 86.7% in 2002-03 in Alberta (Scott et al., 2006). A comprehensive study using data from various parts of Canada between 1998-2003, estimated the overall BLV herd-level prevalence to be 78.3% (Nekouei et al., 2015a), whereas a convenience sample indicated 90% BLV prevalence among Canadian dairy herds (Kuczewski et al., 2019).

Investigation of within-herd BLV prevalence rates among Canadian dairy herds with the convenience samples revealed a median within-herd BLV prevalence of 49% (Kuczewski et al., 2019). This result was much higher than those obtained from specific provinces. For example, in a study conducted with a subset of dairy herds in the Canadian Maritimes, the estimated within-herd prevalence was 30.4% (Nekouei et al., 2015a). Another study conducted in Alberta with 11 dairy herds reported a median within-herd prevalence of 36% (Kuczewski et al., 2021a). However, in Manitoba the within-herd prevalence was high at 60.8% (VanLeeuwen et al., 2006). Limited information is available for Quebec and Ontario, the two provinces with the largest number of dairy herds and cows. The only result from Ontario is of 1992, utilizing a subset of

cows from dairy farms which estimated the within-herd prevalence of 23% (Sargeant et al., 1997), indicating the need for an updated, comprehensive BLV seroprevalence estimation.

1.1.3 Transmission

The natural hosts of BLV includes cattle (*Bos taurus, Bos indicus*) and its closely related species water buffalo (*Bubalus bubalis*), and yak (*Bos grunniens*) (EFSA Panel on Animal Health and Welfare, 2015). However, experimental infection is possible in diverse hosts including sheep (*Ovis aries*), goats (*Capra aegagrus hircus*), and rabbits (*Oryctolagus cuniculus*) (Schwartz and Levy, 1994; EFSA Panel on Animal Health and Welfare, 2015). The broad host range of BLV can be attributed to the expression of cationic amino acid transporter 1 (CAT1)/solute carrier family 7 member 1 (SLC7A1) in these hosts' cells which functions as the receptor for BLV (Bai et al., 2019).

The transmission of BLV virus can occur horizontally or vertically. A free BLV virus is unstable in the environment, therefore natural infection with BLV occurs primarily by exchange of BLV-infected cells present in the bodily fluids such as milk, blood, and colostrum (Rodríguez et al., 2011).

Horizontal transmission

In dairy farms, iatrogenic procedures such as using blood contaminated needles provide ample opportunity for transfer of BLV-infected cells. Reuse of needles have been associated with increased BLV herd prevalence (Kobayashi et al., 2010; Erskine et al., 2012a), indicating the risk of BLV transmission through contaminated needle. A rectal palpation experiment demonstrated a notable BLV seroconversion risk with reuse of palpation sleeves in one facility, whereas, no

significant difference in BLV seroconversion with single sleeves use in a separate facility (Hopkins et al., 1991). Nevertheless, single use of needles or rectal palpation gloves failed to reduce BLV incidence in a separate study (Ruggiero and Bartlett, 2019). This suggests that the invasiveness of the procedure and the extent of infected blood exchange opportunity may contribute to the risk of BLV transfer. Additional potential routes include herd management procedures such as dehorning and tattooing (Hopkins and Digiacomo, 1997). Use of gouge dehorners without sterilization has been associated with increased BLV prevalence in epidemiological studies (Kobayashi et al., 2010; Erskine et al., 2012a). Electric dehorning or sterilizing dehorners after use might minimize the BLV transmission risk.

Hematophagous arthropods may pose BLV transmission risk dependent on the geography and season, which contributes to the insect population and biting incidence. Experimental infection by inoculating mouth parts of hematophagous flies that fed on blood from a BLV-positive cow was able to cause seroconversion in BLV-negative cattle (Panei et al., 2019). However, the role of these flies in transmitting BLV under natural grazing conditions remains to be elucidated. Direct close contact between animals is indicated as a risk, however, the exact mechanism involved is not clear (Ohno et al., 2015). Detection of proviral DNA in saliva and nasal secretions of BLV-infected cattle indicates that close contact between animals may pose a risk (Yuan et al., 2015), but this remains to be validated.

Use of semen from BLV-seropositive bulls is not regarded as a substantial risk as artificial insemination with ejaculates from BLV-seropositive bulls failed to induce infection in BLV-seronegative herds (Monke, 1986). Breeding heifers with BLV-seropositive bulls failed to demonstrate the role of semen in BLV transmission (Benitez et al., 2019a). However, BLV proviral DNA has been detected in the vaginal secretions and smegma of bulls, thus breeding

routes cannot be completely ruled out as a risk for BLV transmission (Yang et al., 2016; Benitez et al., 2019b).

Vertical transmission

Perinatal transmission of BLV can occur from a BLV-infected dam to its calf and the risk is greater if the maternal BLV proviral load is high (Mekata et al., 2015). Evidence of identical BLV genomic sequences in the dams and their infected calves, which were delivered through caesarean section and not fed with maternal colostrum, highlights the possibility of an intrauterine BLV transmission (Sajiki et al., 2017). Embryo transfer doesn't cause BLV transmission, however, if the recipient cow is BLV-infected, the risk of BLV transmission to the developing calf is high (Fukai et al., 1999).

The frequency BLV transmission through milk and colostrum was reported to be much lower than other direct contact routes (Ferrer and Piper, 1981). Intraperitoneal inoculation of leukocytes harvested from the colostrum of a BLV-infected Holstein was able to establish infection in sheep, suggesting the infectious potential of BLV-infected colostrum/milk (Kanno et al., 2014). However, milk and colostrum from BLV-infected dams have preventive role as these anti-BLV maternal antibodies are detected for up to six months in calves (Meas et al., 2002; Nagy et al., 2007; Kobayashi et al., 2010). This can be confirmed by the results from an *in vitro* experiment that demonstrated colostrum containing significantly higher antibody titer than serum but lower proviral load than blood (Konishi et al., 2018). In order to acquire this passive immunity while minimizing the transmission risk, a simple treatment of freeze-thawing of colostrum can be recommended (Kanno et al., 2014).

1.1.4 Pathogenesis

Bovine leukemia virus causes enzootic bovine leukosis (EBL), and primarily targets CD5+ IgM+ B-lymphocytes, wherein it integrates its reverse-transcribed genetic material into the host's genome, forming a provirus and inducing a lifelong, persistent infection (Frie and Coussens, 2015). Additionally, BLV provirus integration in other cells such as T-lymphocytes, monocytes, granulocytes, and mammary epithelial cells have also been reported, however, the tumor cells are only specific to the CD5+ IgM+ B-cells (Buehring et al., 1994; Schwartz et al., 1994).

Bovine leukemia virus and HTLV-1 are closely related, and often these two models are studied together to understand the initial phases following infection with both viruses (Gillet et al., 2007). In the HTLV-1 model, the viral spread occurs through cell-to-cell contact following host entry (Carpentier et al., 2015). After primary infection, the virus replicates either by an infectious cycle or clonal expansion. The infectious cycle involves new target cell infection through cell-to-cell transfer of viral particles, reverse transcription of the viral RNA, integration of the DNA copy of virus into the host chromosome forming a provirus, viral protein expression, and virion budding. The clonal expansion mechanism involves mitotic division of the cells harboring the integrated provirus (Carpentier et al., 2015). Infection with BLV favors proviral integration at highly transcribed host genome regions promoting viral expression and consequent exposure to the host immune response (Gillet et al., 2013). Over time, the viral propagation shifts from expression of active viral particles to clonal expansion escaping the host immune response.

Various theories attempt to explain the progression of BLV/HTLV-1 infection from initial infection to leukemia development. One of them is the potential role of mutations, as observed in Adult T-cell leukemia/lymphoma (ATL) cases in HTLV-1 infection or EBL cases in

BLV infection (Kataoka et al., 2015; Konishi et al., 2021). The oncogenic potential of the *Tax* protein has been thoroughly discussed in BLV and HTLV-1 infections suggesting its role in leukemia induction (Willems et al., 1990). However, this theory is challenged by the fact that the induced malignancies do not express *Tax* protein (Takeda et al., 2004; Merimi et al., 2007). Furthermore, the transcriptional activity of *Tax* protein makes the infected cells vulnerable to cytotoxic immune response, suggesting its minimal role in the later stages of tumor progression (Bangham et al., 2009). Another potential oncogenic factor in HTLV-1 is the HTLV-1 basic leucine zipper (bZIP) factor (HBZ), which is encoded from the minus-strand and consistently expressed in all infected cells (Mitobe et al., 2015). In BLV, antisense transcripts AS1 and AS2 are expressed in both non-malignant and tumor clones as described earlier, which indicates its potential role in tumorigenesis (Durkin et al., 2016). In addition, BLV produces RNA polymerase III-dependent microRNAs, which contribute up to 40% of the microRNA pool in tumor cells (Rosewick et al., 2013). Lastly, BLV/HTLV-1 proviruses integrate near cancer drivers, affecting them through termination of provirus-dependent transcription or by causing disturbance through viral antisense RNA production (Rosewick et al., 2017). This pattern exists not only in tumorous stage, but also in polyclonal non-malignant stages, highlighting that the provirus-induced host gene perturbation contributes in selecting multiple clones during the asymptomatic stage, however, for evolving into full blown leukemia/lymphoma, additional genetic alterations might be required (Rosewick et al., 2017).

1.1.5 Impact of BLV infection

Following a BLV infection, the host's immune system is activated, engaging both humoral and cell-mediated immune responses (Kabeya et al., 2001). This results in persistent

antibody production throughout the host's lifetime. However, a gradual reduction in helper T-cells (CD4+) and cytotoxic T-cells (CD8+), along with disruptions in the proliferation and apoptosis of blood lymphocytes, adversely impacts the immune and vaccination responses in the host (Erskine et al., 2011a; b; Lairmore, 2014; Frie and Coussens, 2015). The suppressed immune system renders BLV-infected animals more vulnerable to secondary infections (Emanuelson et al., 1992). Cattle infected with BLV, exhibiting elevated white blood cells (WBC) and lymphocytes, have a higher incidence of subclinical mastitis compared to BLV-seronegative cows or BLV-seropositive cows with normal WBCs and lymphocytes (Sandev et al., 2004). The severity of mastitis is also higher among BLV-seropositive cows with high proviral loads (Watanabe et al., 2019). Additionally, BLV infections present a classical 30% incidence of persistent lymphocytosis and 5-10% cases of lymphoma among infected animals, adversely impacting animal welfare (Schwartz and Levy, 1994).

The assessment of BLV's impact on the milk production of individual animals and at herd level has varied results which is further discussed in Chapter 2. Apart from milk production, BLV infection influences cow longevity, with BLV-infected cattle reportedly having a higher likelihood of leaving the herd earlier than their non-infected counterparts (Bartlett et al., 2013; Nekouei et al., 2016; Benitez et al., 2020). Another concerning aspect of BLV infection is its association with human breast cancer (Buehring et al., 2015). However, the association indicated was based upon a case-control study, which does not necessarily indicate causation. To elaborate, this study used case samples taken mostly from patients that were undergoing mastectomies for breast cancer, whereas the control samples were mostly from patients that were undergoing reduction mammoplasties. The discrepancy in the population under study represent selection bias which is one of the concerns related with case-control studies. Nevertheless, such

claims need to be taken seriously and the zoonotic potential of BLV should be further clarified with appropriate studies.

1.1.6 Diagnosis

A significant proportion of BLV-infected animals (70%) do not exhibit visible clinical signs (Schwartz and Levy, 1994). In such circumstances, BLV detection becomes challenging without specific clinical tests. Historically, age-dependent normal reference intervals for lymphocyte counts were established in Danish cattle, to screen for and slaughter leukemic cattle (Bendixen, 1963). Although this method was successful in eliminating new EBL cases during a course of five years in Danish herds, cases of leukemia continued to develop and was unable to eradicate the disease. Application of this method using old reference intervals in the current North American dairy herds may be further complicated because of the breed, genetic changes, increased production, and environmental differences. Additionally, with only 30% of BLV-infected cattle demonstrating lymphocytosis, relying entirely on lymphocyte monitoring will not detect all infected animals.

A more reliable BLV diagnostic strategy includes detecting the host's immune response against the virus through serological tests and detecting the proviral genome using polymerase chain reaction (PCR) tests (Polat et al., 2017). Serological assays, such as agar-gel immunodiffusion (AGID) and enzyme-linked immunosorbent assays (ELISA), can be used to detect the antibodies against BLV, commonly anti-gp51 and anti-p24, targeting envelope glycoprotein and viral capsid protein, respectively (Polat et al., 2017). These antibodies are expressed throughout the host's lifetime following BLV infection. ELISA is reliable and flexible as it can be used to screen various sample types including serum, milk, and colostrum.

Commercially available BLV ELISA kits have demonstrated a relative sensitivity of 100% and relative specificity of 95-100%, making ELISA a readily available BLV diagnostic test (Kuczewski et al., 2018).

Another BLV detection method involves detection of a segment of the proviral DNA. Various genes of the BLV proviral genome have been targeted for amplification including LTR regions, *pol* gene, *env* gene, *gag* gene, and *Tax* genes. Among these, the *pol* gene is a conserved region of BLV (Heenemann et al., 2012; Rola-Łuszczak et al., 2013) and therefore used in many PCR based assays. Various PCR methods such as conventional PCR, nested PCR, real-time quantitative PCR (qPCR), and direct blood-based PCR, have been applied to amplify targeted BLV proviral sites (Polat et al., 2017). Experimental studies have indicated that BLV proviruses and antibodies can be detected as early as 24- and 36-days post-infection, respectively (Hutchinson et al., 2020a). This implies proviral detecting methods enables the identification of BLV infection earlier than antibody detection. However, PCR methods require a complicated sample processing and stringent protocols to avoid cross contamination, which increases the testing cost and cannot be performed without proper laboratory facilities.

1.1.7 BLV proviral load

The host genome can get integrated with multiple copies of BLV proviruses. Quantitative PCR (qPCR) methods enables quantification of BLV proviral load, which is expressed as the number of BLV proviruses per denominator such as quantity of DNA or endogenous genes. Multiple approaches to quantifying BLV proviral load are implemented, with differences existing in the choice of target BLV gene for amplification, qPCR assays employed, and methods used in proviral load calculation (Kuczewski et al., 2021b). Categorization of BLV-infected

animals into high (HPL) or low (LPL) proviral loads is rendered to be crucial as HPL cows are considered a higher risk of transmitting the virus than LPL cows (Jimba et al., 2010). Additionally, quantifying BLV proviral load serves as a method to monitor infection status and infectivity in BLV infected animals (Juliarena et al., 2007; Rodríguez et al., 2011). The assessment of proviral load is also commonly used as an indicator of infectivity in HTLV-1 infections (Kaplan et al., 1996). Additionally, some BLV-seropositive cows may test negative in qPCR (not-detected or ND), and may require further investigation (Jimba et al., 2012; Hutchinson et al., 2020a).

Alternative approaches for identifying HPL cows have been explored. Positive associations of BLV proviral load with white blood cells, lymphocytes, and antibody titers (Juliarena et al., 2007; Alvarez et al., 2013; Jaworski et al., 2016) mean that there may be options for veterinarians and farmers for conducting tests to identify and remove HPL cows. Recent studies indicate good sensitivity and specificity in identifying HPL cows based on white blood cells or lymphocytes count (John et al., 2022). However, the proviral load values currently being used to distinguish HPL cows is arbitrary as it is not backed up with clinical or pathological implications. Therefore, further work is required to determine an appropriate BLV proviral load cut-off value for identifying HPL cows.

1.1.8 Progression of BLV infection

Bovine leukosis is assumed to be a gradually progressive disease that initiates as an aleukemic stage following infection, progresses to persistent lymphocytosis stage in 2/3rd of the infected, and may develop lymphoma or lymphosarcoma in 5% of the infected (EFSA Panel on Animal Health and Welfare, 2015). This notion is supported by how BLV establishes its

infection cycle in the host following infection. To elaborate, BLV propagation shifts from active viral replication to clonal propagation to evade the host immune response, which has been mentioned earlier. This duration in shifting of the propagative strategy by BLV is presumably considered as the lag-time in shifting from aleukemic stage to persistent lymphocytosis or lymphoma stage. However, this classical disease model has been challenged by recent studies that have elucidated that persistent lymphocytosis may occur shortly after BLV infection and doesn't necessarily require an additional aleukemic stage (Hutchinson et al., 2020a).

As mentioned earlier, recent studies have highlighted the importance of BLV proviral load for BLV transmission and its control. Therefore, understanding the dynamics of proviral load becomes necessary to understand the overall manifestation of the disease and its monitoring. Longitudinal experimental studies have indicated that the proviral load is established shortly after infection and remains relatively stable over time (Gillet et al., 2013; Forletti et al., 2020; Hutchinson et al., 2020a). However, these results were from experimental studies which may not represent BLV infections occurring naturally. When the dynamics of lymphocytes and proviral load were assessed in naturally BLV-infected cows, the lymphocyte count were relatively stable over time whereas the proviral load demonstrated minimal increments with time (Hutchinson et al., 2021). Interestingly, a subset of cows in the same study underwent rapid increments in both lymphocytes and proviral load, contradicting the results obtained from the statistical models. Such kind of variations are expected because of the individual variations which are not demonstrated in statistical models as these models tend to simplify the individual observations into simpler interpretations. These individual variations may be a result of BLV proviral load differences, as high proviral load cows have higher risk of developing lymphocytosis. Therefore,

it is important to consider the proviral load status of the participant cows while conducting such longitudinal studies.

1.1.9 BLV control

Efforts to control and eradicate bovine leukosis dates back to the 1950s and 1960s when there was no concrete evidence that the disease was caused by a virus (EFSA Panel on Animal Health and Welfare, 2015). Initially, control efforts focused on eliminating persistent lymphocytotic cows and herds, was based on the epidemiological knowledge that infected blood and cells could transmit bovine leukosis. Although, the herd elimination approaches reduced the BLV prevalence significantly, removing individual cows with persistent lymphocytosis deemed ineffective, as the herd prevalence continued to escalate. When BLV was identified as the etiological agent of bovine leukosis, control strategies shifted to stopping the viral transmission by removing BLV-infected animals, implementing management changes, and vaccination.

Several attempts were made for BLV vaccine development, including inactivated viruses, cell lysates, viral subunits, recombinant vaccinia virus, synthetic peptides, DNA vaccines, but were unsuccessful due to inadequate and short-term immune protection (Rodríguez et al., 2011; Gutiérrez et al., 2014). A novel approach using an attenuated BLV virus by deleting genes that removed its pathogenic potential yet retaining antigenicity to induce a long-lasting protection was identified (Abdala et al., 2019). This vaccine strain (pBLV6073DX) was assessed for its efficacy through a farm trial in Argentina. The trail demonstrated encouraging results of inducing sterilizing immunity over a period of 48 months in 28 out of 29 vaccinated heifers, exhibiting extremely low proviral load compared to those exposed to wild-type strains, and indicating non-transmissibility of the strain to sentinels, confirming the overall safety of the vaccine (Suárez

Archilla et al., 2022). It currently awaits industry investments amidst the profitability concerns and therefore, a BLV vaccine may not be commercially available soon.

In the absence of an effective vaccine, BLV control programs are largely focused on test and elimination, test and segregation, or test and management (Rodríguez et al., 2011). Test and elimination methods require identification and culling of all BLV-seropositive animals from the herd. While this may be an effective and fast way of controlling BLV prevalence, it is a costly approach and may not be feasible in high within-herd prevalence situations like North America. In the test and segregate method, BLV-seropositive animals are retained but separated from their BLV-seronegative herd-mates. This may be less expensive than the test and elimination method, but the requirement of extra space to separate a part of the herd and the additional management associated, can make it less practical. In the third method, management changes are implemented to prevent BLV transmission within a herd. This approach has reported mixed success. For example, single use of needles and palpation sleeves over a period of two years failed to reduce the BLV incidence in three dairy herds (Ruggiero and Bartlett, 2019). In contrast, in a separate study, implementation of best management practices such as needles and gloves change, disinfecting instruments between use, colostrum freeze-thawing and more, without elimination or segregation of BLV-seropositive cows enabled reduction of within-herd BLV prevalence in eight out of ten dairy herds (Kuczewski et al., 2021a).

An alternate approach of BLV control is by implementing breeding programs considering genetic factors, which requires selection of animals with specific BoLA class II alleles that associate with lower BLV proviral loads, consequently allowing indication for BLV susceptibility or resistance (Takeshima et al., 2019). While this approach is more feasible than a

mass culling approach, selecting cows for a specific trait without compromising other essential dairy traits and producing adequate number of cows inhabiting such traits is challenging.

In Canada, there is no mandatory BLV control program, however the Canadian Food Inspection Agency (CFIA) has started a voluntary Canada Health Accredited Herd Enzootic Bovine Leukosis (CHAH-EBL) program for Canadian bovine producers (Canadian Food Inspection Agency, 2022). Accreditation through this program enables the Canadian bovine producers to export their product to European countries. However, all associated costs are carried by the farmers and therefore the farmer's participation is limited.

Most countries except Europe have reported increasing BLV prevalence (Bartlett et al., 2020). Multiple factors are responsible for this situation. The relatively low incidence of lymphoma (<5%), which is the most obvious negative consequence of BLV infection, makes most dairy farmers or government agencies reluctant to invest in BLV control strategies. A noticeable disconnect exists among the farmers and veterinarian in implementing BLV control measures even though the financial benefit of implementing BLV control measure on farm are demonstrated (Kuczewski et al., 2019). The concerns regarding the associated cost, logistical inconveniences, and the challenges in continuing the BLV control measures drives the farmers' reluctance to implement control measures (Kuczewski et al., 2022). It is crucial to acknowledge that these processes are time-consuming and require a long-term commitment from farmers (Kuczewski et al., 2021b).

In the context of controlling BLV, the understanding of persistent lymphocytosis became crucial. It is indicated that typically a non-lymphocytotic, BLV-seropositive cow has up to 5% of their circulating lymphocytes with integrated BLV proviruses. This proportion rises up to 25-35% in BLV-seropositive cows that develop persistent lymphocytosis. This variation in the

proportion of BLV proviruses in the peripheral circulation means that a persistently lymphocytotic cow may require lesser amount of blood to establish a new BLV infection in non-infected cows (Buxton and Schultz, 1984). Additionally, this brought forward a concept that not all BLV-infected cows are equally capable of transmitting BLV infection. This concept has been further validated by the BLV proviral load studies that indicate the risk of BLV transmission is higher among the BLV positive cows with a high proviral load. Consequently, BLV proviral load assessment is identified as a robust tool for BLV control.

The high BLV prevalence scenario in North American dairy herds means that implementing test and cull approach on all BLV-infected cows is not financially possible for BLV control. Recent studies in the US using selective removal of high proviral load (HPL) BLV-seropositive cows as the fundamental tool have produced promising results in reducing the BLV prevalence and new infection within the herd (Ruggiero et al., 2019; Taxis et al., 2020, 2023). However, for farmers to embrace this control strategy, it is imperative to convince farmers of the financial advantages associated with removal of HPL cows, particularly in the context of milk production. Furthermore, this approach needs validation using a large number of herds in the Canadian context, as the dairy systems are different between the US and Canada. Additionally, farmers need to get acquainted with the frequency of proviral load monitoring necessary to detect any evolving HPL cow over time. This clarifies the financial implications of HPL monitoring, as qPCR tests can be costly. In order to motivate farmers and veterinarians to embrace this technology, the BLV proviral load categorization needs to be made simpler. And lastly, a robust system of HPL identification using commonly agreed proviral load cut-off is required. In this thesis, I try to address these critical BLV associated questions, which are outlined in the following overview, chapters and objectives.

1.2 Overview of the thesis

The overall aim of this thesis was to evaluate the impact of BLV proviral load on the health and productivity of dairy cows, to examine the efficacy of BLV control programs focused on HPL cow removal, and to establish BLV proviral load cut-offs that can distinguish BLV-infected cows with high proviral loads.

Firstly, the importance of differentiating BLV-seropositive cows based on their BLV proviral load was investigated by evaluating the impact of BLV seropositivity and proviral load on the 305-days milk, fat, and protein production of dairy cows (Chapter 2). This chapter establishes a foundation for appreciating the economic implications of removing HPL cows, as a supplement to previous knowledge of higher risk of transmission in HPL cows (as mentioned above). It sets the context for chapter 3 to implement a voluntary BLV control program in ten dairy herds in Alberta, Canada. This chapter evaluates the impact of selective removal of HPL BLV-seropositive cows on the within-herd BLV prevalence and seroconversions. With categorization of BLV infection based on seropositivity and proviral load, it becomes imperative to explore the changes in proviral load, hematological, and immunological parameters in these groups over time (Chapter 4). These information consequently guide the responses to BLV-infection and frequency of disease monitoring. There is a dire need of defining a HPL cow based on scientific, pathological, and immunological evidence by establishing a clear BLV proviral load cut-off value. Therefore, a study was conducted by leveraging the knowledge of BLV's influences on WBC and lymphocyte count to direct a cut-off value of BLV-proviral load that accurately classifies a HPL cow (Chapter 5). Finally, the key findings of this doctoral research, its implication, limitations, and future directions are discussed (Chapter 6).

1.3 Research hypothesis and objectives

The hypothesis and objectives driving the chapters in this doctoral thesis are outlined as below:

Chapter 2 Hypothesis: The HPL BLV-seropositive cows produce less 305-d kg milk (M305), fat (F305), and protein (P305) production compared to LPL BLV-seropositive and BLV-seronegative cows.

Objective: To assess the difference in the actual 305-d kg milk (M305), fat (F305), and protein (P305) production between BLV-seropositive (further differentiated into HPL and LPL) and BLV-seronegative cows.

Chapter 3 Hypothesis: Selective removal of high proviral load BLV-infected animals aids in reducing BLV prevalence and incidence rate within the herd.

Objective: To assess the impact of selective removal of high proviral load BLV-infected animals over a 3-year period on the within-herd BLV prevalence and incidence rate of new BLV infections in ten Canadian dairy herds.

Chapter 4 Hypothesis: The proviral load, WBC, lymphocyte, antibody titer, and CD21+ increases and CD3+, CD4+, CD8+, and WC1+ decreases with time in a BLV-infected cow.

Objective: To evaluate the variability of BLV proviral load, lymphocyte count, WBC count, BLV antibody titer, and immune cell proportions (CD3+, CD4+, CD8+, CD21+, and WC1+) in BLV-seropositive (further categorized based on proviral load as high (HPL), moderate (MPL),

low (LPL), and not-detected (ND; antibody-positive, provirus-negative) and BLV-seronegative adult dairy cows within a 1.5 year timeframe.

Chapter 5 Hypothesis: BLV-infected cows in HPL and MPL group have similarities in terms of higher proviral load, WBC count, lymphocyte count, antibody titer, and CD21+ percentage, and lower CD3+, CD4+, CD8+, and WC1+ percentage when compared to LPL, ND, and BLV-seronegative cows. Strong positive correlation of BLV proviral load with WBC and lymphocyte count will provide information to dictate a cut-off for HPL BLV-seropositive cow, that is more robust than the ones currently being used.

Objectives: i) To evaluate the impact of BLV infection on hematological (WBC, lymphocyte, monocyte, and granulocyte counts) and immunological (BLV antibody titer, CD3+, CD4+, CD8+, CD21+, and WC1+ cell population) parameters by comparing cows based on BLV seropositivity (BLV-positive and BLV-seronegative) and BLV proviral load status [high (HPL), moderate (MPL), low (LPL), and not detected (ND)].

ii) To determine cut-off values of BLV proviral load and antibody titer for distinguishing high proviral load cows based on their white blood cell and lymphocyte counts.

CHAPTER 2: Effects of bovine leukemia virus seropositivity and proviral load on milk, fat, and protein production of dairy cows.

2.1 Abstract

The objective was to evaluate the effects of bovine leukemia virus (BLV) infection, as determined by BLV seropositivity and proviral load, on 305-d milk, fat, and protein production of dairy cows. A cross-sectional study was conducted among 1,712 cows from 9 dairy herds in Alberta, Canada. The BLV status was assessed using an antibody ELISA, whereas BLV proviral load in BLV-seropositive cattle was determined with quantitative PCR. Dairy Herd Improvement 305-d milk, fat, and protein production data were obtained for all enrolled cattle. Differences in these milk end points were assessed in 2 ways: first, by categorizing cows based on BLV serostatus (i.e., BLV positive or negative), and second, by categorizing based on BLV proviral load (i.e. BLV-negative, low proviral load [LPL] BLV positive, and high proviral load [HPL] BLV-positive). A mixed-effect multivariable linear regression model was used to assess differences in milk parameters. We found that BLV positivity, adjusted for parity and natural log-transformed somatic cell count (SCC), was not associated with reduction in 305-d milk, fat, or protein production. However, significant reductions in 305-d milk, fat, and protein yield occurred in HPL cows, but not in LPL cows, compared to BLV-negative cows, when adjusted for parity number and natural log-transformed SCC. In summary, BLV proviral load may predict effects of BLV infection on milk, fat, and protein production.

Key words: BLV, bovine leukosis, proviral load, production

2.2 Introduction

Bovine leukosis, caused by bovine leukemia virus (BLV), is an endemic viral disease in dairy and beef herds of North America. Controlling bovine leukosis in this region is challenging, as several factors hinder elimination of BLV. First, herd- and animal-level BLV prevalence in the US and Canadian dairy herds are 90 and 40%, respectively, and have been at these levels for the last 20 years (VanLeeuwen et al., 2005; LaDronka et al., 2018; Kuczewski et al., 2019). Second, in North America there are insufficient organized BLV control programs, supported by the livestock industry or government. Finally, in the majority of the infected cattle, BLV causes a subclinical infection, making the disease difficult to detect without laboratory testing and for producers to appreciate its effects (Schwartz and Levy, 1994).

BLV belongs to the family *Retroviridae* (ICTV, 2022) which mostly spreads horizontally, primarily through transmission of infected B-lymphocytes in blood; however, transmission also occurs through milk, colostrum, and intrauterine infection (Rodríguez et al., 2011; Sajiki et al., 2017). Following entry in the host, the virus creates a DNA copy of its genomic RNA and integrates this into the DNA of infected cells in the host, thus it is termed a provirus (Goff, 2013). Clinical manifestation of BLV infection is not apparent in the majority of the cases, as only one-third of infected cattle develop persistent lymphocytosis, due to a gradual polyclonal increment in BLV-infected cells over time (Florins et al., 2007; EFSA Panel on Animal Health and Welfare, 2015). The lymphocytosis phase can further evolve into lymphosarcoma, which occurs in 5% of infected cattle (Schwartz and Levy, 1994; EFSA Panel on Animal Health and Welfare, 2015). Although the majority of infected cattle appear healthy, infected individuals have higher likelihood of getting culled before reaching maximum lifetime production (Bartlett et al., 2013; Nekouei et al., 2016). One reason for increased risk of culling of

infected cattle may be increased susceptibility to secondary infections due to virus-induced disruption of immune function (Frie and Coussens, 2015). Another reason could be lower-than-expected milk production, although negative influence of BLV infection on milk production has not been established in many studies (Kale et al., 2007; Tiwari et al., 2007; Sorge et al., 2011). In contrast, other studies have reported a negative association of BLV positivity with milk production at a herd-level (Ott et al., 2003; Erskine et al., 2012b) or an individual level (Nekouei et al., 2016; Norby et al., 2016). These discrepancies in study outcomes may be due to several factors, including limitations inherent in cross-sectional nature of the studies, small sample sizes, and variations in data analyses (Nekouei et al., 2016).

Cattle infected with BLV can have variable numbers of BLV proviruses in their white blood cells (WBC); therefore, a distinction can be made between cattle with high proviral load (HPL) and low proviral load (LPL). As HPL cows have been linked with higher probability of transmitting the virus, removing this category of infected individual can be explored as a means of controlling within-herd transmission of BLV (Juliarena et al., 2016; Ruggiero et al., 2019; Kuczewski et al., 2021b). Although effects of proviral load on milk, fat, or protein production have apparently not been studied, BLV-positive cows that are lymphocytotic for 2 years have significantly lower milk and fat production than those that are non-lymphocytotic (Da et al., 1993). As HPL cows are at higher risk of being lymphocytotic (Ohno et al., 2015), it can be hypothesized that production of milk and its components may be lower in this population.

The objective of this study was to assess the difference in the actual 305-d kg milk (M305), fat (F305), and protein (P305) production between BLV-seropositive (further differentiated into HPL and LPL) and BLV-seronegative cows.

2.3 Materials and methods

2.3.1 Herd selection

Dairy producers were contacted after they expressed interest in a separate 3-year bovine leukemia virus control project. Ten dairy herds were evaluated if they met the following eligibility criteria: 1) willingness to remove HPL cows within 3 mo after identification; 2) farm located within 3-h driving distance from Calgary, Canada; 3) availability of a free-stall barn to house adult cattle; and 4) implementation of a fly control program. Upon completion of the project's first year, additional criteria, 5) DHI participation through Canada's DHIA Lactanet (Sainte-Anne-de-Bellevue, QC, Canada); and 6) authorization to use milk production records allowed 9 dairy herds to be enrolled for this cross-sectional study, as 1 herd was not a DHI participant. All procedures were approved by the Veterinary Sciences Animal Care Committee of the University of Calgary (Calgary, AB, VSACC AC17-0242, AC20-0095, and AC21-0210).

2.3.2 Data collection

Sampling and laboratory tests occurred between July 2020 and February 2021. For each herd, actual 305-d milk, fat, and protein yields, and current lactation average SCC data (start of lactation to sample collection month) of each cow were retrieved in 2023 for the parity in which the BLV sampling occurred. Only cows that were BLV tested and whose milk production records were available were included in the final analysis. Individual cow data collected from Lactanet Canada included M305, F305, P305, parity number, breed, and SCC. Data for M305, F305, and P305 were determined by DHI at the end of the corresponding lactation.

2.3.3 Sample collection and processing

Milk samples (25-40 mL) of all lactating cows were collected aseptically during routine milking by hand stripping into a clean 50-mL container containing a preservative tablet (Broad Spectrum Microtabs II, Advanced Instruments, Norwood, MA). Blood samples of all cows that were dry on the day of sampling were collected by venipuncture of coccygeal blood vessels using a serum tube with clot activator and a tube with K3-EDTA (Vacutainer, BD, Franklin Lakes, NJ). All samples were transported to the University of Calgary (Calgary, AB, Canada) laboratory in a cooler with cool packs, stored at 4°C, and processed within 48 h after sample collection.

Milk samples were centrifuged at 5,000 x g, 20 min, 4°C for skim milk separation and stored at –20°C until tested. Blood samples with clot activator were used for serum separation after centrifugation at 1,500 x g for 10 min at 10°C, and serum aliquots were stored at –20°C. Milk and serum samples were used to determine BLV infection status using an antibody ELISA (milk: Bovichek BLV ELISA kit, Biovet Inc., Montreal, QC, Canada; serum: Leukosis Serum X2 Ab Test, IDEXX Laboratories, Inc., Westbrook, ME), using the manufacturers' protocols. However, farm G (Table 2-1) used Lactanet to determine BLV status of the individual animals. The ELISA results from milk and serum were interpreted as BLV positive, BLV suspected or BLV negative, according to the manufacturer's protocol. During a follow-up visit, blood samples were collected from cows identified as BLV positive based on milk ELISA test results. When blood samples were taken at the first visit from all cows in the herd or from the nonlactating cows, those samples were tested using the serum ELISA. A 200-µL aliquot of the blood sample with EDTA from all BLV-positive cows was stored at –80°C for a quantitative (q) PCR testing.

2.3.4 BLV proviral load determination

Genomic DNA was extracted from EDTA-treated blood samples using a Quick-DNA Miniprep Plus Kit (Zymo Research, Irvine, CA), following manufacturer's instructions. DNA concentration was measured using a Qubit 3.0 Fluorometer and dsDNA BR Assay Kit (Invitrogen, Waltham, MA). DNA samples were normalized to ≤50 ng/µL and used for proviral load quantification using a BLV SS1 qPCR assay (CentralStar Cooperative Inc., East Lansing, MI). This assay is a multiplex probe-based system that uses standard curves generated using plasmids containing the BLV polymerase gene, and bovine Beta-Actin gene target sequences to calibrate the qPCR and determine BLV proviral load (Taxis et al., 2020). Each qPCR reaction contained 2 µL of DNA, 12.5 µL of PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Inc., Coralville, IA), 1.25 µL of 20X BLV SS1 Primer Mix, and 9.25 µL of nuclease-free water. Duplicates of each sample were analysed only once using the following protocol: 95°C for 10 min, followed by 40 cycles of each 95°C for 15 s and 60°C for 1 min in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The BLV proviral load was calculated by dividing the number of BLV copies by the number of Beta-Actin copies and interpreted as a ratio. Samples that were BLV ELISA positive but were negative in the qPCR results, or where provirus detection values were lower than 0.25, were categorized as LPL, and those with values ≥0.25 were classified as HPL. Additionally, proviral load categories were also classified as LPL (<0.25), moderate proviral load (MPL; 0.25-0.50), and HPL (≥0.50), to determine whether differences in proviral load should be grouped into 2 or 3 categories.

2.3.5 Data management

The outcome variables used for this study were M305, F305, and P305 (continuous variables) of the individual cow. Three different approaches were used. In the first, BLV status of each cow was used as a binary exposure variable (BLV positive or BLV negative). Two ELISA results classified as suspect according to the ELISA assay's instruction were excluded to remove misclassification bias. In the second approach, BLV proviral load of each cow was used as a categorical exposure variable (BLV negative, LPL [<0.25] and HPL [≥0.25]). In the last approach, BLV proviral load was additionally categorized into 4 groups: BLV negative, LPL (<0.25), MPL (≥0.25 to <0.50), and HPL (≥0.50). Explanatory variables such as parity number, breed, calving season, and SCC were assessed for association with M305, F305, and P305. Parity was categorized into 4 categories as 1, 2, 3 and ≥4. Breed of cow was categorized as Holstein Friesian, Jersey, Ayrshire, or Brown Swiss. Calving season was represented as winter, spring, summer, or fall. Current lactation SCC was calculated as the average of the SCC of a cow in the current lactation up to the month of sample collection. A natural logarithmic transformation was conducted to generate LnSCC.

2.3.6 Statistical analyses

Data from individual cows were exported into Microsoft Excel (Microsoft Corp., Redmond, WA) and analyzed using Stata 15.1 (StataCorp LLC, College Station, TX). Associations of M305, F305, and P305 with each explanatory variable was determined using a regression model, and variables' associations at $P < 0.20$ were included in a mixed-effect multivariable linear regression model. Multicollinearity among all explanatory variables were assessed through Spearman's rank correlation and those correlated ($r \geq 0.60$) were further

investigated for biological plausibility before being included in the model. For models with BLV status as the exposure variable, 2-way interaction terms were created between BLV status and parity, BLV status and breed, BLV status and calving season, and BLV status and LnSCC to assess combined effects of those variables in the model. For models with BLV proviral load as the exposure variable, 2-way interaction terms of proviral load with parity, breed, LnSCC, and calving season (for F305) were created. Farm was included as a random effect. In the multivariable model, stepwise elimination was used to retain significant variables ($P \leq 0.05$). The likelihood ratio test was used to compare models with and without interaction terms; however, when the difference was not significant ($P > 0.05$), interaction terms were excluded. Confounding variables were defined as those variables that, if removed from the model, changed the coefficient of exposure variable (BLV, LPL or HPL) by >20%. Confounding variables were retained in the model. BLV status or proviral load were retained in the model as they were the exposure variables. The distribution of the residuals was assessed visually for normality and homoscedasticity. The R^2 value was calculated as the square of the correlation between observed response and the predicted response of the final model. Finally, a likelihood ratio test was conducted between models containing proviral load as 3 or 4 categories.

2.4 Results

The initial dataset contained 1,909 cattle tested for BLV antibodies via ELISA, of which 654 (34.3%) were BLV positive and 1,253 were BLV negative, whereas 2 cows were BLV suspected and not included in the analysis (Table 2-1). Median milking group size was 193 cows (range: 81-402 cows), and a median within-herd BLV prevalence of 33.9% was found (range: 9.2-52.2%). Furthermore, 195 cows (23 HPL, 31 LPL, and 141 BLV-negative cows) did not

have milk production data and were removed from the analysis (Table 2-1). Therefore, the final data set for the analysis contained 1,712 cows. The majority of the data set represented Holstein Friesian breed (n = 1,680 cows), followed by Brown Swiss (n = 12 cows), Jersey (n = 10 cows), and Ayrshire (n = 10 cows). Descriptive summaries of the outcome variables (M305, F305, and P305) and predictor variables (proviral load, parity, LnSCC) is presented in Table 2-2.

2.4.1 Effect of BLV seropositivity on M305, F305, and P305

The explanatory variables did not show multicollinearity. In all univariate models with M305, F305, or P305 as the outcome variable, BLV status, parity, and breed were included ($P < 0.20$), whereas LnSCC ($P > 0.20$) was forced into the mixed-effect multivariable linear regression model for further analysis. Calving season was included ($P = 0.09$) only in the F305 model. Adding the interaction terms of BLV and parity, BLV and breed, BLV and LnSCC, and BLV and calving season (for F305) did not improve the model ($P > 0.05$) from the model without the interaction terms in the likelihood ratio test; therefore, no effect modification by parity, breed, LnSCC, or calving season was observed. However, confounding effects of parity and LnSCC (M305 and P305 models) and parity only (F305 model) were observed in the final models, as removal of these variables changed the coefficients of BLV variable by >20%. Breed and LnSCC were forced in the model irrespective of their observed confounding effects, because these variables are typically strongly correlated with the outcome variables, and removing them would result in loss of valuable information.

The M305, F305, and P305 was not different between BLV-positive and BLV-negative cows when adjusted for parity (F305) or parity and LnSCC (M305 and P305; Table 2-3).

2.4.2 Effect of BLV proviral load on M305, F305, and P305

Explanatory variables did not show multicollinearity. Parity and breed ($P < 0.20$) were included, and LnSCC ($P > 0.20$) was forced into all the 3 models, whereas calving season ($P < 0.20$) was considered only for the F305 model. Inclusion of the interaction terms of proviral load with parity, breed, LnSCC, and calving season (for F305 model) did not result in different coefficients ($P > 0.05$) from the model without interaction terms. Only parity showed a confounding effect. Breed and LnSCC were forced in all 3 models.

Adjusted for parity, M305, F305, and P305 were 294, 13, and 9 kg lower, respectively, for HPL than for BLV-negative cows (Table 2-4). No significant difference in M305, F305, and P305 was observed for LPL compared to BLV-negative cows.

Variance explained by the variables was 28% for the M305 model and 27% for the F305 and P305 models. The intraclass correlation coefficient (ICC) was 0.23, 0.17, and 0.22 for the M305, F305, and P305 models, respectively.

2.4.3 Effect of BLV proviral load as four categories on M305, F305, and P305

When BLV proviral load was categorized into 4 groups (BLV negative, LPL, MPL, and HPL), only MPL cows produced less milk, fat, and protein than BLV-negative cows (Table 2-5). However, keeping the BLV proviral load as 3 or 4 categories did not make the models significantly different for M305 ($P = 0.48$), F305 ($P = 0.95$), or P305 ($P = 0.90$).

2.5 Discussion

This study evaluated the effects of BLV seropositivity and BLV proviral load on M305, F305, and P305 in dairy cows at the individual animal level. We found that BLV positivity,

regardless of proviral load status, was not associated with M305, F305, or P305. However, when BLV-positive cows were further categorized based on BLV proviral load, HPL cows had significantly lower M305, F305, and P305 than BLV-negative cows.

Earlier studies have examined associations between BLV infection and milk production in individual cows, using seropositivity. Similar to our findings, some studies reported no difference in milk production between cows that were positive and negative for BLV (Tiwari et al., 2007; Sorge et al., 2011). However, in contrast, other studies reported lower milk production in BLV-positive cows (Nekouei et al., 2016; Norby et al., 2016). A possible explanation for this discrepancy is that these studies included equal representation of cows from different parities using larger number of herds, which may have increased the power of the studies and allowed them to detect differences in milk production due to BLV infection. Additionally, the study by Nekouei et al. (2016) used a historical cohort, allowing them to integrate the gradual progression of BLV infection and its effect on milk production, which was not possible in a cross-sectional study such as ours.

When BLV-positive cows were further categorized into HPL and LPL, and compared with BLV-negative cows, a decrease in milk, fat, and protein production was detected in HPL cows but not in LPL cows. This provided evidence that BLV status (positive vs. negative) alone cannot be used to assess the effects of the disease. A BLV-positive cow could have been infected recently or months or even years ago; therefore, it is reasonable to assume that effects of infection due to differences in duration may be very different in these cattle (Kobayashi et al., 2019). Additionally, when grouping all BLV-positive animals in 1 category, LPL and HPL cows are grouped together as BLV-positive cows, and the influence of proviral load is not detectable.

Historically, high BLV ELISA optical density values (Norby et al., 2016) or persistent lymphocytosis, a clinical advanced stage of BLV infection (Da et al., 1993), have been associated with declines in milk and fat production. We can compare these results against our findings of lower milk, fat, and protein production in BLV HPL cows because high BLV ELISA optical density values and persistent lymphocytosis are associated with high proviral loads (Juliarena et al., 2007; Jimba et al., 2010).

No consensus currently exists on the threshold to define HPL cows. Various studies use a variety of definitions such as ≥0.50 BLV copies/cell (Taxis et al., 2020), >100,000 copies/μg of DNA (Juliarena et al., 2007), >1% of infected cells (Alvarez et al., 2013), >500 copies/50 ng of DNA (Mekata et al., 2018) or ≥100,000 copies/10^5 cells (Ruggiero et al., 2019). In our study, we defined HPL as ≥0.25 (ratio of BLV copies per Beta-Actin copy), which includes proviral loads between 0.25 and 0.5. We did not observe any difference between models using BLV proviral load as 3 or 4 categories, as described in the Methods section. Therefore, categorizing HPL cows as those with ≥0.25 was justifiable.

Milk production data are prone to selection bias as records of cows with abnormally low milk production or cows that will get culled are excluded (Norby et al., 2016); this may have influenced our analysis. One way this could be addressed is through testing cows for BLV infection and proviral load when they are removed from the herd; however, this is logistically challenging. Additionally, milk production data may be subjected to survivor bias, as only cows that are producing well are likely to remain in the herd, irrespective of their BLV status or proviral load. Therefore, milk production from a BLV-positive cows or HPL cows might not be an exact representation of BLV infection, as these cows have survived selection and are superior in terms of milk production. Another instance of survivor bias would occur if HPL cows were

intentionally removed before the end of their lactation, making their 305-d milk data unavailable. However, we do not have specific information on producer's culling strategies. Moreover, in the data analysis, cows from LPL and BLV-negative groups were also excluded due to missing information. Therefore, any survivor bias from such circumstances are assumed to be minimal.

Several aspects of the analysis were taken into consideration but were ultimately excluded from the current results. One of these was the inclusion of only Holstein Friesian cows. When we excluded Brown Swiss, Jersey, and Ayrshire cows (n = 32), the resulting *P*-value of our analysis was even smaller, indicating a higher level of significance. Additionally, we explored the potential effect of milking frequency, by categorizing milkings as twice-a-day, thrice-a-day, and robotic milking. This failed to demonstrate any interaction or confounding effect in our analysis and, therefore, was not part of the final model.

Proviral load assessment has recently become an important component of BLV control programs, as HPL cows pose a higher BLV transmission risk and removal of these cows can reduce on-farm BLV prevalence (Ruggiero et al., 2019; Taxis et al., 2020; Kuczewski et al., 2021b). High BLV proviral load is also associated with development of lymphoma and lymphocytosis (Jimba et al., 2010; EFSA Panel on Animal Health and Welfare, 2015). Our study could provide additional motivation for farmers to control BLV in their herds by examining differences in milk production caused by BLV infection and proviral load. In a Canadian dairy system, where dairy farmers are paid based on butter fat and protein, findings from our study implied that there are incentives for controlling BLV in a dairy herd. However, it is important to note that the results from our study were based on only 9 herds in Alberta, Canada, and therefore, generalization of these findings should be performed with caution.

2.6 Conclusions

Understanding the effects of bovine leukosis on animal-level production is challenging when information on BLV infection is limited. Therefore, BLV proviral load information was used to assess differences in milk, fat, and protein production associated with BLV infection. We found no differences in M305, F305, or P305 when comparisons were made between BLV-seropositive and BLV-negative cows. However, consideration of BLV proviral load showed significant reductions of M305, F305, and P305 in HPL cows compared to BLV-negative cows. Therefore, considering BLV proviral load is important when assessing impacts of bovine leukosis.

Table 2-1. Details of the dairy farms participating in the study of bovine leukemia virus (BLV) in Canadian dairy herds

Farm	No. cows tested	No. cows included in the analysis	Breed[1]	Daily milking frequency	Within-herd BLV prevalence (95% CI)	LPL[2] point estimate (95% CI)	HPL[3] point estimate (95% CI)
A	180	167	Mixed	Thrice	33.9 (26.2 to 41.6)	0.06 (0.04 to 0.09)	0.37 (0.30 to 0.44)
B	402	359	HF	Thrice	52.2 (47.5 to 57)	0.07 (0.06 to 0.08)	0.42 (0.39 to 0.44)
C	215	195	HF	Twice	44.7 (36.3 to 53)	0.08 (0.05 to 0.10)	0.41 (0.37 to 0.44)
D	193	179	HF	Twice	47.7 (38.8 to 56.6)	0.08 (0.05 to 0.10)	0.38 (0.35 to 0.41)
E	81	77	HF	Robotic	13.6 (6.3 to 20.8)	0.12 (0.004 to 0.24)	0.40 (0.29 to 0.50)
F	142	129	Mixed	Twice	9.2 (4.8 to 13.5)	0.12 (–0.14 to 0.37)	0.37 (0.29 to 0.45)
G	299	271	HF	Thrice	19.7 (14.5 to 25)	0.06 (0.03 to 0.09)	0.48 (0.42 to 0.54)
H	216	175	Mixed	Twice	43.1 (34.8 to 51.3)	0.06 (0.04 to 0.08)	0.48 (0.43 to 0.53)
I	181	160	HF	Twice	10.5 (5.9 to 15.1)	0.04 (0.01 to 0.06)	0.48 (0.26 to 0.71)

[1]HF = Holstein Friesian, Mixed = mix of Holstein Friesian, Jersey, Brown Swiss, or Ayrshire breeds.

[2]LPL = low proviral load: ≥0 to <0.25.

[3]HPL = high proviral load: ≥0.25.

Table 2-2. Summary statistics of variables used for assessing association of bovine leukemia virus (BLV) status and BLV proviral load (BLV copies per Beta-Actin copy) with 305-d milk (M305), fat (F305), and protein (P305) production of cows in participating dairy herds

Variable	**No. cows**	**Mean**	**SD**	**Minimum**	**25th percentile**	**50th percentile**	**75th percentile**	**Maximum**
M305 (kg)	1,712	11,816	2,316	3,368	10,163	11,670	13,396	20,448
F305 (kg)	1,712	481	94	107	418	469	539	826
P305 (kg)	1,712	384	70	102	336	379	431	623
Proviral load	600	0.22	0.20	0	0.02	0.20	0.37	0.80
Parity	1,712	2.3	1.4	1	1	2	3	9
LnSCC[1]	1,712	3.9	1.2	1.1	3	3.6	4.6	9

[1] = Log-normal transformed average of SCC in current lactation (cells/mL × 1,000).

Table 2-3. Mixed-effect multivariable analysis model for measuring association of bovine leukemia virus (BLV) positivity (+: positive, –: negative) with 305-d milk, fat and protein production (kg; n=1,712)[1]

	305-d milk production				**305-d fat production**				**305-d protein production**			
Item	Coefficient	SE	*P*-value	95% CI	Coefficient	SE	*P*-value	95% CI	Coefficient	SE	*P*-value	95% CI
Fixed effects												
Intercept	10,927	345	<0.001	10,251 to 11,603	445	13	<0.001	420 to 470	354	11	<0.001	333 to 374
BLV status												
BLV–	Referent				Referent				Referent			
BLV+	–57	100	0.57	–252 to 139	–4	4	0.3	–13 to 4	–1	3	0.66	–7 to 5
Parity			<0.001				<0.001				<0.001	
1	Referent				Referent				Referent			
2	1,768	109	<0.001	1,555 to 1,981	70	5	<0.001	60 to 79	59	3	<0.001	52 to 65
3	2,433	124	<0.001	2,189 to 2,676	100	5	<0.001	90 to 111	74	4	<0.001	67 to 82
≥4	2,968	128	<0.001	2,718 to 3,218	119	6	<0.001	109 to 130	85	4	<0.001	77 to 92

Breed			<0.001				<0.001				<0.001	
Holstein Friesian	Referent				Referent				Referent			
Jersey	–2,246	556	<0.001	–3,335 to –1,157	–44	24	0.07	–90 to 3	–46	17	0.007	–80 to –13
Ayrshire	–1,790	551	<0.001	–2,870 to –709	–98	24	<0.001	–145 to –52	–65	17	<0.001	–98 to –31
Brown Swiss	–323	507	0.52	–1,316 to 671	–12	22	0.58	–55 to 30	20	16	0.21	–11 to 50
LnSCC[2]	–166	35	<0.001	–236 to –97	–6	2	<0.001	–9 to –3	–5	1	<0.001	–7 to –2
Random effect variance												
Herd	868,267	418,109		337,880 to 2,231,231	1,136	553		437 to 2,951	790	381		307 to 2,033

[1]R^2 = 0.28 (305-d milk [M305] model), 0.27 (305-d fat [F305] and 305-d protein [P305] models). Intraclass correlation coefficient = 0.23 (M305 model), 0.17 (F305 model), and 0.22 (P305 model).

[2]Log-normal transformed average of SCC in current lactation (cells/mL × 1,000).

Table 2-4. Mixed-effect multivariable analysis model for measuring association of bovine leukemia virus (BLV) proviral load with 305-d milk, fat and protein production (kg; n=1,712)[1]

Item	305-d milk production				305-d fat production				305-d protein production			
	Coefficient	SE	*P*-value	95% CI	Coefficient	SE	*P*-value	95% CI	Coefficient	SE	*P*-value	95% CI
Fixed effects												
Intercept	10,931	345	<0.001	10,256 to 11,607	445	13	<0.001	420 to 470	354	10	<0.001	333 to 374
BLV status[2]			0.01				0.04				0.01	
BLV–	Referent				Referent				Referent			
LPL	113	116	0.33	–113 to 340	2	5	0.76	–8 to 11	4	4	0.27	–3 to 11
HPL	–294	129	0.02	–548 to –41	-13	6	0.02	–24 to –2	–9	4	0.03	–17 to –0.9
Parity			<0.001				<0.001				<0.001	
1	Referent				Referent				Referent			
2	1,780	108	<0.001	1,567 to 1,992	70	5	<0.001	61 to 79	59	3	<0.001	53 to 66

3	2,442	124	<0.001	2,199 to 2,684	101	5	<0.001	90 to 111	75	4	<0.001	67 to 82
≥4	2,982	128	<0.001	2,732 to 3,232	120	6	<0.001	109 to 131	85	4	<0.001	77 to 93
Breed			<0.001				<0.001				<0.001	
Holstein Friesian	Referent				Referent				Referent			
Jersey	–2,333	555	<0.001	–3,421 to –1,245	–47	24	0.05	–93 to 0.06	–49	17	0.004	–83 to –15
Ayrshire	–1,831	550	0.001	–2,909 to –753	–100	24	<0.001	–146 to –53	–66	17	<0.001	–99 to –32
Brown Swiss	–375	506	0.46	–1,366 to 617	–14	22	0.52	–57 to 29	18	16	0.25	–13 to 49
LnSCC[3]	–169	35	<0.001	–238 to –99	–6	2	<0.001	–9 to –3	–5	1	<0.001	–7 to –2
Random effect variance												
Herd	866,770	417,351		337,326 to 2,227,195	1,131	551		435 to 2,939	787	380		306 to 2,026

[1]R^2 = 0.28 (305-d milk [M305] model), 0.27 (305-d fat [F305] and 305-d protein [P305] models). Intraclass correlation coefficient = 0.23 (M305 model), 0.17 (F305 model), and 0.22 (P305 model).

[2]BLV–: BLV-negative cows. BLV-positive cows further categorized into low proviral load (LPL: ≥0 to <0.25) and high proviral load (HPL: ≥0.25).

[3]Log-normal transformed average of SCC in current lactation (cells/mL × 1,000).

Table 2-5. Mixed-effect multivariable analysis model for measuring association of bovine leukemia virus (BLV) proviral load on 305-d kg milk, fat, and protein production (kg), when BLV proviral load was categorized into 4 categories (n=1,712)[1]

Item	305-d milk production				305-d fat production				305-d protein production			
	Coefficient	SE	*P*-value	95% CI	Coefficient	SE	*P*-value	95% CI	Coefficient	SE	*P*-value	95% CI
Fixed effects												
Intercept	10,932	345	<0.001	10,257 to 11,607	445	13	<0.001	420 to 471	354	10	<0.001	333 to 374
BLV status[2]			0.03				0.09				0.04	
BLV–	Referent				Referent				Referent			
LPL	115	116	0.32	–112 to 341	2	5	0.76	–8 to 11	4	4	0.27	–3 to 11
MPL	–335	141	0.02	–612 to –58	–13	6	0.03	–25 to –1	–9	4	0.04	–17 to –0.3
HPL	–151	239	0.53	–619 to 317	–12	10	0.24	–32 to 8	–8	7	0.28	–22 to 6
Parity			<0.001				<0.001				<0.001	
1	Referent				Referent				Referent			
2	1,782	108	<0.001	1,569 to 1,994	70	5	<0.001	61 to 79	59	3	<0.001	53 to 66
3	2,435	124	<0.001	2,192 to 2,679	101	5	<0.001	90 to 111	74	4	<0.001	67 to 82

≥4	2,982	127	<0.001	2,732 to 3,232	120	6	<0.001	109 to 131	85	4	<0.001	77 to 93
Breed			<0.001				<0.001				<0.001	
Holstein Friesian	Referent				Referent				Referent			
Jersey	–2,329	555	<0.001	–3,417 to –1,241	–47	24	0.05	–94 to –0.04	–49	17	0.004	–83 to –15
Ayrshire	–1,841	550	0.001	–2,920 to –763	–100	24	<0.001	–146 to –53	–66	17	<0.001	–99 to –33
Brown Swiss	–375	506	0.46	–1,366 to 617	–14	22	0.52	–57 to 29	18	16	0.25	–13 to 49
LnSCC[3]	–169	35	<0.001	–238 to –99	–6	2	<0.001	–9 to –3	–5	1	<0.001	–7 to –2
Random effect variance												
Herd	866,915	417,419		337,383 to 2,227,562	1,131	551		435 to 2,939	787	380		306 to 2,026

[1]R^2 = 0.28 (305-d milk [M305] model), 0.27 (305-d fat [F305] and 305-d protein [P305] models). Intraclass correlation coefficient = 0.23 (M305 model), 0.17 (F305 model), and 0.22 (P305 model).

[2]BLV–: BLV-negative cows. BLV-positive cows further categorized into low proviral load (LPL: ≥0 to <0.25), moderate proviral load (MPL: ≥0.25 to <0.5), and high proviral load (HPL: ≥0.5).

[3]Log-normal transformed average of SCC in current lactation (cells/mL × 1,000).

CHAPTER 3: Removing bovine leukemia virus infected animals with high proviral load leads to lowering within-herd prevalence and new case reduction.

3.1 Abstract

Bovine leukosis is prevalent in the North American dairy industry and its effect on animal health and production is widely documented. However, not all bovine leukemia virus (BLV) infected animals transmit the virus equally. Animals with high BLV proviral loads (HPL) are associated with higher transmission risks and therefore, their removal may reduce transmission and eventually within-herd prevalence. We aimed to evaluate the impact of selectively removing HPL cows on the within-herd BLV prevalence and incidence rate of new BLV infection in ten dairy herds. Annual blood and/or milk samples were collected from adult cows over three years. ELISA tests determined the BLV positivity of animals and BLV SS1 quantitative PCR assays estimated proviral loads in blood of BLV-positive animals. Herd managers were encouraged to consider the proviral load when making culling decisions and implement BLV control practices. HPL cows had the highest relative risk of removal indicating the farmers prioritized HPL cows for culling. The within-herd BLV prevalence decreased significantly in four herds whereas the BLV incidence rate decreased in nine herds. Evaluation of changes in proviral load over the three years indicated a relative stable level of proviral load, suggesting a single proviral load test in an adult cow may suffice to make culling decisions.

Key words: bovine leukosis, prevalence, proviral load, control, transmission

3.2 Introduction

Bovine leukosis, a common infectious disease of bovines, is caused by Bovine Leukemia Virus (BLV), a retrovirus. The virus is transmitted horizontally through blood, milk or colostrum (Hopkins and Digiacomo, 1997). While 22 European countries (European Commission, 2020), as well as Australia and New Zealand (Voges, 2009, Queensland Government Department of Agriculture and Fisheries, 2016), have successfully eradicated BLV from their dairy herds, North America still faces significant challenges in controlling the disease. In this region, nearly 90% of the dairy herds are infected, with an average of 40% BLV-infected animals per herd (Nekouei et al., 2015a; LaDronka et al., 2018; Kuczewski et al., 2019). This high level of BLV prevalence, together with the lack of a commercial vaccine or an organized BLV control program, has led to a gradual increase in BLV prevalence.

BLV-infected animals often do not display clinical signs, only about a third of the infected exhibit persistent lymphocytosis and approximately 5% of the infected animals will develop lymphoma (Schwartz and Levy, 1994; EFSA Panel on Animal Health and Welfare, 2015). Additionally, there are significant economic and production related consequences. One major implication is the suppression of the host's immune system (Frie and Coussens, 2015), increasing the likelihood of production, longevity, and welfare deficits. To elaborate, reduction in 305-days milk, fat, and protein in BLV-infected animals, particularly in cows with high BLV proviral load (Shrestha et al., 2024), higher culling rates of positive animals (Bartlett et al., 2013; Nekouei et al., 2016), and tumors leading to carcass condemnation (Agriculture and Agri-Food Canada, 2023) are observed. With many countries residing to importing cattle from BLV-free herds only, and growing consumer awareness on animal welfare, it is economically justified to

focus on reducing BLV infection in herds and implement control measures to mitigate its impact (Kuczewski et al., 2019).

Controlling BLV infection in high prevalent regions is a complex and time-consuming process that requires a multifaceted approach, including testing for BLV-infected animals, segregating BLV-positive individuals, implementing management practices to minimize virus transmission, and selectively culling infected animals (Kuczewski et al., 2021a). Successful BLV elimination from various countries has demonstrated that this process still requires a long, collaborative approach at the national level (Nuotio et al., 2003; Acaite et al., 2007). In North America, the within-herd prevalence is often very high (LaDronka et al., 2018; Kuczewski et al., 2019), making it impractical to eliminate all infected animals at once. Additionally, the presence of a milk quota system in Canada further complicates the situation. Therefore, there is a need of a control strategy that can be embraced by dairy farmers, which is practically and economically feasible. Quantifying the BLV proviral load, which is the number of BLV genome copies integrated into the host's genome, is being used in BLV risk assessment (Jimba et al., 2010). This provides an opportunity to identify BLV-infected cows with a high proviral load (HPL), that are considered to have higher risk of transmitting the virus (Mekata et al., 2015; Juliarena et al., 2016), and of developing lymphocytosis and enzootic bovine leukosis (Ohno et al., 2015; Kobayashi et al., 2020). Targeted removal of HPL BLV-infected animals with the aim to minimize virus transmission and reduce BLV prevalence to a manageable level is considered a reasonable approach in high prevalence situations. Based on this concept, two pilot studies conducted in the USA demonstrated encouraging results of reducing the within-herd BLV prevalence (Ruggiero et al., 2019; Taxis et al., 2020, 2023). The question remains if a similar strategy is leading to BLV prevalence reduction within a Canadian dairy production system.

Therefore, the objective of this study was to assess the impact of selective removal of high proviral load BLV-infected animals over a 3-year period on the within-herd BLV prevalence and incidence rate of new BLV infections in ten Canadian dairy herds.

3.3 Materials and methods

A three-year study was conducted by enrolling ten dairy herds that were interested to participate in a bovine leukosis control program from Alberta, Canada. All procedures involved with animals were approved by the Veterinary Sciences Animal Care Committee of the University of Calgary (Calgary, AB, Canada, VSACC AC17-0242, AC20-0095, and AC21-0210).

3.3.1 Study design

In the first year (2020), all adult cows (lactating, dry, and close-to-calving heifers) were screened for the presence of BLV antibodies in their milk or blood using an ELISA test (Figure 3-1). Blood samples from BLV-ELISA positive cows were used to determine the BLV proviral load using a qPCR. All test results were shared with the producers, and they were encouraged during farm visits to implement BLV control measures and cull cows with highest BLV proviral load within 3-months after obtaining the results. However, implementation of BLV control practices and culling were at the producer's discretion. In the second and third year, BLV-antibody screening was conducted only on previously BLV-negative and non-tested cows (replacement heifers), and BLV proviral load was determined on all BLV-seropositive cows. BLV control focused management changes in herds were captured through informal discussions during farm visits. At the end of the study, change in within-herd BLV prevalence and incidence risk was assessed on ten herds over the three years of study.

3.3.2 Herd recruitment

Interested dairy producers were contacted either in-person during the Western Canadian Dairy Seminar (WCDS) 2020, Red deer, AB, Canada, through email, or phone. Each interested farmer received a herd risk assessment form, and their responses were screened to match to the eligibility criteria: i) willingness to remove HPL cows within 3 months of identification, ii) farm was located within 3-hour driving distance from Calgary, iii) free-stall herd, and iv) implemented a fly control program. Lastly, a consent form was signed between the owners of the selected farms and the research team at the University of Calgary. Initially, eleven dairy herds that met the eligibility criteria were selected. Later, one herd was removed from the study due to technical difficulties with the robotic milk sampling system leading to unreliable results. The details of the ten herds (A-J) used for this study along with the BLV control related management practices being implemented at the start of the study are summarized in Table 3-1. Only four herds were aware of their BLV prevalence status: B (40%), E (20%), G (30%), and J (30%). Herd G and J were regularly monitoring their cows through a BLV ELISA test with Lactanet (Sainte-Anne-de-Bellevue QC, Canada). Two herds had to downsize due to a milk quota cut. The number of cows removed were: n = 64 cows in year three (herd H) and n = 16 cows in year two and n = 37 cows in year three (herd J).

3.3.3 Sample collection and processing

A milk sample (25-40 mL) was collected aseptically from each lactating cow during routine milking by hand stripping into a 50-mL container with a preservative tablet (Broad Spectrum Microtabs II, Advanced Instruments, Norwood MA, USA). In herds using an automatic milking system (AMS), milk samples were collected by the automatic milk sampler.

Blood samples (serum and EDTA; Vacutainer, BD, Franklin Lakes NJ, USA) were taken via the coccygeal vein. The samples were transported in a cooler, stored at 4°C, and processed within 48 hours of collection.

Skim milk was separated by centrifugation of milk samples at 5,000 x g, 20 min, 4°C and stored at –20°C until further analysis. Serum was separated by centrifugation at 1,500 x g, 10 mins, 10°C, and stored at –20°C. A 200-µL EDTA-mixed whole blood aliquot was stored at –80°C until further analysis.

3.3.4 BLV ELISA test

BLV infection status was determined using an antibody ELISA on milk (Bovichek BLV ELISA kit, Biovet Inc., Montreal QC, Canada) or serum samples (Leukosis Serum X2 Ab Test, IDEXX Laboratories, Inc., Westbrook ME, USA), using the manufacturer's protocol. All the BLV testing was conducted at the University of Calgary laboratories, except for Farm G (for all study years) and Farm J (Year 1 only), that used Lactanet to test their cows for BLV status. The BLV ELISA results were interpreted as BLV-positive, BLV-suspected, or BLV-negative according to the manufacturer's protocol. An ELISA-positive cow was considered to remain positive for its lifetime, and therefore, was not re-tested.

3.3.5 BLV proviral load quantification

Genomic DNA was extracted from the frozen EDTA-mixed whole blood samples of all BLV-positive cows using a Quick-DNA Miniprep Plus Kit (Zymo Research, Irvine CA, USA) as per the manufacturer's protocol. DNA concentration was measured using a Qubit 3.0 Fluorometer and dsDNA BR Assay Kit (Invitrogen, Waltham MA, USA). DNA samples were

normalized with nuclease-free water to ≤50 ng/µL and BLV SS1 quantitative PCR (qPCR) assay (CentralStar Cooperative Inc., East Lansing MI, USA) was used for the BLV proviral load quantification as previously described (Shrestha et al., 2024). BLV proviral load was interpreted as i) high proviral load or HPL (≥0.50), ii) moderate proviral load or MPL (0.25 to 0.50), iii) low proviral load or LPL (>0 to <0.25), and iv) not detected or ND (ELISA-positive, no proviral load detected).

3.3.6 Data analysis

The proportion of HPL cows of the total cows tested per herd and of the total BLV-infected cows in a herd was assessed for all sampling years. The relative risk (RR) of removal of cows was determined for BLV-positive, HPL, MPL, LPL, and ND groups in comparison to BLV-negative group for each herd for the first and second year. For example, the RR of HPL removal in herd A in the first year was determined as = (number of HPL cows removed in first year/ number of HPL cows identified in first year) / (number of BLV-negative cows removed in first year/ number of BLV-negative cows identified in first year).

Within-herd BLV prevalence on a farm was determined following each sampling by calculating the proportion of ELISA-positive cows relative to the total number of adult cows tested. The changes in within-herd prevalence over the three time-points was analyzed for each farm using the χ^2 test for trend in OpenEpi 3.01 (Dean et al., 2013). The change was interpreted as significantly different when $P < 0.05$.

The incidence rate of new infection was calculated only for the second and third year of the study as we did not have BLV-status information before year one. It was determined as the ratio of the total number of new BLV ELISA-positive cows during the one-year period to the

total number of cows that were at-risk during the same period per 100 cows. Some ELISA-negative cows left the herd (left), while some new young replacement cows were introduced (replaced) during the one-year period. Therefore, the at-risk population was corrected by subtracting half the number of these absent cows (left) (Ruggiero et al., 2019) and adding half the number of the replacement cows (replaced) to the number of ELISA-negative cows from the previous testing year:

Corrected at-risk population = number of ELISA-negative cows from previous testing – (0.5 × left) + (0.5 × replaced)

Change in BLV proviral load of cows was assessed over the three years using a multilevel mixed-effects model. A categorical variable (group) was generated based on the first-year test results of the cows: ND (reference group), LPL, MPL, HPL, and BLV-negative. The BLV proviral load variable (continuous) contained many zeroes in its value, therefore, 0.0001 was added to each proviral load results and a natural logarithmic transformation was applied. Year (measured) was introduced as the predictor. The interaction of year and group was introduced into model to assess potential effect modification whereas, cow ID nested into herd was used as the random effect. Models were compared using Bayesian information criterion (BIC). If significant, individual rates of change were obtained for each group and compared using linear combinations. Statistical significance was determined at a 5% level. All the statistical procedures were analyzed using Stata 15.1 (StataCorp LLC, College Station, TX, USA).

All graphical plots were generated using GraphPad Prism version 10.0.2 for Windows, GraphPad Software, Boston, Massachusetts US, www.graphpad.com and Stata 15.1 (StataCorp LLC, College Station, TX, USA).

3.4 Results

The sampling period started July 10, 2020 and ended January 19, 2023 (Supplementary material, Table S3-1).

3.4.1 Proportion of HPL cows

The details of the number of BLV-positive cows categorized in different proviral load groups by their lactation number demonstrates a larger proportion of HPL cows being in older age-group (≥3 lactation number) (Table 3-2). The median (max-min) number of HPL cows per herd was 7 (1-22), 5 (1-24), and 2 (0-10) in the first, second, and third year, respectively. The median (max-min) proportion of HPL cows of the total cows tested per herd was 3.1% (0.7-7.4 %), 2.5% (0.6-6.4%), and 1% (0-6%) in the first, second, and third year, respectively. On the other hand, the median (max-min) proportion of HPL cows of the total BLV-infected animals was 10.5% (3.3-19%), 10.4% (2.5-30.8%), 5.7% (0-15.3%) in the first, second and third year, respectively.

3.4.2 Relative risk (RR) of removal of cows from different groups

The median (max-min) RR of removal of BLV-positive cows compared to BLV-negative cows was 1.46 (0.33-2.39) and 1.49 (0.89-2.42) in the first and second year, respectively (Table 3-3). On further categorization of BLV-positive cows, the median (max-min) RR of removal of cows from HPL, MPL, LPL, and ND groups was 1.79 (0-3.37), 1.31 (0-1.63), 1.43 (0-2.42), and 1.2 (0.74-3.23) in the first year, and 1.69 (0-3.28), 1.71 (1.28-3.26), 1.34 (0.47-1.96), and 1.22 (0.84-4.1) in the second year, respectively.

3.4.3 HPL removal

The proportion of HPL cows removed was different in the 10 herds (Figure 3-2). Throughout the study period, most herds removed some HPL cows but never all HPL cows that were identified. The exceptions were herd D (n = 5) in year one and herd F (n = 4) in year two that removed all identified HPL cows, and herd A (n = 2), E (n = 1), F (n = 1), and I (n = 2) in the year one, and herd D (n = 2) in year two that didn't remove any HPL cows.

3.4.4 BLV prevalence

The number of cows tested in the first, second, and third year were 2,085, 2,089, and 2,015, respectively. One sample in the first and two samples in the second year classified as BLV ELISA-suspect and qPCR-negative and were removed from the analysis to avoid misclassification bias. While retesting these animals in the following year, one tested BLV-positive in the second year, whereas in the third year one tested BLV-positive and the other one tested BLV-negative. The median within-herd BLV prevalence in the first, second, and third year was 28.9 (9.2-52.2%), 27.3 (8.7-50.1%), and 23.2 (5-51.8%), respectively. Over the three years, there was a significant decrease ($P < 0.05$) in BLV within-herd prevalence on herd A, D, G, and J, whereas BLV prevalence increased in only herd E (Figure 3-3). The median (max-min) BLV proviral load values of in herd A, D, E, G, and J were 0.56 (0.50-0.83), 0.53 (0.50-0.63), 0.54 (0.5-0.7), 0.59 (0.50-0.76), and 0.58 (0.51-0.77), respectively. Herd B had the highest BLV prevalence and herd F had the lowest BLV prevalence over all three years.

3.4.5 Incidence rate of new BLV infection

The median combined incidence rate of new BLV infection for all ten farms was 14.4 (4.4-30.4) per 100 cow-years on the second year, and 8.1 (0-32) per 100 cow-years on the third year. The incidence rate decreased from second to third year on all farms except herd B (Figure 3-4).

3.4.6 BLV proviral load

The distribution of BLV proviral load in BLV-seropositive cows in all ten herds in year 1, 2, and 3 demonstrated a decrease in frequency of cows demonstrating higher proviral load values with time (Supplementary materials, Figure S3-1). The mixed-effects model with the interaction of group and year was established as the most parsimonious model in BIC comparison. HPL cows demonstrated the highest proviral loads ($P < 0.001$) among all five groups followed by MPL cows (Table 3-4). Using linear combinations, assessment of individual rates of change among groups revealed that the proviral load were relatively stable among HPL, MPL, and BLV-negative groups ($P > 0.05$), whereas increments overtime was noticed only in ND and LPL groups ($P < 0.001$) (Supplementary materials, Table S3-2).

3.5 Discussion

We assessed the impact of selective removal of HPL cows on the BLV within-herd prevalence and incidence rate in ten dairy herds in Alberta, Canada for three years. A significant reduction in BLV prevalence was observed in four herds (A, D, G, and J), whereas the incidence rate reduced in nine herds (Figure 3-3).

Two previous USA studies implemented identification and removal of HPL cows as a major intervention for controlling bovine leukosis (Ruggiero et al., 2019; Taxis et al., 2020; Taxis et al., 2023). BLV within-herd prevalence decreased in both studies which is similar to our findings in four herds. However, there were differences to our study based on location, number of herds, testing frequency, parameters used for culling, and management measures to control BLV. The larger number of herds in our study reflects the variety of herd size and management practices of the Canadian dairy industry, although not all different types of farms could be included in this study. In Canada, a pilot BLV control program was implemented but that was focused on BLV risk assessment and implementing best management practices (Kuczewski et al., 2021b). We opted for an annual testing strategy which seemed more practical to implement on larger scale. Including lymphocyte counts as an affordable option for predicting HPL cows (Ohno et al., 2015; John et al., 2022) could be used as an additional tool in BLV elimination strategies, but we were not able to include that in the current study. Segregation of HPL cows was limited by the farm's infrastructure, only herd J segregated HPL cows.

The reduction in BLV prevalence and incidence in herd D, G, and J can be attributed to removal of most (>70%) of the HPL cows as they have higher risk of transmitting the virus (Mekata et al., 2018). On contrary, this couldn't be stated for herd A as they only removed 1 out of the 9 HPL cows that were identified. We can speculate that these changes occurred due to management changes. Some herds might have increased awareness regarding the bovine leukosis scenario in their herd after enrolling in this study and receiving the test results. This could have prompted them to make management changes. For instance, herd A implemented a single needle and rectal palpation gloves use policy, using milk/colostrum exclusively from BLV-negative cows to feed calves, discontinuing purchasing cows and use bulls for breeding purpose. Such

adjustments in herd management practices are effective in reducing BLV-within herd prevalence and seroconversions (Kuczewski et al., 2021b). Additionally, we identified HPL cows; therefore, the farmers might have taken precautions in minimizing contact of these high-risk cows to other non-infected cows within the herd. Herd G had a significant reduction in BLV prevalence, but the BLV incidences were comparable. This could occur due to aggressive culling but replacing with BLV-positive young stocks (9/17 and 6/16 newly infected cows were young replacement stock in year two and three, respectively). Downsizing in herd H and J by prioritizing BLV-infected or HPL cows for removal could have assisted in reducing their BLV incidence. An increase in BLV incidence in herd B could be attributed to its high BLV prevalence which enables wider BLV exposure and higher likelihood of infection for BLV-negative cows (Hutchinson et al., 2020b).

HPL cows had the highest RR of removal which means that herd managers prioritized removal of HPL cows. This was also recommended throughout the study. Additionally, lower production and health in HPL cows may have contributed to their removal (Ohno et al., 2015; Kobayashi et al., 2020; Shrestha et al., 2024). Higher proportion of HPL cows being of older age-group could also be a reason for HPL cows having higher RR of removal (Table 3-2). A long-term follow-up on this specific group and assessment of culling reasons could assist in clarifying the decision that led to HPL removal, which was beyond the scope of this study. A median 3% proportion of HPL cows/herd in our study means that removing HPL cows is a more practical BLV control approach than removing all BLV-infected cows.

Assessment of the rate of change in BLV proviral load over time demonstrated that increments were not enough to change a HPL cow to an LPL or vice-versa. This indicates that frequent qPCR tests might not be necessary to determine proviral load status in a BLV-positive

cow which is similar to previous findings (Hutchinson et al., 2021). Since the cost of testing is regarded as a major hindrance for farmers and veterinarians to engage in BLV control (Kuczewski et al., 2022), not requiring repeated testing makes inclusion of proviral load testing more accessible in BLV elimination programs.

Our study lacks baseline data to compare BLV prevalence and incidence rate changes before and after removing HPL cows. Incorporating a control farm would have been ideal for such studies. However, practically and ethically it is difficult to test the cows for an infection and not communicate the results to the farmers.

In Canada, commercial labs offering BLV proviral load tests do not exist. Availability of this diagnostic facility enables farmers to voluntarily identify and remove HPL animals. Given Canada's high BLV prevalence, HPL cow focused BLV control program seems rational.

Table 3-1. Information on herd details and bovine leukosis related control practices obtained from the risk assessment conducted at the initiation of study.

Herd	No. cows tested	Breed	Milking frequency	Buy animal	Breeding type	Change needles	Change palpation gloves	Pasteurize colostrum
A	180	Mixed	Thrice	Yes	AI + bull	Never	Never	Never
B[1]	402	HF	Thrice	Yes	AI	Always	Never	Sometimes
C	215	HF	Twice	No	AI	Sometimes	Always	Never
D	193	HF	Twice	No	AI	Always	Sometimes	Never
E	81	HF	AMS	Yes	AI + bull	Sometimes	Sometimes	Never
F	142	Mixed	Twice	No	AI	Never	Never	Never
G	299	HF	Thrice	No	AI	Always	Sometimes	Never
H[2]	216	Mixed	Twice	No	AI	Sometimes	Never	Never
I	181	HF	Twice	No	AI	Always	Always	Never
J	176	Mixed	Twice	No	AI	Always	Always	Sometimes

[1] = Used automatic milking system (AMS) after completing third year sampling, [2] = Used AMS in third year sampling

HF = Holstein, Mixed = mix of different breeds.

Table 3-2. Number of bovine leukemia virus (BLV)-seropositive cows differentiated by their proviral load categories and lactation number (1, 2, and ≥3) over three study years.

		Year 1				**Year 2**				**Year 3**			
		BLV	Lactation no.			BLV	Lactation no.			BLV	Lactation no.		
Herd	Proviral load	prevalence	1	2	≥3	prevalence	1	2	≥3	prevalence	1	2	≥3
(herd size)	category[1]	(%)				(%)				(%)			
A	ND	33.9	2	1	9	28.4	2	3	9	24.1	–	3	11
(n = 180)	LPL		3	7	20		7	2	11		5	5	10
	MPL		3	2	12		4	1	7		2	–	10
	HPL		–	–	2		–	1	6		1	1	3
B	ND	52.2	4	5	19	50.1	3	6	15	51.7	2	3	20
(n = 402)	LPL		20	25	41		19	32	40		22	34	54
	MPL		6	28	40		8	17	37		5	10	42
	HPL		2	3	17		2	1	21		1	2	6
C	ND	44.7	1	4	4	42.7	6	2	3	37.6	5	2	5
(n = 215)	LPL		13	9	19		18	7	22		3	14	22

	MPL		7	12	16		5	11	16		6	5	13
	HPL		2	3	6		–	3	3		–	4	6
D	ND	47.7	–	2	9	41.7	3	2	8	30.3	1	1	4
(n = 193)	LPL		9	14	19		14	9	18		9	11	13
	MPL		7	9	19		4	5	15		–	3	9
	HPL		1	2	2		1	1	–		–	–	2
E	ND	13.6	–	–	–	26.1	1	1	–	22.3	–	–	2
(n = 81)	LPL		1	5	–		6	4	1		3	7	4
	MPL		3	1	–		1	3	4		–	–	5
	HPL		1	–	–		1	1	–		–	–	–
F	ND	9.2	1	–	–	8.7	–	–	1	5	–	–	–
(n = 142)	LPL		2	–	2		2	2	1		1	1	2
	MPL		1	3	3		–	1	2		–	1	1
	HPL		–	–	1		–	1	3		–	–	1
G	ND	19.7	–	–	5	15.4	1	1	2	11.6	2	–	3
(n = 299)	LPL		1	6	21		5	3	17		4	4	15

	MPL		1	2	12		2	1	7		1	–	4
	HPL		–	–	11		1	–	4		–	–	1
H	ND	43.1	3	1	6	39.9	–	2	4	38.8	–	2	4
(n = 216)	LPL		12	9	25		19	9	17		7	7	16
	MPL		3	8	10		5	2	10		2	3	9
	HPL		1	4	11		2	1	10		–	2	7
I	ND	10.5	–	1	3	10.9	1	1	1	8.6	2	–	1
(n = 181)	LPL		6	1	4		3	2	5		1	1	6
	MPL		1	–	1		4	1	1		3	1	2
	HPL		–	–	2		–	–	2		–	–	–
J	ND	23.9	2	3	4	18.1	5	–	4	11.4	–	4	2
(n = 176)	LPL		2	6	9		5	3	2		–	4	2
	MPL		–	5	3		1	3	5		–	–	1
	HPL		–	1	7		–	–	1		–	–	1

[1]HPL = High proviral load (≥0.5), MPL = Moderate proviral load (≥0.25 to <0.5), LPL = Low proviral load (≥0 to <0.25 BLV copies/WB), and ND = Not detected = BLV ELISA-positive and not detected in quantitative PCR test.

Table 3-3. Relative risk (RR) of removal of cows based on their bovine leukemia virus (BLV)-seropositivity and proviral load categories in the year one and two of study.

	RR in Year 1					RR in Year 2				
	Proviral load categories[1]				BLV ELISA-	Proviral load categories[1]				BLV ELISA-
Herd	**HPL**	**MPL**	**LPL**	**ND**	**Positive cows**	**HPL**	**MPL**	**LPL**	**ND**	**Positive cows**
A	0	1.13	1.38	0.80	1.15	0.93	3.26*	1.96	0.93	1.81*
B	2.08*	1.63*	1.59*	0.74	1.54*	2.33*	1.65*	1.43	1.16	1.57*
C	2.37*	1.49	1.18	1.24	1.43	2.08	1.56	1.24	1.13	1.39
D	3.37*	1.49	1.60*	0.92	1.57*	0	1.77*	1.33	2.10*	1.55*
E	0	0.92	0	–	0.33	1.28	1.28	0.47	1.28	0.89
F	0	0.92	2.42*	3.23*	1.49	2.91*	1.94	1.75	2.91*	2.24*
G	2.15*	1.58	1.48	1.19	1.61*	3.28*	2.87*	1.80*	4.10*	2.42*
H	1.50	1.00	1.37	1.20	1.29	1.04	1.59	1.36	2.26*	1.42
I	0	0	1.21	1.99*	1.12	1.30	1.30	1.04	0.86	1.11
J	3.14*	1.57	2.22*	2.79*	2.39*	2.52*	1.96*	1.01	0.84	1.30

[1]HPL = High proviral load (≥0.5), MPL = Moderate proviral load (≥0.25 to <0.5), LPL = Low proviral load (≥0 to <0.25), and ND = Not detected = BLV ELISA-positive and not detected in quantitative PCR test. BLV ELISA-positive cows include the combination of HPL, MPL, LPL, and ND cows.

* Indicates statistically significant RR value = 95% CI doesn't include 1.

Table 3-4. Results of multilevel mixed-effects model demonstrating the change in bovine leukemia virus (BLV) proviral load in five proviral load categories of cows over the three years (number of observations = 1553).

	Coef.	**SE**	**P-value**	**95% CI**
Fixed effects				
Intercept	–9.7	0.2	<0.001	–10.2 to –9.3
Group[1]				
ND	Ref.			
LPL	5.7	0.3	<0.001	5.2 to 6.2
MPL	8.6	0.3	<0.001	8.1 to 9.2
HPL	9.2	0.3	<0.001	8.5 to 9.9
BLV ELISA-negative	5.7	0.4	<0.001	4.9 to 6.5
Year	0.5	0.1	<0.001	0.3 to 0.7
Group##Year				
LPL	–0.1	0.1	0.26	–0.3 to 0.1
MPL	–0.5	0.1	<0.001	–0.7 to –0.3
HPL	–0.6	0.1	<0.001	–0.8 to –0.3
BLV ELISA-negative	–0.5	0.1	<0.001	–0.8 to –0.2
Random effect				
Herd	0.04	0.04		0.01 to 0.3
Cow ID	3.1	0.2		2.8 to 3.4

Residuals	0.6	0.03	0.5 to 0.6

[1]HPL = High proviral load (≥0.5), MPL = Moderate proviral load (≥0.25 to <0.5), LPL = Low proviral load (≥0 to <0.25), and ND = Not detected = BLV ELISA-positive and not detected in quantitative PCR test.

Note: Each result of the outcome variable BLV proviral load was added with 0.001 and transformed using a natural log-transformation. The results are presented without back-transformation. The study cows nested within the herds were included as random effects. $P <$ 0.05 indicate significant result.

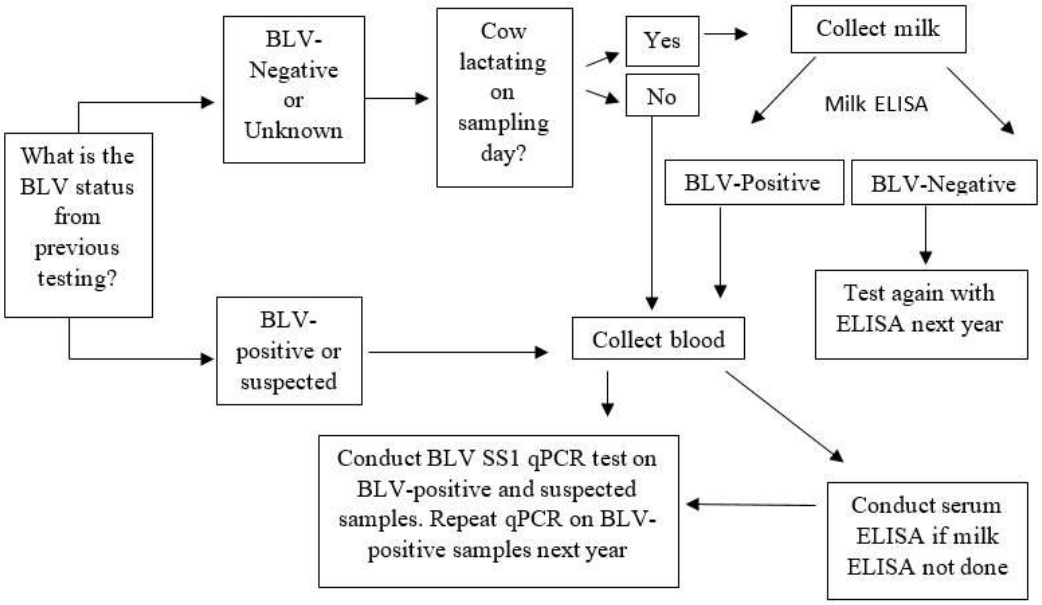

Figure 3-1. Sampling strategy

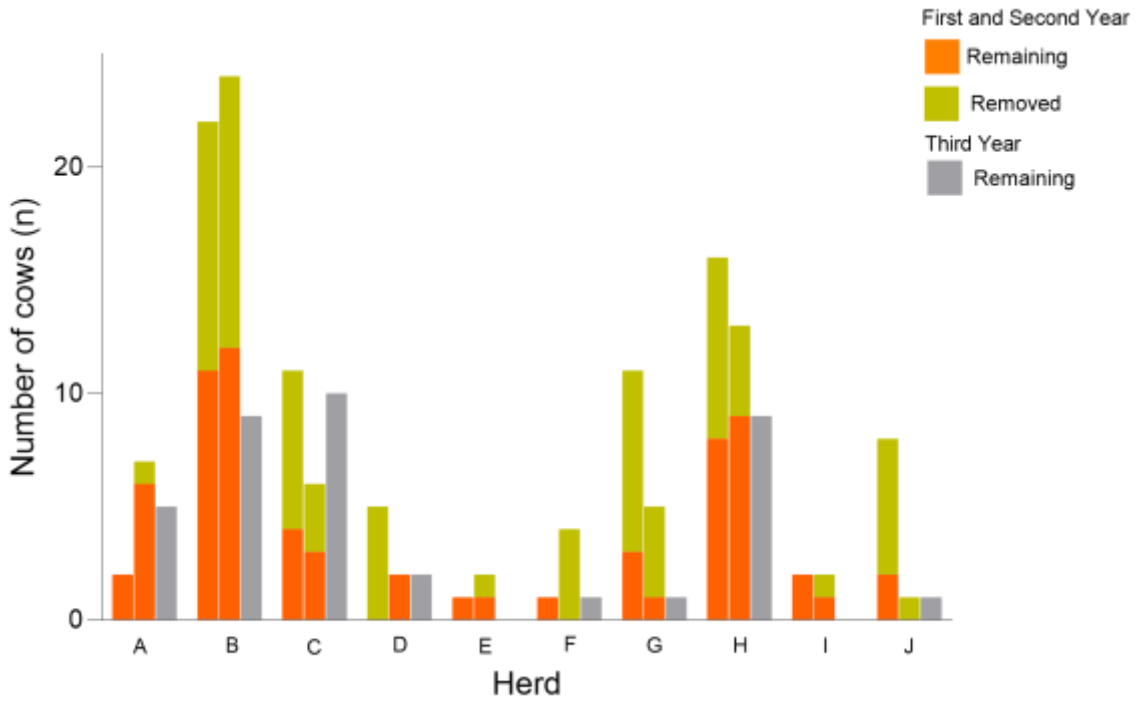

Figure 3-2. Bar diagrams demonstrating the number of high proviral load or HPL (≥0.5) cows removed and remaining (stacked together) in the first, second, and third year of the study in herd A-J. For the third year, information on the number of HPL cows removed was not available when the study concluded. Herd E and I had no HPL cows identified in the third year.

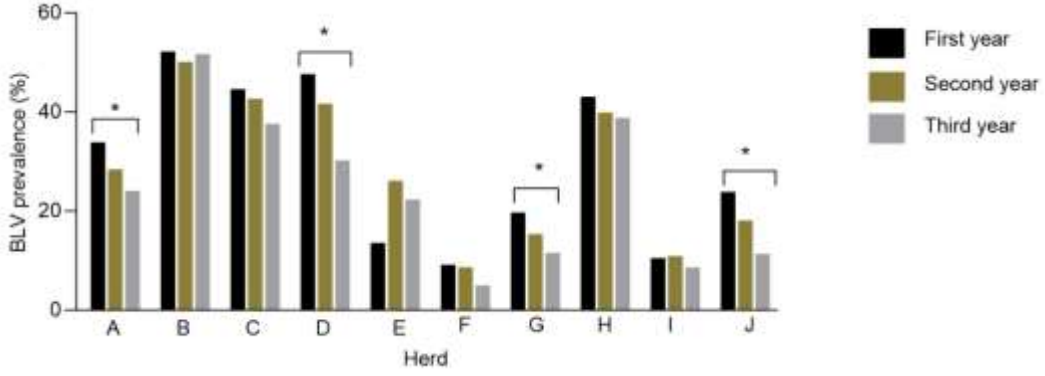

Figure 3-3. Change in within-herd prevalence (obtained of performing ELISA tests) of bovine leukosis in the enrolled herds (A-J) over the three years. Significant reduction in prevalence was observed in herd A, D, G, and J in the Chi-squared (χ^2) test for trend (* = $P < 0.05$).

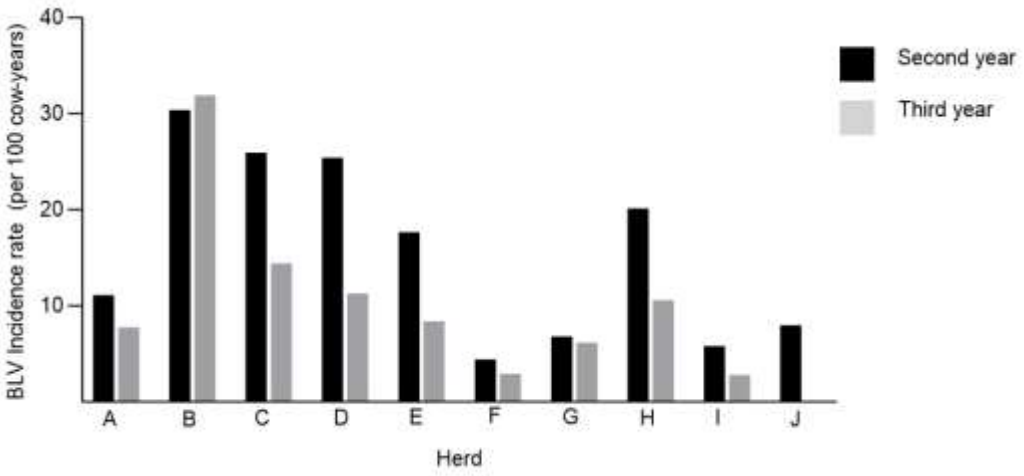

Figure 3-4. Incidence rate (per 100 cow-years) of new bovine leukemia virus (BLV) infection in the second and third year of study among the ten dairy herds (A-J). The newly infected cows may include existing cows, replacement heifers, or bought-in cows (herd A, B, and E).

CHAPTER 4: Dynamics of proviral load, antibody titer, and immune cells in bovine leukemia virus-infected and non-infected dairy cows

4.1 Abstract

Bovine leukemia virus (BLV) causes widespread infection in the dairy cows of North America. The proviral load of BLV is currently being used in assessing BLV transmission and the impact in production caused by BLV infection. Recent longitudinal studies have indicated a relatively stable BLV proviral load and lymphocyte counts in BLV-infected animals over time, however, these studies have not considered BLV proviral load status which may cause individual differences. The objective of our study was to assess the variability of white blood cells (WBC), lymphocytes, BLV proviral load, antibody titer, and immune cells (CD3+, CD4+, CD8+, CD21+, and WC1+) among BLV-seropositive (further categorized based on proviral load as high (HPL), moderate (MPL), low (LPL), and not detected (ND; e.g., antibody-positive, provirus-negative)), and BLV-seronegative dairy cows through a longitudinal study. Blood samples were taken from forty-six Holstein dairy cows from a single herd every three months at seven time-points to assess the parameters mentioned in the study objective. Mixed-effects regression model was used to assess the changes in parameters over time. There was no significant change in WBC estimates, proviral load, and CD4+ cell proportion over time for all groups. Antibody titer, CD8+, and CD21+ cell proportion significantly increased, whereas lymphocyte count, CD3+, and WC1+ cell proportion significantly decreased over time for all groups. The results from this study can be used to inform decisions regarding frequency of various tests used in BLV control programs.

Key words: longitudinal study, BLV, white blood cell, lymphocyte, CD3+, CD4+, CD8+, CD21+, WC1+

4.2 Introduction

Bovine leukemia virus (BLV), a deltaretrovirus, establishes a life-long infection in a host through integration of the viral genome into the host's genome and formation of a provirus (Goff, 2013). Usually, the clinical signs of BLV-infection are not visible, yet BLV causes immune-suppression, production losses, and reduced longevity in dairy cows (Bartlett et al., 2013; Frie and Coussens, 2015; Nekouei et al., 2016). Confirmation of a BLV-infection requires detection of anti-BLV antibodies or BLV proviral DNA. The latter method provides an opportunity to detect and quantify BLV proviral load using qPCR methods which is expressed as the number of BLV proviral copies per denominators such as white blood cells (WBC), microgram of genomic DNA, or similar (Kuczewski et al., 2021b). Recently, it became clear that BLV-infected animals with a high proviral load (HPL) are associated with higher risk of BLV transmission or developing lymphoma, and a reduction in milk, fat, and protein production (Juliarena et al., 2016; Kobayashi et al., 2020; Shrestha et al., 2024). Therefore, the identification and removal of HPL BLV-infected cows is studied to reduce BLV prevalence on farm (Ruggiero et al., 2019; Taxis et al., 2020; Chapter 3).

The acceptance of a disease monitoring technology by dairy farmers is incumbent upon the cost and ease of use, as these barriers often discourage engagement of farmers and veterinarians in BLV control practices (Kuczewski et al., 2022). Proviral load testing through qPCR can be expensive, therefore, adoption of this test may depend on how frequent this test needs to be conducted for disease monitoring. Recent infection trials indicated that the proviral load is established shortly after infection (Forletti et al., 2020; Hutchinson et al., 2020a) and remain relatively stable over time (Hutchinson et al., 2021). This indicated that an annual proviral load test should suffice for monitoring BLV progression (Hutchinson et al., 2021).

However, these studies did not differentiate BLV proviral load status of cows which may influence the varied progression in proviral loads. Usually, BLV-infected cows are classified using proviral load values as high (HPL), moderate (MPL), or low (LPL) proviral loads. Additionally, some cows are ELISA-positive but qPCR-negative or not detected (ND). Whether ND cows remain qPCR-negative over longer periods is unknown.

Cows in the HPL category have a high probability of being immunocompromised as many develop a persistent lymphocytosis, have proliferated B-cells (CD21+), and reduced T-cell proportions (CD3+, CD4+, CD8+) (Ohno et al., 2015; Farias et al., 2018) while at the same time BLV proviral load is positively associated with WBC counts and antibody titers (Juliarena et al., 2007; Alvarez et al., 2013). However, how and if variations in these parameters over time can be expected in adult milk producing animals is unclear and is relevant when proviral load or other parameters will be used for BLV control. Therefore, the objective of this study was to evaluate the variability of the proviral load, lymphocyte count, WBC count, BLV antibody titer, and immune cell proportions (CD3+, CD4+, CD8+, CD21+, and WC1+) in BLV-seropositive and BLV-seronegative adult dairy cows within a 1.5-year timeframe.

4.3 Materials and methods

4.3.1 Experimental animals

Forty-six Holstein Friesian dairy cows with known BLV antibody and proviral load status were selected at a dairy farm located in Alberta, Canada with a herd size of 402 cows. This farm operated in a free-stall system, where cows were milked three times a day. Farm selection was based on the willingness of the farmer to participate in this study in which the cows required sampling every three-months at seven time-points (T1-T7). The enrolled cows were categorized

into five groups according to their BLV antibody status and proviral load of BLV-seropositive cows at the starting point of the study: high proviral load (HPL; ≥0.5), moderate proviral load (MPL; 0.25 to 0.5), low proviral load (LPL; >0 to <0.25), not detected (ND; antibody-positive, proviral load-negative), or BLV-seronegative (NEG), with 5-10 cows enrolled in each group (Table 4-1). At T4, six additional antibody negative cows were enrolled in NEG group as a replacement for those that seroconverted or were removed from the herd. All procedures involving animals were approved by the Veterinary Sciences Animal Care Committee of the University of Calgary (VSACC AC20-0095 and AC21-0210).

4.3.2 Sampling scheme

Sampling was conducted every three months between June 2021 and December 2022, except T5 (four months interval) and T6 (two months interval) (Table 4-2). Blood samples were collected using a 10-mL serum tube containing clot activator and a 10-mL K3-EDTA containing tube (Vacutainer, BD, Franklin Lakes NJ, USA) via the caudal tail vein. The samples were transported in a cooler containing ice packs to the University of Calgary laboratory, stored at 4°C and processed within 48 hrs of collection.

Serum was harvested by centrifugation at 1500 x g for 10 min at 10°C and stored at -20°C. EDTA-mixed blood was aliquoted for three different purposes: i) A 200 µl EDTA-mixed blood sample was stored in a 1.5-mL microcentrifuge tube at –80°C, for proviral load determination. ii) A 1 mL aliquot was set aside in a 1.5-mL microcentrifuge tube for WBC and lymphocyte count. iii) Remaining EDTA-mixed blood was used to isolate peripheral blood mononuclear cells (PBMC). A 4 mL blood diluted in equal volume of 1x phosphate buffer saline (PBS) was poured in a SepMate-15 tube (StemCell Technologies, Vancouver BC, Canada)

containing a layer of Lymphoprep density gradient (StemCell Technologies, Vancouver BC, Canada). Separation of PBMCs were conducted following the manufacturer's protocol and resuspended in a cold 1 mL mixture of 10% dimethyl sulphoxide (DMSO) in horse serum (Gibco, Grand Island NY, USA), and stored in a 2-mL cryovial in nitrogen tank until further processing.

4.3.3 White blood cells (WBC) and lymphocyte count

The HemaTrue automatic veterinary hematology analyzer (Heska Corporation, Loveland CO, USA) was utilized to determine the amount of WBC and lymphocytes (cells/µL) following manufacturer's instructions. A cow was defined as persistently lymphocytotic if >5,200 lymphocytes/µL were detected in at least 75% of the observations (5/7 observations) (Hutchinson et al., 2020a; John et al., 2022).

4.3.4 BLV proviral load measurement

Frozen EDTA-mixed blood samples were used for BLV proviral load determination using the BLV SS1 qPCR assay (CentralStar Cooperative Inc., East Lansing MI, USA). Briefly, genomic DNA, extracted from the frozen EDTA-mixed blood sample (Quick-DNA Miniprep Plus Kit, Zymo Research, Irvine CA, USA) and normalized to ≤50 ng/µL (Qubit 3.0 Fluorometer and dsDNA BR Assay Kit, Invitrogen, Waltham MA, USA), was used in the assay. Following completion of the amplification cycle, BLV proviral load was calculated as the number of BLV proviruses per Beta-Actin copy, and expressed as a ratio.

4.3.5 Antibody titer determination

BLV antibody status and antibody titers in BLV-seropositive cows were determined using the Leukosis Serum X2 Ab Test (IDEXX Laboratories, Inc., Westbrook ME, USA) following manufacturer's protocol, the iMark Microplate Absorbance Reader (Bio-Rad, Hercules CA, USA) was used to read the optical densities (OD). An end-point dilution approach was implemented. Eight 2-fold serial dilutions were prepared by mixing 50 µL of original serum or diluted serum with 50 µL 1x PBS in a 96-well microtiter plate. All serum dilutions were tested for antibody presence. The highest dilution of a serum sample that still contained detectable antibodies ('positive' or 'suspected'), was interpreted as the antibody titer. This value was calculated as 2^n, where n = the highest dilution with detectable BLV antibodies.

4.3.6 Flow cytometry for B- and T-cell estimation

Samples from 28 cows that were present throughout the study period T1-T7 were used for analyzing B-cell (CD21+) and T-cell (CD3+, CD4+, CD8+, and WC1+) proportions in PBMCs using the respective antibodies to prepare the multi-label mix (supplemental data, table S4-1). The cryovial containing frozen PBMCs were thawed at 37°C in water bath and transferred into a tube containing 9 mL 1x HBSS (Gibco, Grand Island NY, USA) at room temperature. The tube was centrifuged at 1200 rpm for 5 minutes at 18°C, and the cell pellet resuspended in 1 mL 1x HBSS. Viability count of the PBMC cells was performed using Acridine Orange/Propidium Iodide (Logos Biosystems, Inc., Annandale VA, USA) and a LUNA cell counting slide in a LUNA-FL dual fluorescence cell counter (Logos Biosystems Inc., Annandale VA, USA). The mean ± SD viability of the PBMCs was 91% ± 12%, 10^5 live cells were used for the analysis. For each sample, an unstained control and a multi-label staining was conducted. To differentiate

live and dead cells, staining with Ghost dye Violet 510 (Cytek Biosciences, Fremont CA, USA) was performed on multi-label samples only. This followed a wash (5% bovine serum albumin [BSA] for Fc blocking), fixation (1% paraformaldehyde), and a second wash step. Cells were incubated with 20 µL of the respective antibody mix (M1, M2, M3, or unstained, see supplemental data table S4-1). Unstained controls were incubated with 5% BSA. Multi-label samples were incubated with M1, except samples from T1 (n = 20), T5 (n = 13), and T6 (n = 28), that were incubated with M2 and M3 separately. Cells were incubated in the dark for 30 mins at room temperature, followed by 15 mins incubation on ice. After a final wash, cells were resuspended in 200 µL 5% BSA and analyzed in the Attune NxT Flow Cytometer (Thermo Fisher Scientific Inc., Waltham MA, USA) by acquiring ≥30,000 events per sample. The data were analyzed using FlowJo v10.8.1 Software (BD Life Sciences, Ashland OR, USA) and target population differentiation was based on single color controls, unstained control, and Fluorescence Minus One (FMO) controls as shown in Figure 4-1. Briefly, parent cells were gated for lymphocytes and single cells population followed by live cells FMO control. Gatings based on FMO controls for CD3+, CD21+, and WC1+ cell population was applied to estimate the percentages of CD3+, CD21+, and WC1+ target cells, respectively. The proportions of CD4+ and CD8+ cells were differentiated by applying the CD4+ vs CD8+ FMO control on CD3+ cells as the parent population.

4.3.7 Data management and analysis

Enrolled cows from each of the five groups were assigned a unique ID for identification purposes (Table 4-1).

Nine continuous outcome variables were analyzed: WBC and lymphocyte count, BLV proviral load, antibody titer (AT), and percentages of CD3+, CD4+, CD8+, CD21+, and WC1+ cells in PBMC population. To visually assess the distribution and changes of these variables from T1-T7, graphs were built for each combination of group and variable in Graphpad Prism (v10.0.2 for Windows, GraphPad Software, Boston MA USA, www.graphpad.com). In order to evaluate the changes in each of these outcomes as a function of group and time-point, mixed-effects regression models fitted using maximum-likelihood were used. Since some proviral load contained zero cells (ND), a value of 0.0001 was added to all proviral load results. A natural log-transformation was applied to measure outcome variables (WBC, lymphocyte, proviral load, AT, CD3+, and WC1+) to improve model fit. Animals were categorized into five groups to represent their BLV-status: HPL, MPL, LPL, ND (reference group), and NEG. Time (measured) and group (categorical) were introduced into models as fixed-effects. Interaction of group and time was included to evaluate potential effect modification. Random slopes for time were tested in all models but findings were independent on their presence (results not shown). Consequently, we chose to utilize only cow ID as the random effect in all models to address the issue of non-independence among multiple observations within the same cows.

Models were compared using likelihood ratios and the Bayesian information criterion (BIC). Individual rates of change were obtained for each group and compared using linear combinations. Statistical significance was considered at the 5% level. Stata 15.1 (StataCorp LLC, College Station, TX, USA) was used for the analyses.

4.4 Results

The age of enrolled cows ranged from 2-7 years. Few cows were removed from the herd at T3 [HPL (n = 2), LPL (n = 1), NEG (n = 1)], T4 [(HPL (n = 1), MPL (n = 3), ND (n = 1)], T5 [MPL (n = 1), LPL (n = 1)], and T7 [(MPL (n = 1)], making their data unavailable. Some cows from the NEG group seroconverted and became BLV-seropositive at T2 (n = 3), T3 (n = 2), and T4 (n = 1). The descriptive summaries of the WBC count, lymphocyte count, proviral load, BLV antibody titer, T-cell (CD3+, CD4+, CD8+, and WC1+), and B-cell proportions (CD21+) in the PBMCs of the enrolled cows from T1-T7 are available in Table 4-2.

4.4.1 White blood cell count

The median (min; max) WBC count (×1,000 cells/μL) in HPL, MPL, LPL, ND, and NEG group were 20.1 (10.3; 41.2), 14.5 (7.7; 35.8), 10.1 (6.7; 19.1), 8.5 (4.6; 12.5), and 8.9 (5.7; 14.5), respectively. The highest level of variability in WBC count was detected among HPL and MPL group (Figure 4-2). Cow 03 had the highest WBC count (41.2) but was removed after T2. There was no significant interaction between group and time. Additionally, log-transformed WBC counts did not significantly change over time for any group. Increased log-transformed WBC counts were observed for HPL (0.8) and MPL (0.5) groups compared to ND cows (supplemental data, table S4-2).

4.4.2 Lymphocyte count

Lymphocyte count (×1,000 cells/μL) [Median (min; max)] was highest for HPL [14.7 (6.1; 33)], followed by MPL [9 (4.9; 20.4)], LPL [5.6 (1.7; 14.2)], NEG [4.1 (1.7; 9.4)], and ND [3.3 (1.8; 5.4)] groups. Similar to WBC counts, lymphocyte counts highest level of variability

was detected in the HPL and MPL groups. Cow 03 had the highest lymphocyte count (33), but was removed after T2 (Figure 4-2). All cows from HPL and MPL group, three from LPL (cow 19, 21, and 23), and one from NEG group (cow 44) exhibited persistent lymphocytosis. Interaction effect of time and group was not significant in the model. Log-transformed lymphocyte counts were significantly higher in LPL (0.5), MPL (1), and HPL (1.4) groups in comparison to ND group. Log-transformed lymphocyte counts significantly decreased over time for all groups (supplemental data, table S4-2).

4.4.3 BLV proviral load

The proviral loads for ND group were not detectable over all time-points (Figure 4-2 and 4-3). The median (min; max) proviral load for HPL, MPL, LPL, and NEG group (cows seroconverted during the study) were 0.52 (0.26; 1.0), 0.36 (0.13; 0.57), 0.15 (0.0002; 0.36), and 0.12 (0; 0.41), respectively. Cow 01 and 03 (both HPL) had the highest proviral load (0.95 and 0.98, respectively) and were removed from the herd after T4 and T2, respectively. There were fluctuations in proviral load which caused HPL cows demonstrate MPL level (n = 5), MPL cows demonstrate LPL level (n = 6), MPL cows demonstrate HPL level (n = 3), and LPL cows demonstrate MPL level (n = 2) proviral load at some time-points. Among the seroconverted NEG cows, only cow 34 had no detectable (ND) proviral load throughout T2-T7. Mixed-effects regression model revealed no interaction effect of time and group. Compared to ND group, all groups had higher log-transformed proviral loads, with HPL (8.6) and MPL (8.1) groups having the highest values. The log-transformed proviral load did not change with time for all groups (supplemental data, table S4-3).

4.4.4 Antibody titer (AT)

The median (min; max) AT for the five groups were: MPL = 32 (8; 256), HPL = 32 (4; 128), LPL = 16 (8; 128), ND = 8 (1; 64), and NEG = 16 (2; 64) (for cows that seroconverted). The variability of AT was at the highest level for HPL and MPL cows as detected for WBC and lymphocyte counts. Cow 11 (MPL) had an abrupt increase in AT from T2 (n = 64) to T3 (n = 256), after which it was removed from the herd. As with other models, no interaction of group and time was observed. Log-transformed AT values were higher in LPL, MPL, and HPL groups compared to ND group. Significant increase in log-transformed AT was observed for all groups over time (supplemental data, table S4-3).

4.4.5 B- and T-cells

The proportion of CD3+ cell [median (min; max)] was the lowest in HPL group [3 (0.5; 17)] followed by MPL [4 (0.2; 42)], LPL [6 (0.6; 31)], NEG [14 (2; 36)], and ND [18 (2; 65)] groups. The proportion of CD4+ cell was the lowest in HPL [7 (0.7; 82)] followed by MPL [9 (1; 88)], NEG [17 (5; 85)], LPL [17 (4; 88)], and ND [24 (7; 86)] groups. Similar to CD4+, lowest CD8+ cell proportion was observed in HPL [1 (0.6; 4)], followed by MPL [2 (1; 8)], NEG [4 (0.8; 14)], LPL [4 (1; 14)], and ND [6 (1; 18)] groups. In case of WC1+ cells, the lowest cell proportions were observed in HPL [0.9 (0.2; 4.3)] group followed by MPL [1 (0.2; 28)], LPL [2 (0.5; 6)], NEG [3 (0.6; 13)], and ND [3 (0.4; 14)] groups. The proportion of CD21+ cell was the highest in MPL [49 (12; 68) followed by LPL [47 (21; 62)], HPL [46 (13; 72)], NEG [38 (15; 70)], and ND [34 (15; 45)] groups. The CD3+ cell population data demonstrated a congregation at T5 (MPL and ND), T7 (NEG and HPL), similarly for CD4+ cells at T4 (HPL) (Figure 4-4). A

very high WC1+ cell proportion (28) was observed for Cow 13 in MPL group at T1 after which it decreased (≤1.1).

In the mixed-effects regression model, the interaction effect of time and group was not significant to assess the variations in any of the five immune cell (CD3+, CD4+, CD8+, CD21+, WC1+) proportions. Log-transformed CD3+ cell proportions were significantly lower for the HPL (–1.6), MPL (–1.3), and LPL (–0.5) groups whereas CD21+ cell proportions were significantly higher for the MPL (18), HPL (16), and LPL (13) groups compared to the ND group (supplemental data, table S4-4). The cell proportions of CD4+ and log-transformed WC1+ demonstrated significantly lower results for HPL (–10.6), and HPL (–1.4) and MPL (–1) group, respectively, compared to the ND group (supplemental data, table S4-5). For CD8+ cells, no differences occurred between the groups (supplemental data, table S4-5). An increase over time for all groups was observed for CD8+ and CD21+ cell proportions whereas a decrease over time for all groups was observed for log-transformed CD3+ and WC1+ cell proportions. For CD4+ cell proportions no change was observed with time for all groups.

4.5 Discussion

Our objective was to evaluate the dynamics of blood cell count (WBC and lymphocytes), proviral load, AT, and immune cells (B- and T-cells) over time among dairy cows differentiated based on BLV seropositivity and proviral load status (HPL, MPL, LPL, ND, and NEG). There were no changes in WBC count, proviral load, and CD4+ cell populations over time for all groups. However, lymphocytes, CD3+, and WC1+ cell populations decreased with time, and AT, CD8+, and CD21+ increased with time for all groups.

The stability in proviral loads observed in our study is in contrast to another longitudinal study that reported increments in proviral load when naturally BLV-infected cattle were sampled every six-months (Hutchinson et al., 2021). The discrepancy observed could be attributed to variations in the study design, as the latter study used secondary dataset from a separate intervention study which required culling cows with high proviral loads and/or lymphocytosis with the aim of reducing within-herd BLV prevalence (Ruggiero et al., 2019). This may have imposed a bias by limiting sampling points for specific cow groups that were removed from the herd. Nevertheless, the study used higher number of cows than our study, increasing the power of their study. The authors (Hutchinson et al., 2021) emphasized that the observed increments in proviral load were minimal to have any significant impact and recommended that monitoring proviral load every six months might be more frequent than necessary. The relative stability of BLV proviral load results over a 1.5-year period in our study aligns with the recommendation that frequent proviral load testing may not be required for reliable BLV monitoring.

Longitudinal studies evaluating animals from the point of infection might offer better insight into how BLV infection evolves over time. Four independent studies have looked into the dynamics of BLV proviral load following infection through experimental approaches (Jimba et al., 2012; Gillet et al., 2013; Forletti et al., 2020; Hutchinson et al., 2020a). The duration of the study varied from 70 to 250 days post-infection, with variations in the frequency of sampling. Taken together, these studies revealed that the BLV proviral load reaches its peak shortly after infection, following which it may decline, remain stable, increase, or remain low. The exact point of infection in cows was not known in our study. Therefore, it is difficult to identify the observed patterns in our case. However, a modest assessment of proviral load patterns in the NEG group cows that seroconverted in our study could be made, although the numbers are low.

All seroconverted NEG cows exhibited a relatively stable proviral load over time, except cow 35. However, comparing natural BLV-infection results to experimental infections should be done with caution as the experimental infections were conducted with inocula which may not necessarily mimic the exposure to this virus during a natural infection.

Even though the mixed-effect regression model suggested a relatively stable proviral load over time, individual cow dynamics demonstrated fluctuations at several time-points where the proviral load of a cows significantly changed, for example, in HPL cows MPL level proviral load were detected etcetera. Individual variations in a subset of cows can be anticipated even though the model suggests less variability, as a statistical model simplifies the overall observations to represent an overall pattern rather than matching individual observations (Hutchinson et al., 2021). Additionally, these proviral load groupings are based on arbitrary numbers and we do not know the clinical implications of these variations. For example, if the proviral load of a HPL cow decreases to that of a MPL cow, will the cow no longer be at transmission risk or are both HPL and MPL groups equally risky. To address these questions, an assessment of these groups based on clinical and immunological impacts, using larger number of animals will be required. This will be addressed in Chapter 5 of this thesis.

The cows in the ND group continued to test provirus-negative throughout all time-points. The failure to detect proviruses could be due to the sensitivity of the qPCR assay, a robust cytotoxic immune response of the host limiting proviruses, or the virus population being limited to an organ and not being circulated in the blood (Klintevall et al., 1994; Hutchinson et al., 2020a). Since proviruses in these cows could not be detected throughout all time-points, while keeping a consistent qPCR approach, the latter two reasons could be more applicable in our case,

suggesting the host's immune response play a vital role in the expression and establishment of BLV proviral load.

We observed a decrease in lymphocytes over time, which is in contrast to a previous study indicating stable lymphocyte counts (Hutchinson et al., 2021). We had a lower number of cows assigned into five different groups, this may have an impact on the power of our study and thus contributing to discrepancies with Hutchinson et al. (2021). Interestingly, the overall WBC count remained stable, despite lymphocytes being a component of WBCs. White blood cells include monocytes and granulocytes (neutrophils, basophils, and eosinophils), besides lymphocytes. Bovine leukemia virus specifically targets CD5+ B-lymphocytes (Schwartz et al., 1994). Therefore, the changes in lymphocytes observed could likely be attributed to a direct influence of BLV, considering its targeted impact on this subset of WBCs. The previous notion that persistent lymphocytosis occur as a result of gradual BLV progression has been challenged by the recent finding indicating persistent lymphocytosis can occur shortly after infection (Hutchinson et al., 2020a). In our study, all HPL and MPL cows demonstrated persistent lymphocytosis on all time points. While we do not know exactly when these cows were infected, it supports the notion of less variability in lymphocyte counts over time, especially in BLV-infected cows with high proviral loads. Three cows from the LPL group and one from the NEG group exhibited persistent lymphocytosis, despite not demonstrating high proviral load throughout the sampling period. Previously, it was also reported that BLV-infected cows with high proviral load may demonstrate normal lymphocyte count (Juliarena et al., 2007). Combining these two results, it could be inferred that lymphocyte counts are not reliable indicators of BLV-infection or proviral load status.

Antibody titers increased with time for all groups. Following an experimental infection, antibody titers against BLV peaked followed by a decline among LPL cows, whereas it remained consistently high among HPL cows (Forletti et al., 2020). As explained above, this may occur due to the differences in the inocula used in experimental infections which may not replicate a natural infection. Cows in the ND group had consistently low AT while cows in HPL, MPL, and LPL groups had higher AT, suggesting the humoral response in BLV-infected animal may be attributed to the antigenic stimulation caused by the level of BLV proviruses (Gutierrez et al., 2012; Jaworski et al., 2016). Our results also align with Gurierrez et al. (2012), who observed majority of BLV-infected cows with weak BLV antibody levels having undetected or low proviral loads.

The host's immune system is significantly impacted by BLV, as evidenced by the alterations in B-cell and T-cell homeostasis (Frie and Coussens, 2015). In previous BLV-infection studies, abnormalities in B- and T- cells were assessed by comparing BLV-seronegative animals with BLV-seropositive animals further categorized as PL and non-PL or HPL and LPL (Erskine et al., 2011b; Farias et al., 2018). In our study, we introduced additional BLV proviral load categories (MPL and ND) to understand their role on the changes in host's immune system. Previous study indicated elevated B-cells and lower T-cells in HPL cows compared to LPL cows or BLV-negative cows (Farias et al., 2018), however the temporal dynamics of these parameters has not been studied before. Our study demonstrates an overall increase in the proportion of B-cell (CD21+) with time for all groups, indicating BLV's potential role in reducing cellular apoptosis and enhancing cellular proliferation (Erskine et al., 2011; Frie and Coussens, 2015). Among T-cells, CD3+ and WC1+ cell proportions decreased, CD8+ cell proportions increased, whereas CD4+ cell proportions remained stable over time for all groups.

These findings indicate that BLV infection and proviral load induce significant alterations in the antibody production, cytokine production and cytotoxic activity, confirming deleterious impacts of BLV on the host immune system.

A relatively high level of variability of WBC, lymphocytes, and AT were observed among HPL and MPL cows in our study. Individual cow differences such as age, stage of lactation, and immune system may have contributed to this diversity. In some instances, the enrolled cows had an abrupt increase in proviral load, lymphocytes, and WBC (Cow 01 and 03) or just in AT (Cow 11), which seemed to coincide with their removal from the herd. Whether there was a health implication associated to that high rise that led to its culling was not assessed. Apart from the proviral load results from T1 and T5, no other results were shared with the farmer that could inform the culling decisions. Misclassification bias could have impacted the flow cytometry results in our study. At certain time-points, the data seemed to congregate to a single value, for example CD3+ cell population at T5 (MPL and ND), T7 (NEG and HPL), and CD4+ cell population at T4 (HPL) (Figure 4-4). We assume that different factors that impact day-to-day sample processing and analysis, such as ambient temperature during sampling and transport, pipetting errors, and sample storage conditions might have contributed to these findings. Therefore, we expect those to be artifacts. Analyzing all samples on a single day and at stable temperatures would remove such variations, however travel distances, climatic circumstances, and processing capacity limit our abilities to process the samples differently.

Overall, our study indicates that there is limited value to repeat tests for proviral load, WBC count, and CD4+ cell population during BLV control efforts, in most of the cases the findings will persist over time. The occurrence of persistent lymphocytosis in cows from HPL

and MPL group in our study demonstrates that even a single lymphocyte test indicating high values is representative of the haematological status of that animal.

Table 4-1. Categorization of the study animals into five groups based on their bovine leukemia virus (BLV) ELISA and proviral load status at enrolment.

Group	ID	BLV ELISA status at enrolment	BLV proviral load (BLV copies/Beta-Actin copy) at enrolment	Number of enrolled cows(n)	Cow ID
High proviral load	HPL	+	≥0.50	8	01-08
Moderate proviral load	MPL	+	≥0.25 to <0.50	10	09-18
Low proviral load	LPL	+	>0 to <0.25	8	19-26
Not detected	ND	+	Not detected	6	27-32
BLV-seronegative[a]	NEG	–	Not tested	14[b]	33-46

WBC = White blood cells

[a]Some of the BLV-seronegative cows seroconverted during the study.

[b] 8 cows were enrolled at time-point 1, 6 cows were added at time-point 4

Table 4-2. White blood cells, lymphocytes, proviral load, antibody titers, CD3+, CD4+, CD8+, CD21+, and WC1+ results from the study cows at the seven time-points (T1-T7).

	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
Sampling date	**June 2021**	**Sep 2021**	**Dec 2021**	**Mar 2022**	**July 2022**	**Sep 2022**	**Dec 2022**
Parameters							
WBC ($\times 10^3$ cells/µL)	10 (8; 17)	12 (9; 17)	12 (9; 15)	11 (9; 14)	9 (8; 12)	10 (8; 14)	11 (9; 13)
Lymphocytes ($\times 10^3$ cells/µL)	6 (4; 12)	6 (4; 11)	7 (4; 9)	5 (4; 8)	5 (3; 7)	5 (3; 9)	5 (3; 8)
Proviral load (BLV copies/Beta-Actin copy)	0.31 (0.07; 0.49)	0.32 (0.03; 0.50)	0.23 (0.06; 0.35)	0.19 (0.01; 0.29)	0.19 (0.01; 0.33)	0.26 (0.05; 0.40)	0.24 (0.03; 0.35)
Antibody titer (number of dilutions)	16 (8; 32)	16 (8; 32)	32 (16; 64)	32 (16; 64)	16 (8; 32)	32 (16; 64)	16 (8; 32)
CD3+ cells (%)	14 (6;24)	8 (5;14)	16 (5;21)	13 (4;17)	5 (3;12)	15 (8;24)	3 (1;5)
CD4+ cells (%)	25 (9; 66)	12 (7; 19)	63 (10; 83)	42 (9; 69)	9 (5; 15)	9 (5; 20)	10 (7; 56)

CD8+ cells (%)	4 (2; 7)	5 (3; 7)	4 (2; 6)	3 (2; 5)	2 (1.8; 4)	5 (2; 9)	1.4 (1; 3)
CD21+ cells (%)	27 (20; 48)	42 (34; 53)	41 (34; 47)	44 (37; 49)	43 (39; 52)	46 (34; 53)	48 (36; 62)
WC1+ cells (%)	3 (2; 5)	5 (2; 6)	4 (2; 5)	3 (1; 4)	2 (1; 3)	2 (1; 3)	0.8 (0.5; 1.2)

T = Time-point, WBC = White blood cells, BLV = Bovine leukemia virus, Numbers in table are [Median (Q1; Q3)]

The number of samples used at T = 1 were n = 40 (for WBC and lymphocyte count), n = 32 (for proviral load and antibody titer analysis), and n = 28 (for CD3+, CD4+, CD8+. CD21+, and WC1+ analysis)

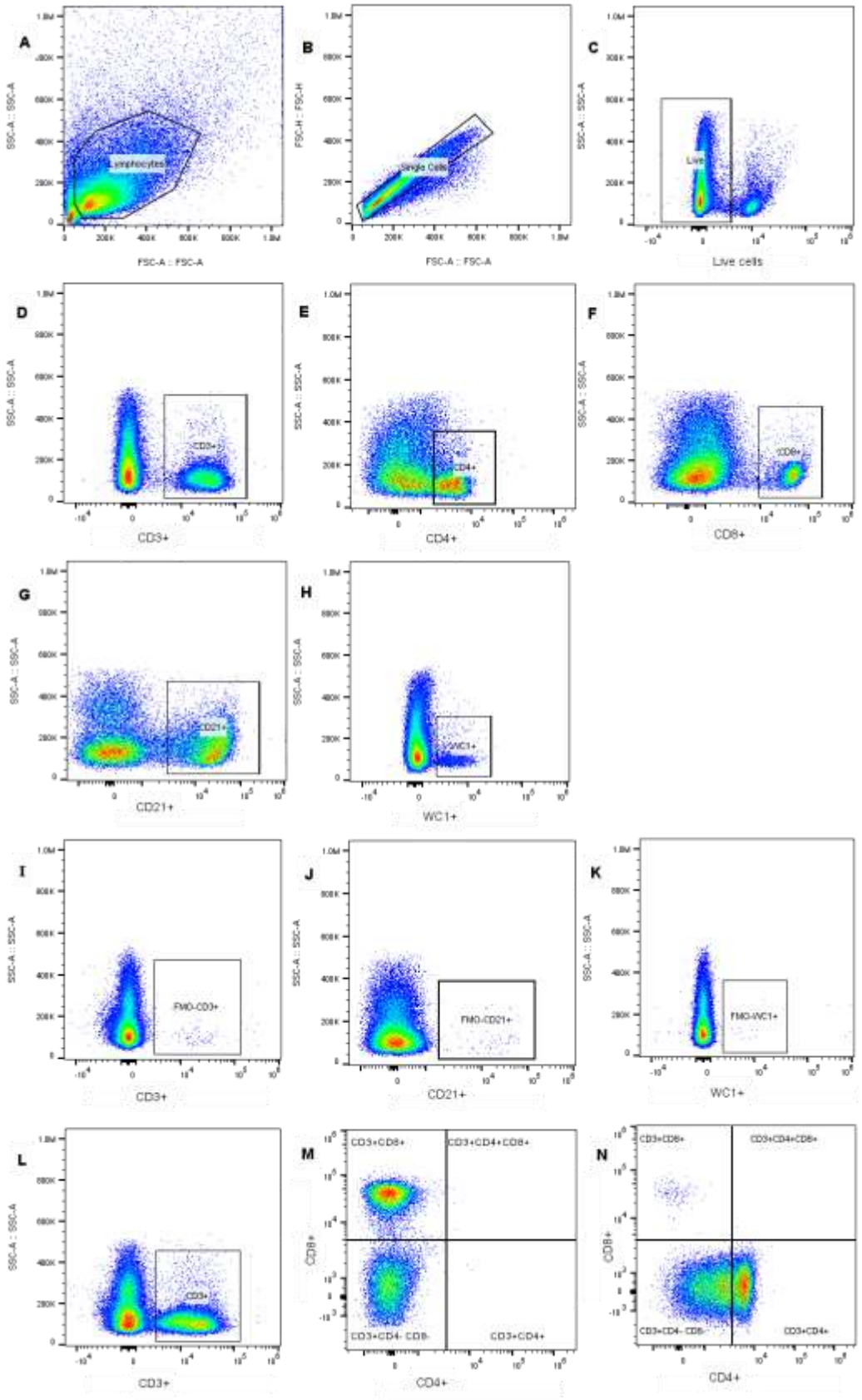
A
Lymphocytes
SSC-A :: SSC-A
FSC-A :: FSC-A
B
Single Cells
FSC-H :: FSC-H
FSC-A :: FSC-A
C
Live
SSC-A :: SSC-A
Live cells
D
CD3+
SSC-A :: SSC-A
CD3+
E
CD4+
SSC-A :: SSC-A
CD4+
F
CD8+
SSC-A :: SSC-A
CD8+
G
CD21+
SSC-A :: SSC-A
CD21+
H
WC1+
SSC-A :: SSC-A
WC1+
I
FMO-CD3+
SSC-A :: SSC-A
CD3+
J
FMO-CD21+
SSC-A :: SSC-A
CD21+
K
FMO-WC1+
SSC-A :: SSC-A
WC1+
L
CD3+
SSC-A :: SSC-A
CD3+
M
CD3+CD8+
CD3+CD4+CD8+
CD3+CD4- CD8-
CD3+CD4+
CD8+
CD4+
N
CD3+CD8+
CD3+CD4+CD8+
CD3+CD4- CD8-
CD3+CD4+
CD8+
CD4+

Figure 4-1. Gating strategy for flow cytometry using FlowJo v10.8.1 Software (BD Life Sciences, Ashland OR, USA). A) The parent cells were gated to differentiate lymphocyte population, followed by B) Single-cells gating, and C) live cells gating. Single color control gating are demonstrated for D) CD3+, E) CD4+, F) CD8+, G) CD21+, and H) WC1+. Fluorescence Minus One (FMO) control gating are demonstrated for I) FMO-CD3+, J) FMO-CD21+, K) FMO-WC1+. L) Parent CD3+ cell population were further differentiated into M) FMO-CD4+, and N) FMO-CD8+.

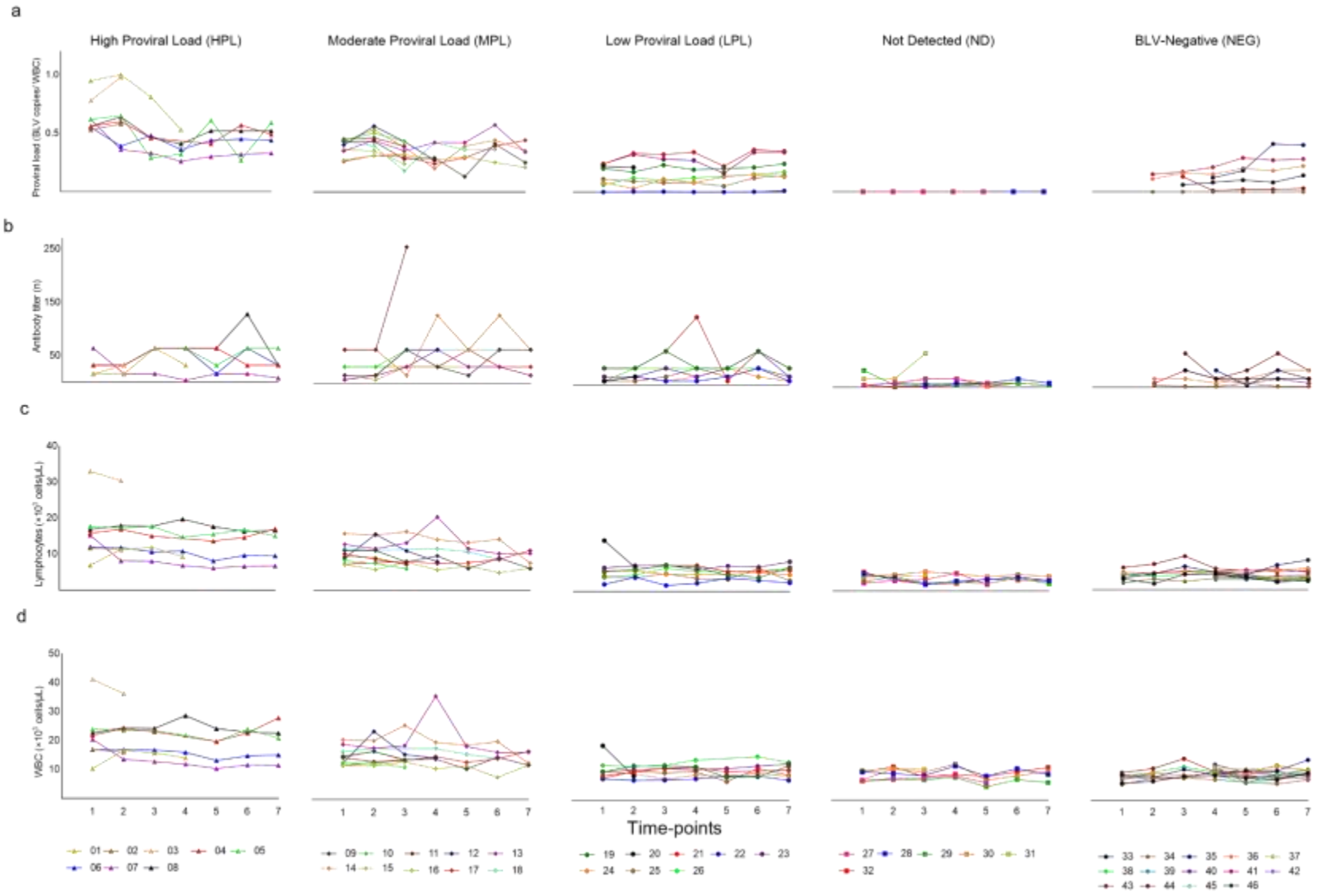
a
High Proviral Load (HPL)
Moderate Proviral Load (MPL)
Low Proviral Load (LPL)
Not Detected (ND)
BLV-Negative (NEG)
Proviral load (BLV copies/ WBC)
b
Antibody titer (n)
c
Lymphocytes (×10³ cells/μL)
d
WBC (×10³ cells/μL)
Time-points
01 02 03 04 05
06 07 08
09 10 11 12 13
14 15 16 17 18
19 20 21 22 23
24 25 26
27 28 29 30 31
32
33 34 35 36 37
38 39 40 41 42
43 44 45 46

Figure 4-2. Dynamics of a) bovine leukemia virus (BLV) proviral load, b) antibody titer, c) lymphocyte count, and d) white blood cell (WBC) count over time in individual cows. Each line indicates measurement of a single cow (01-46). Graphs in each column represent cows belonging to one of the five groups based on BLV-seropositivity and proviral load (BLV copies/Beta-Actin copy): high proviral load (HPL: ≥0.5), moderate proviral load (MPL: ≥0.25 to <0.50), low proviral load (LPL: >0 to <0.25), not detected (ND: BLV-seropositive and proviral load-negative), or BLV-seronegative (NEG), based on results at the initiation of the study. The numbers 1-7 indicate the time-points the samples were collected. Each time-point was three months apart, except time-point 5 (four months) and time-point 6 (two months).

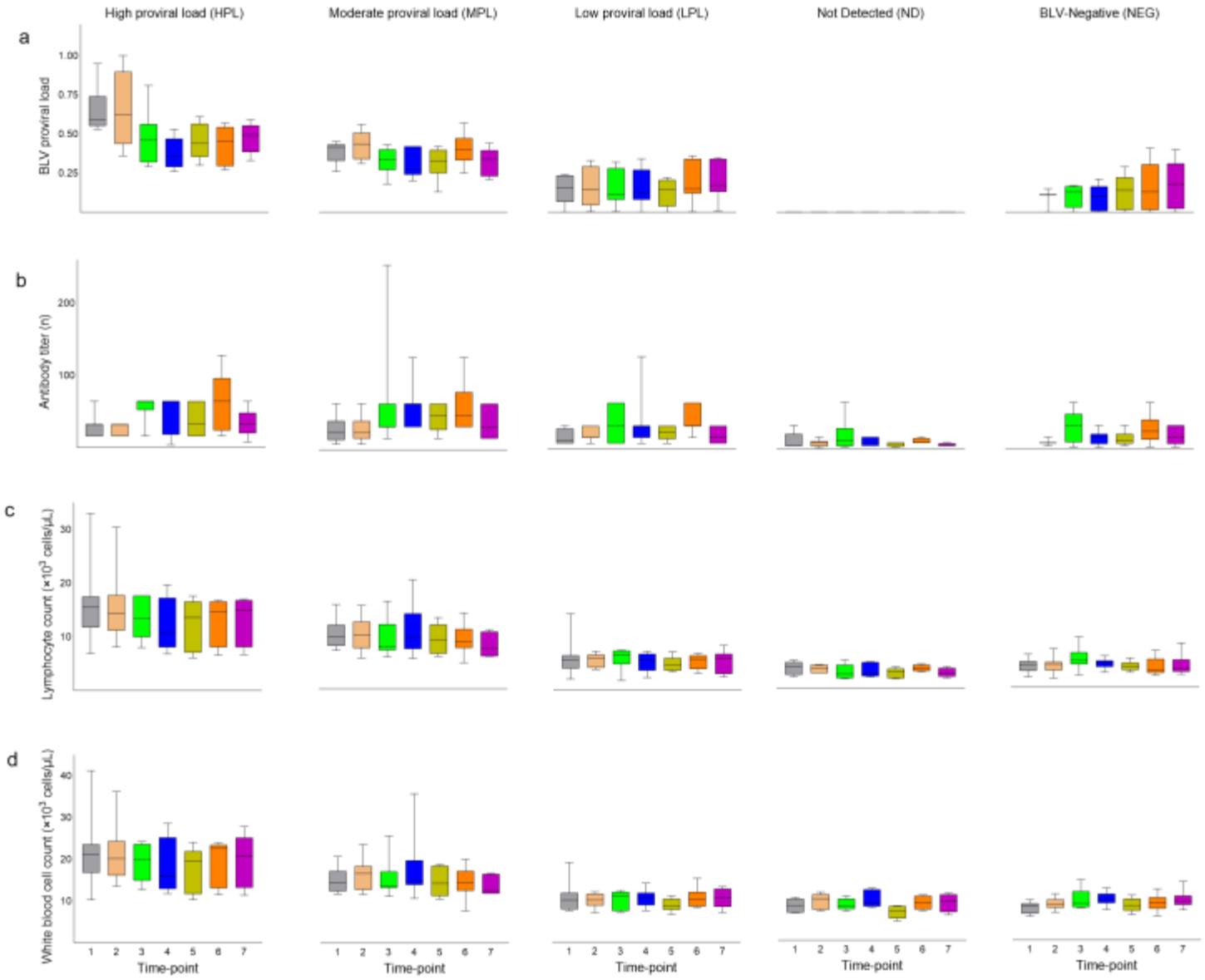
High proviral load (HPL)
Moderate proviral load (MPL)
Low proviral load (LPL)
Not Detected (ND)
BLV-Negative (NEG)
a
BLV proviral load
1.00
0.75
0.50
0.25
b
Antibody titer (n)
200
100
c
Lymphocyte count (×10³ cells/μL)
30
20
10
d
White blood cell count (×10³ cells/μL)
40
30
20
10
1 2 3 4 5 6 7
Time-point

Figure 4-3. This figure represents the simplified version of figure 4-2 by using box-plots to demonstrate changes in a) bovine leukemia virus (BLV) proviral load, b) antibody titer, c) lymphocyte count, and d) white blood cell (WBC) count over time. Graphs in each column represent cows belonging to one of the five groups based on BLV-seropositivity and proviral load (BLV copies/Beta-Actin copy): high proviral load (HPL: ≥0.5), moderate proviral load (MPL: ≥0.25 to <0.50), low proviral load (LPL: >0 to <0.25), not detected (ND: BLV-seropositive and proviral load-negative), or BLV-seronegative (NEG), based on results at the initiation of the study. The numbers 1-7 indicate the time-points the samples were collected. Each time-point was three months apart, except time-point 5 (four months) and time-point 6 (two months).

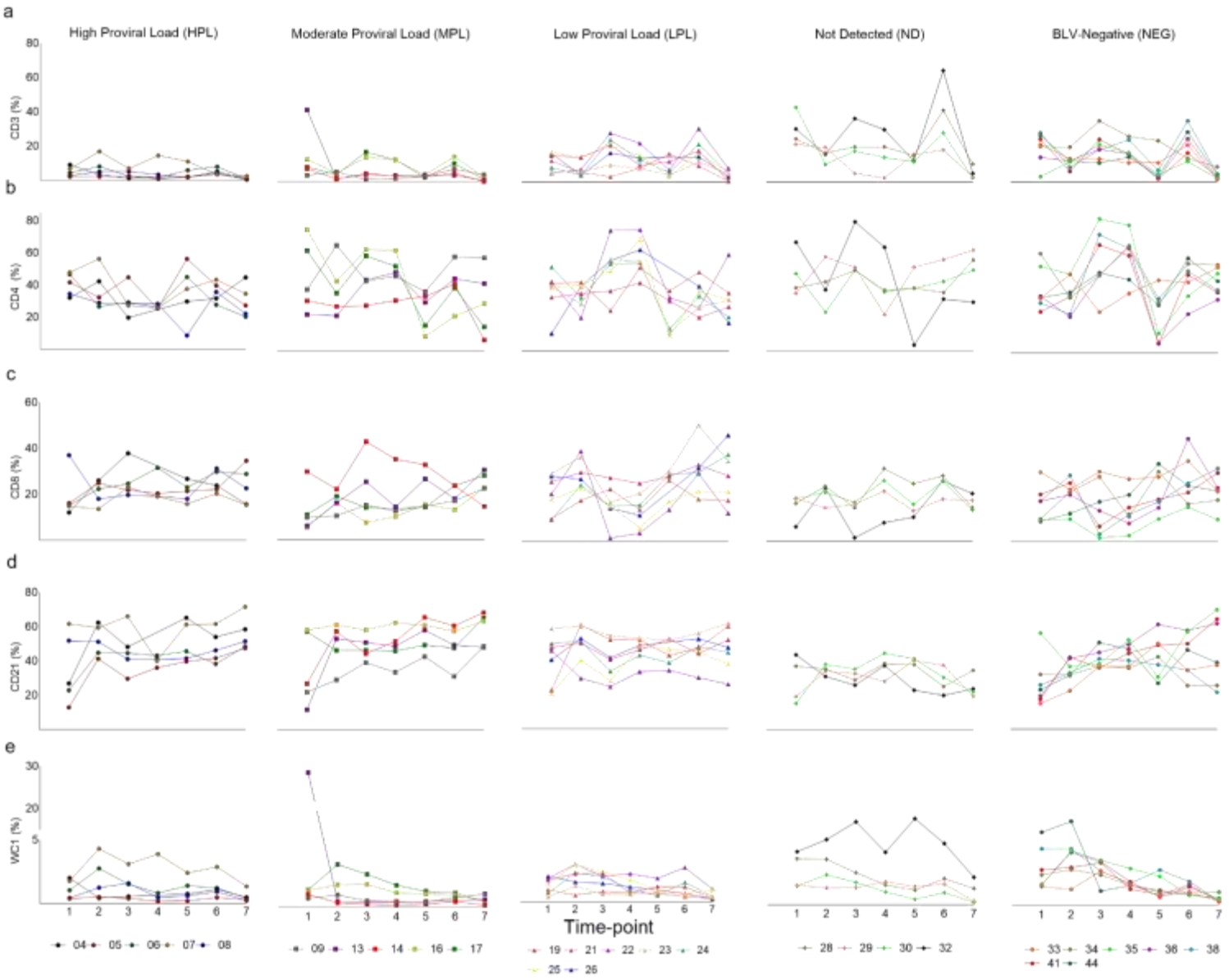
a
High Proviral Load (HPL)
Moderate Proviral Load (MPL)
Low Proviral Load (LPL)
Not Detected (ND)
BLV-Negative (NEG)
CD3 (%)
b
CD4 (%)
c
CD8 (%)
d
CD21 (%)
e
WC1 (%)
Time-point
04 05 06 07 08
09 13 14 16 17
19 21 22 23 24 25 26
28 29 30 32
33 34 35 36 38 41 44

Figure 4-4. Dynamics of a) CD3+, b) CD4+, c) CD8+, d) CD21+, and e) WC1+ cell population over time in individual cows. Each line indicate measurement of a single cow (01-46). Graphs in each column represent cows belonging to one of the five groups based on BLV-seropositivity and proviral load (BLV copies/Beta-Actin copy): high proviral load (HPL: ≥0.5), moderate proviral load (MPL: ≥0.25 to <0.50), low proviral load (LPL: >0 to <0.25), not detected (ND: BLV-seropositive and proviral load-negative), or BLV-seronegative (NEG), based on results at the initiation of the study. The numbers 1-7 indicate the time-points the samples were collected. Each time-point was three months apart, except time-point 5 (four months) and time-point 6 (two months).

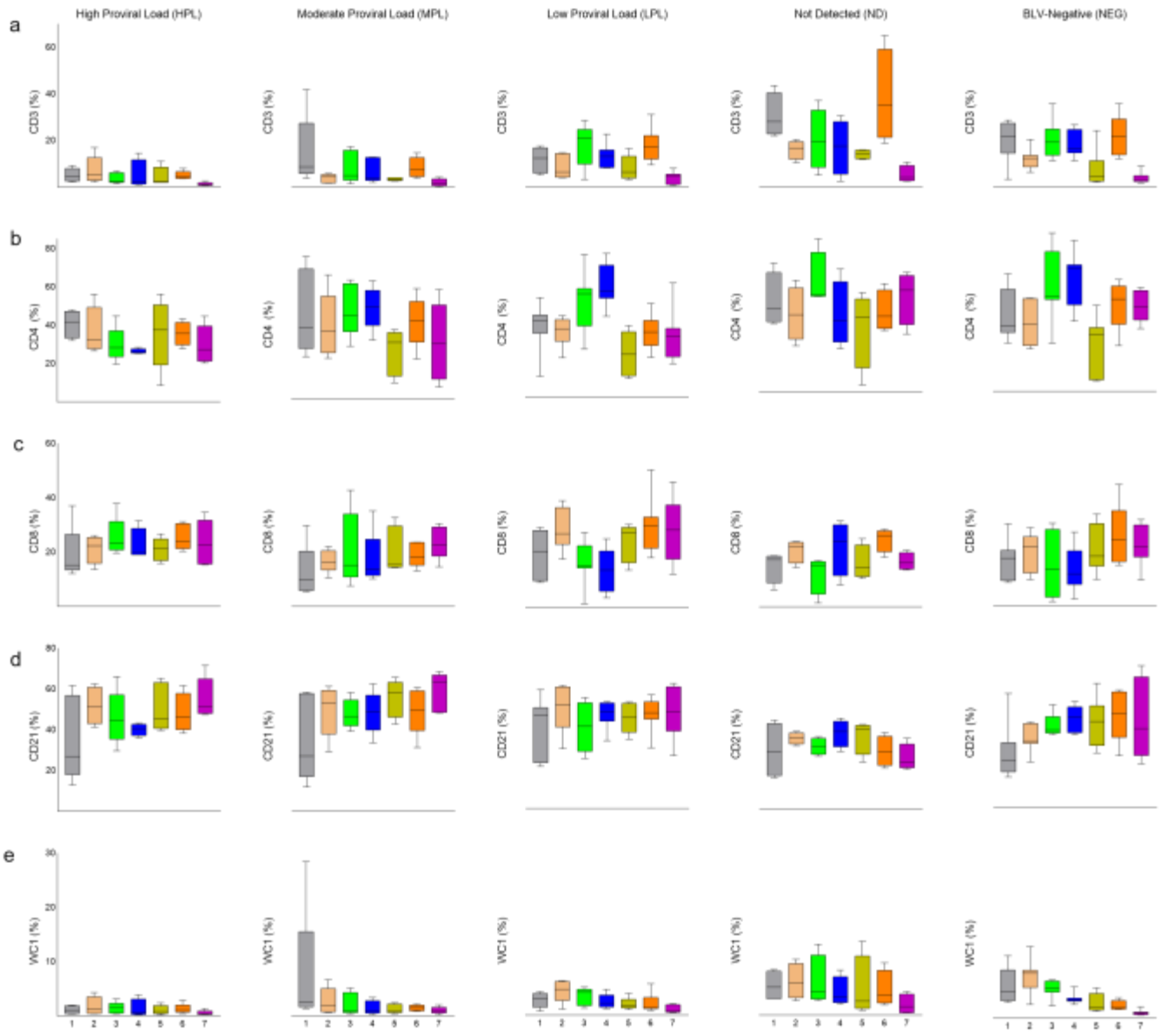
a
b
c
d
e
High Proviral Load (HPL)
Moderate Proviral Load (MPL)
Low Proviral Load (LPL)
Not Detected (ND)
BLV-Negative (NEG)
CD3 (%)
CD4 (%)
CD8 (%)
CD21 (%)
WC1 (%)

Figure 4-5. This figure represents the simplified version of figure 4-4 by using box-plots to demonstrate a) CD3+, b) CD4+, c) CD8+, d) CD21+, and e) WC1+ cell population dynamics over time. Graphs in each column represent cows belonging to one of the five groups based on BLV-seropositivity and proviral load (BLV copies/Beta-Actin copy): high proviral load (HPL: ≥0.5), moderate proviral load (MPL: ≥0.25 to <0.50), low proviral load (LPL: >0 to <0.25), not detected (ND: BLV-seropositive and proviral load-negative), or BLV-seronegative (NEG), based on results at the initiation of the study. The numbers 1-7 indicate the time-points the samples were collected. Each time-point was three months apart, except time-point 5 (four months) and time-point 6 (two months).

CHAPTER 5: The association between hematological and immunological parameters to determine bovine leukemia virus proviral load in BLV-infected bovines

5.1 Abstract

Bovine leukemia virus (BLV) infection in cattle is diagnosed using ELISA for antibody-detection and qPCR for BLV proviral detection and quantification. There is a lack of consensus on the proviral load categories and the proviral load cut-off for high proviral load (HPL) cow identification. The first objective of this study was to evaluate the impact of BLV infection on hematological (WBC, lymphocyte, monocyte, and granulocyte counts) and immunological (BLV antibody titer, CD3+, CD4+, CD8+, CD21+, and WC1+ cell population) parameters by comparing cows based on BLV seropositivity (BLV-positive and BLV-seronegative) and BLV proviral load status [high (HPL), moderate (MPL), low proviral load (LPL), and not detected (ND)]. The second objective was to determine cut-off values of BLV proviral load and antibody titer for distinguishing HPL and LPL cows. A cross-sectional study was conducted with 429 cows selected from ten dairy herds categorized into five groups. Blood samples from participant cows were used for analyzing blood cells (white blood cell, lymphocyte, monocyte, and granulocyte), antibody titers, and flow cytometry on immune cells (CD3+, CD4+, CD8+, CD21+, WC1+ cell proportions). Reference level of WBCs and lymphocytes were generated using measurements from BLV-seronegative cows and the higher values were used to establish optimal proviral load and antibody titer cut-offs. There were significant differences among the five groups for all parameters except granulocytes, CD4+, and CD21+ cell proportions. Use of high WBC (>12.6 $\times 10^3$ cells/µL) and high lymphocytes (>6.3 $\times 10^3$ cells/µL) indicated an

optimal HPL cut-off at 0.25 (sensitivity = 85.5% and specificity = 86.7%) and 0.21 (sensitivity = 91% and specificity = 91.7%), respectively. Whereas, antibody titers cut-offs were similar (n = 32) for both high WBC (sensitivity = 70.6% and specificity = 62.8%) and high lymphocyte (sensitivity = 66.7% and specificity = 64.8%) methods. Our results indicate a lower HPL cut-off based on blood parameter information which warrants further investigation using epidemiological and pathological data to establish HPL cut-offs.

Key words: enzootic bovine leukosis, high proviral load, threshold, PCR, blood cell count

5.2 Introduction

Enzootic bovine leukosis (EBL) causes a widespread infection in dairy operations in North America with nearly 90% of the herds infected (LaDronka et al., 2018; Kuczewski et al., 2019). EBL is caused by bovine leukemia virus (BLV), a deltaretrovirus, which primarily targets CD5+ B-cells mostly leading to subclinical infections, however, BLV infections could have clinical implications such as persistent lymphocytosis and lymphoma in 30% and 5% of the infected animals, respectively (Schwartz and Levy, 1994). Because of the low mortality and lack of evident clinical signs, controlling bovine leukosis hasn't been a priority in North America. However, BLV causes immunosuppression in cows which subsequently leads to early removal from herds, or reduced milk, fat, and protein production (Bartlett et al., 2013; Frie and Coussens, 2015; Shrestha et al., 2024). Additionally, BLV impairs the welfare of infected animals which is concerning to the consumers and might amplify the negative perceptions towards the dairy industry (Bartlett et al., 2014).

Historically, BLV detection and control was implemented by monitoring elevated lymphocytes level, detection of lymphomas in post-mortem examinations, and antibody detection in serological tests (EFSA Panel on Animal Health and Welfare, 2015). Recently, assessing BLV proviral loads using qPCR-based methods indicated that not all infected animals pose high transmission risk. Thus, adjusting BLV control strategies from removing all BLV-infected animals to removing the subset with a high proviral load could make BLV control easier to implement (Jimba et al., 2010; Juliarena et al., 2016; Ruggiero et al., 2019). Animals with a high proviral load (HPL) demonstrate more often a persistent lymphocytosis (Juliarena et al., 2007; John et al., 2022). An association of a higher white blood cell counts (WBC) and higher antibody titers with increasing BLV proviral loads were reported (Alvarez et al., 2013; Jaworski

et al., 2016). Additionally, proliferation of B-lymphocytes (CD21+) and exhaustion of T-lymphocytes (CD3+) and its CD4+ and CD8+ sub-populations were observed in HPL cows (Farias et al., 2018). These evidence explain the health and production implications of BLV infection, especially those with a high proviral load.

As mentioned in Chapter 4, BLV-infected animals are usually categorized as high (HPL), moderate (MPL), low (LPL), or not-detected (ND) based on proviral load values. However, these categorizations are based on arbitrary numbers (Juliarena et al., 2007; Ruggiero et al., 2019; John et al., 2022). There needs to be health or immunological relevance to support the BLV categorizations, as arbitrary categorizations may discourage farmers and veterinarians from using this tool. High WBC and lymphocyte count were used to identify a HPL cow (Juliarena et al., 2007; John et al., 2022), however these studies used a predetermined arbitrary HPL cut-off (100,000 copies of provirus or 0.5 BLV copies/WBC), and therefore a more rational approach is warranted. Using BLV antibody titers could be another alternative to identify a HPL cow as BLV proviral load values and antibody titers are associated (Jaworski et al., 2016). Therefore, we had two main objectives in this study. The first objective was to evaluate the impact of BLV infection on hematological (WBC, lymphocyte, monocyte, and granulocyte counts) and immunological (BLV antibody titer, CD3+, CD4+, CD8+, CD21+, and WC1+ cell population) parameters by comparing cows based on BLV seropositivity (BLV-positive and BLV-seronegative) and BLV proviral load status (HPL, MPL, LPL, and ND). The second objective was to determine cut-off values of BLV proviral load and antibody titer for distinguishing HPL cows based on their white blood cell and lymphocyte counts.

5.3 Materials and methods

5.3.1 Sample collection and processing

The dairy cows enrolled in this study were participants in a three-year bovine leukosis control project conducted across ten dairy herds (A–J) in Alberta, Canada as described in detail in Chapter 3 of this thesis. Briefly, the study involved annual screening of all adult cows with unknown BLV-serostatus or known BLV-seronegative status with an ELISA test in milk (Bovichek BLV ELISA kit, Biovet Inc., Montreal QC, Canada) or blood (Leukosis Serum X2 Ab Test, IDEXX Laboratories, Inc., Westbrook ME, USA) samples. Blood samples were obtained from cows that were confirmed BLV-seropositive, to quantify the BLV proviral load using the BLV SS1 qPCR test (CentralStar Cooperative Inc., East Lansing MI, USA). The results were classified in groups as i) high proviral load or HPL (≥0.50), ii) moderate proviral load or MPL (0.25 to 0.5), iii) low proviral load or LPL (>0 to <0.25), iv) not detected or ND (ELISA-positive, no proviral load detected), and v) NEG (ELISA-negative).

In the second year of the project, a subset of 429 cows were selected from these herds ensuring representation from each of the five groups. Blood samples (1 serum and 1 EDTA tube) were collected from each of these cows using the caudal tail vein. The samples were transported in a cooler with ice pack to the University of Calgary laboratory and processed within 48 hrs of collection. Sample processing was conducted as described in the "Sampling scheme" under methodology section of Chapter 4. Briefly, two aliquots of serum sample (stored at –20 °C and –80 °C) and three aliquots of EDTA-mixed blood sample was harvested. Of the two serum aliquots, one was used to determine the BLV serostatus of cows that were previously seronegative (n = 106), while the other aliquot was used for antibody titer determination in a subset of BLV-seropositive cows (n = 257) using the end-point dilution approach (methods in

Chapter 4). Of the three EDTA-mixed blood aliquots, one aliquot (4-mL) was used for peripheral blood mononuclear cells (PBMC) separation and stored in liquid nitrogen, second aliquot (1-mL) was used for determining the WBC and lymphocyte count ($\times 10^3$ cells/μL) using the HemaTrue automatic veterinary hematology analyzer (Heska Corporation, Loveland CO, USA), and the third aliquot (200-μl stored at –80 °C) was used for BLV proviral load determination (methods in Chapter 4). Monocytes and granulocytes count ($\times 10^3$ cells/μL) results were additionally obtained as these results were available together with WBC and lymphocyte count results. PBMC separation, WBC, and lymphocyte count was performed on all study cows (n = 429), whereas BLV proviral load was estimated only on BLV-seropositive cows (n = 348). Based on the ELISA and qPCR results, the selected cows were assigned into one of the five groups: HPL (n = 46), MPL (n = 100), LPL (n = 146), ND (n = 56), and NEG (n = 81).

A subset of PBMC samples (n = 45) stored in liquid nitrogen were selected from four targeted herds, ensuring equal number of selected cows from each of the five groups (n = 9 cows/group). Flow cytometry analysis was conducted on frozen PBMC cells to determine B-cells (CD21+), T-cells (CD3+), and its subsets (CD4+, CD8+, and WC1+ cell population) proportions in PBMC. The details on the cell processing, antibody staining, flow cytometry analysis, gating strategies, and target cell population estimation are described under methodology section of Chapter 4. The mean ± SD viability of the PBMC cells was 92.6% ± 7.3%.

5.3.2 Statistical analysis

To address the first objective evaluating the impact of BLV infection and proviral load, we considered ten outcome variables: WBC, lymphocyte, monocyte, and granulocyte counts, and percentages of CD3+, CD4+, CD8+, CD21+, and WC1+ cells in the PBMC population, and

finally antibody titers. These variables were assessed across the five groups of cows, except for antibody titer, which was assessed in four groups (HPL, MPL, LPL, and ND). Correlations of BLV proviral load with the ten outcome variables was assessed using Spearman's rank correlation test. The distribution of the outcome variables was assessed for normality using the Shapiro Wilk's test. If the variables followed a normal distribution (CD4+, CD8+, and CD21+ cells), mean comparisons of the groups was conducted using an ANOVA, followed by a Tukey-Kramer HSD (Honestly Significant Difference) post-hoc test. Variables non-normal distributed (WBC, lymphocytes, monocytes, granulocytes, antibody titers, CD3+, and WC1+ cells), a Kruskal-Wallis test was utilized to compare the means between groups, followed by Dunn's pairwise comparison test with Bonferroni correction to compare mean rank of each column with the mean rank of every other column. The statistical significance was determined at $P < 5\%$. Data from NEG cows (n = 81) was utilized to establish reference values for WBC and lymphocyte counts using the formula: mean cell count ± 2 SD (John et al., 2022). The upper range values were used as thresholds to categorize cows into four groups based on the cell counts: normal WBC, high WBC, normal lymphocyte, and high lymphocyte. Receiver operator characteristic (ROC) analysis was performed to establish optimal cut-offs for BLV proviral load in all BLV-seropositive cows (n = 348), and for antibody titers in a subset of BLV-seropositive cows (n=257) by using high WBC and high lymphocyte thresholds. Area under the curve (AUC) analysis was conducted for all four analyses. All statistical procedures were performed using Stata 15.1 (StataCorp LLC, College Station, TX, USA) and all graphs were built in Graphpad Prism (v10.0.2 for Windows, GraphPad Software, Boston MA USA, www.graphpad.com). All protocols involving animals were approved by the Veterinary Sciences Animal Care Committee of the University of Calgary (VSACC AC20-0095 and AC21-0210).

5.4 Results

The ten dairy herds enrolled in this study had the median (min-max) within-herd BLV prevalence of 27.3 % (8.7-50.1%). The median (min-max) number of cows selected from each herd was 39 (26-64). The majority of cows were of Holstein Friesian breed (n = 379), followed by Jersey (n = 2), Ayrshire (n = 4), and Brown Swiss (n = 5). Breed information was not available for 39 cows. The median lactation number of the cows was 3, ranging from 1 to 9. The sampling period started on June 3, 2021, and ended on February 8, 2022. Table 5-1 summarizes the distribution of BLV proviral load and the ten outcome variables.

5.4.1 Hematological Parameters

5.4.1.1 White blood cell count (WBC)

A significant positive correlation was observed between WBC count and proviral load ($\rho = 0.83$, $P < 0.001$). The median (max-min) WBC count ($\times 10^3$ cells/μL) was the highest in HPL cows [21.2 (10.3-47.4)], followed by MPL [15.5 (8.7-22.9)], LPL [9.5 (5.5-19.4)], NEG [8.6 (4.6-14.3)], and ND [8 (2.5-13)] cows. The five groups were significantly different from each other with regards to the mean of log transformed WBC. Dunn's comparison test revealed significant differences between all groups except LPL vs NEG and ND vs NEG (Figure 5-1).

5.4.1.2 Lymphocyte count

A significant correlation was observed between lymphocyte count and BLV proviral load ($\rho = 0.88$, $P < 0.001$). The median (max-min) lymphocyte count ($\times 10^3$ cells/μL) for the five groups were: HPL [15.4 (6.9-35)], MPL [9.7 (5-16.3)], LPL [4.7 (1.6-14.2)], NEG [3.6 (1.7-

7.4)], and ND [3.2 (1.4-6.3)]. Significant differences in the mean of log transformed-lymphocyte count were observed among the groups. Pairwise comparisons using Dunn's test revealed significant differences between all groups, except NEG vs ND (Figure 5-1).

5.4.1.3 Monocyte count

A significant positive association was observed between monocyte count and proviral load ($\rho = 0.68$, $P < 0.001$). Monocyte count results could not be obtained for five cows (one from each of the five groups). The median (min-max) monocyte counts ($\times 10^3$ cells/μL) for the five groups were: HPL [1.4 (0.8-2.9)], MPL [1.1 (0.6-2.3)], LPL [0.8 (0.4-1.7)], NEG [0.8 (0.4-1.4)], and ND [0.7 (0.3-1.3)]. The groups were significantly different to each other in terms of the mean of log transformed monocytes. Post-hoc analysis revealed significant differences between groups, except LPL vs ND, NEG vs ND, and LPL vs NEG (Figure 5-1).

5.4.1.4 Granulocyte count

Granulocytes demonstrated a weak, positive association with proviral load ($\rho = 0.1$, $P = 0.04$). Similar to monocyte count, granulocyte count results could not be obtained for five cows. The median (min-max) granulocyte counts ($\times 10^3$ cells/μL) varied among the five groups as: HPL [4.4 (2.1-9.6)], MPL [3.9 (1.3-9.9)], LPL [3.9 (1.6-8)], NEG [4 (2-8.1)], and ND [3.6 (0.8-7.8)]. No difference in the mean of log transformed-granulocyte count was observed among the groups (Figure 5-1).

5.4.2 Immunological Parameters

5.4.2.1 Antibody titer

Determination of antibody titer was implemented in four groups: HPL (n = 43), MPL (n = 75), LPL (n = 93), and ND (n = 46). A positive correlation was observed between antibody titer and BLV proviral load ($\rho = 0.57$, $P < 0.001$). The median (max-min) antibody titer values (number of dilutions) varied among the four groups as: MPL [32 (8-512)], HPL [32 (8-256)], LPL [16 (1-256)], and ND [8 (2-32)]. Significant differences were observed among the groups while comparing means of log transformed-antibody titers. Pairwise comparisons demonstrated significant differences between groups, except HPL vs MPL (Figure 5-1).

5.4.2.2 B- and T-cells

The summary of the B-cells (CD21+), T-cells and its subsets (CD3+, CD4+, CD8+, and WC1+) using flow cytometry are presented below and their distributions are graphically presented in Figure 5-2. CD3+ cell proportions in the PBMC population were negatively associated with BLV proviral load ($\rho = -0.58$, $P < 0.001$). The median (max-min) CD3+ % was the highest for LPL [20.7 (17.1-44.3)], followed by ND [18.7 (13.9-41.1)], NEG [16 (9.1-58.3)], MPL [10.6 (5.5-25.7)], and HPL cows [9.2 (2.8-27.2)]. Comparisons of the mean log transformed-CD3+ cell proportions revealed significant differences among the groups. Post-hoc comparisons indicated differences between HPL vs LPL, HPL vs ND, and MPL vs LPL groups (Figure 5-2).

There was no association of the proportion of CD4+ cells in PBMCs with BLV proviral load ($\rho = -0.21$, $P = 0.21$). The mean ± SD of CD4+ cell % varied among the five groups: NEG (39.7 ± 8.4), ND (33.2 ± 12.6), MPL (29.3 ± 13), LPL (25.8 ± 13), and HPL (23.8 ± 13.5).

ANOVA analysis followed by the Tukey HSD pairwise comparisons failed to demonstrate any differences between the groups (Figure 5-2).

Negative correlation between CD8+ cell proportions in PBMCs and BLV proviral load was observed ($\rho = -0.54$, $P < 0.001$). The CD8+ cell % (mean ±SD) was: ND (34.4 ± 8.1), LPL (29.1 ± 15.7), MPL (25.2 ± 11.1), NEG (24.3 ± 8.3), and HPL (15.5 ± 11.3). The groups were different in their CD8+ proportions in the ANOVA analysis. Post-hoc analysis revealed difference between HPL vs ND groups (Figure 5-2).

A strong negative correlation of WC1+ cells proportion in PBMCs was observed with BLV proviral load ($\rho = -0.81$, $P < 0.001$). The distribution of WC1+ cell % [median (max-min)] varied between the groups: ND [3.5 (1.8-6.8)], NEG [3.4 (1.5-6.6)], LPL [3 (0.6-4.4)], MPL [1.6 (1.1-2.6)], and HPL [0.9 (0.4-1.5)]. Differences in the mean of log transformed-WC1+ cell proportions were observed between the groups. Post-hoc analysis revealed differences between HPL vs ND, HPL vs NEG, HPL vs LPL, MPL vs ND, and MPL vs NEG groups (Figure 5-2).

There was no correlation of CD21+ cells in the PBMCs with BLV proviral load ($\rho = 0.24$, $P = 0.15$). The CD21+ cell% (mean ± SD) of the five groups were as follows: MPL (48.3 ± 17.6), HPL (37.7 ± 18.1), LPL (34.6 ± 14.4), ND (32.9 ± 9.9), and NEG (30.2 ± 14.7). There were no differences among the groups regarding the mean of CD21+ cell%(Figure 5-2).

5.4.3 Determination of proviral load and antibody titer cut-offs

5.4.3.1 Reference levels for white blood cell and lymphocyte

The NEG cows used for determining the reference levels of WBC and lymphocyte were primarily in lactation 1-4 (n = 77), while two were in their 5th and two in their 8th lactation. The majority of these NEG cows were of Holstein Friesian breed (n = 71), one was Brown Swiss,

whereas breed information was not available for the remaining cows (n = 9). Reference ranges were estimated to be 5-12.6 ($\times 10^3$ cells/μL) for WBC and 1.2-6.3 ($\times 10^3$ cells/μL) for lymphocyte count. Based on the upper limits of the reference ranges, the cows in the five groups were further classified into normal and high WBC and lymphocyte counts (Table 5-2). Three HPL cows exhibited normal WBC count (<12.6 $\times 10^3$ cells/μL), whereas none of the HPL cows had lymphocyte count in the normal range (<6.3 $\times 10^3$ cells/μL). Seventy-five percent of the MPL cows had high WBC count, whereas 93% of MPL cows exhibited high lymphocyte count.

5.4.3.2 BLV proviral load cut-off

Receiver operator characteristic curves for identification of high WBC count with BLV proviral load resulted in the area under the curve (AUC) of 0.93 (95% CI: 0.91-0.96, $P = 0.01$) (Figure 5-3). An optimal cut-off was established at 0.25 with a sensitivity of 85.5% (95% CI: 81.8-89.2%), a specificity of 86.7% (95% CI: 83.1-90.2%), a positive predictive value (PPV) of 80.8% (95% CI: 76.7-85%), and a negative predictive value (NPV) of 90.1% (95% CI: 87-93.2%). Increase in the proviral load cut-off value led to decrease in sensitivity and increase in specificity.

Similarly, using proviral load values to identify high lymphocyte with ROC curves resulted in an AUC of 0.97 (95% CI: 0.96-0.99, $P = 0.007$) (Figure 5-3) with an optimal cut-off at 0.21 with a sensitivity of 91% (95% CI: 88-94%), a specificity of 91.7% (95% CI: 88.8-94.6%), a PPV of 91% (95% CI: 88-94%), and a NPV of 91.7% (95% CI: 88.8-94.6%). As the proviral load cut-offs increased, sensitivity decreased, and specificity increased.

5.4.3.3 Antibody titer cut-off

Receiver operator characteristics curves for identifying high WBC count using antibody titer resulted in an AUC of 0.74 (95% CI: 0.68-0.79, $P = 0.03$) (Figure 5-3), with an optimal antibody titer cut-off at 32 with a sensitivity of 70.6% (95% CI: 65.1-76.2%), a specificity of 62.8% (95% CI: 56.9-68.8%), a PPV of 58.3% (95% CI: 52.3-64.4%), and a NPV of 74.4% (95% CI: 69.1-79.7%). An increase in the antibody titer cut-off values led to a decrease in sensitivity and an increase in specificity.

Similar to identification of high WBC, an optimal cut-off to identify high lymphocyte using antibody titer was established at 32, with a sensitivity of 66.7% (95% CI: 60.9-72.4%), a specificity of 64.8% (95% CI: 59-70.6%), a PPV of 66.7% (95% CI: 60.9-72.4%), and a NPV of 64.8% (95% CI: 59-70.6%). Increase in antibody titer cut-off led to decrease in sensitivity and increase in specificity. The ROC curves resulted in an AUC of 0.74 (95% CI: 0.68-0.80, $P = 0.03$) (Figure 5-3).

5.5 Discussion

The first objective of our study was to explore the impact of BLV infection and currently used proviral load status (HPL, MPL, LPL, ND, and NEG) on the hematological and immunological parameters in dairy cows. White blood cell, lymphocyte, and monocyte counts were significantly different between the groups classified based on BLV infection status, highlighting the impact of BLV infection and proviral load on host's immune system (Frie and Coussens, 2015). BLV-seropositive cows, especially HPL and MPL cows had higher values of these three parameters, and strong positive associations of BLV proviral load with these parameters, which is in line with previous studies (Alvarez et al., 2013; John et al., 2022). BLV

infection induces a strong humoral and cytotoxic T-cell responses in the host that persist long-term (Florins et al., 2007), which is why most BLV infections remain subclinical. However, persistent lymphocytosis is observed in 30% of the infected animals, which also have a higher risk of increased proviral loads (Schwartz and Levy, 1994; Juliarena et al., 2007). This can be related to our study as all HPL cows and 93% of the MPL cows had high lymphocytes (Table 5-2). Granulocytes were weakly associated with proviral load in our study and no differences observed among groups (except HPL vs ND), in contrast to the monocyte fraction. These results were similar to a previous finding of no differences in neutrophils (a component of granulocytes) concentrations observed between BLV-seropositive and seronegative cows (Swenson et al., 2013). Neutrophils, which are the first responders to most infections, have a relatively shorter lifespan than monocytes. Monocytes can differentiate to macrophages and contribute to innate as well as adaptive immune responses (Abbas and Lichtman, 2011). Elevated monocytes in HPL and MPL cows indicates the impact BLV proviral load may have on the expression of surface antigens and the phagocytic activity of monocytes and monocyte-derived macrophages (Werling et al., 1998).

We determined an updated reference level for WBC and lymphocyte count based on BLV-seronegative cows. Older veterinary hematology reference intervals might not reflect the current production system, genetics, and disease status, and therefore requires updating (George et al., 2010). Recently, a study in the Canadian Maritimes determined reference values for these two parameters based on BLV-seronegative dairy cows (John et al., 2022). The upper limit of the reference intervals in that study for WBC (>10.8 $\times 10^3$ cells/μL) and lymphocyte (>5.2 $\times 10^3$ cells/μL) count was lower than what we observed (>12.6 $\times 10^3$ cells/μL for WBC and >6.3 $\times 10^3$ cells/μL for lymphocyte counts). While both these studies had similarities regarding sample size,

BLV-status, geography, timing, and lactation number of the enrolled cows, we speculate these variations occurred due to differences in the way the automatic cell counting systems score certain cell types. The study by John et al. (2022) used a Sysmex XT 2000i hematology analyzer (Sysmex Canada, Inc., Mississauga ON, Canada) while we used a HemaTrue automatic veterinary hematology analyzer (Heska Corporation, Loveland CO, USA).

We also observed a significant effect of BLV infection and associated proviral load on immunological parameters. Antibody titers were relatively high in HPL and MPL groups, and lower in ND group. In our longitudinal study (Chapter 4) we observed a consistently high BLV antibody titer among the MPL group and a low titer in the ND groups, which is similar to this study. This confirms a positive association between BLV proviral load and antibody titer (Gutierrez et al., 2012; Jaworski et al., 2016). We investigated the impact of BLV infection on the immune cell fractions (B- and T-cells). The proportion of T-cells (CD3+) and its subsets (CD8+ and WC1+) in HPL cows were lower compared to ND cows, but similar to NEG cows (except WC1+) and MPL cows. No differences in CD21+ or CD4+ cell proportions were observed among the groups which is in contrast to a previous finding that observed significantly high CD21+ and low CD3+ and CD4+ in HPL cows when compared to LPL and BLV-negative cows, and low CD8+ in HPL cows compared to LPL cows (Farias et al., 2018). BLV infection causes exhaustion of T-cells and proliferation of B-cells as BLV proviruses interfere with the natural cycle of cellular apoptosis and proliferation (Erskine et al., 2011b; Frie and Coussens, 2015). Therefore, with the increase in BLV proviral load, depleted T-cell and proliferated B-cells are expected. However, our results failed to demonstrate these variations. This could have happened due to a lack of power with lower number of samples in this study, expected day-to-

day variations in sample processing, or artifacts produced while working with frozen PBMC cells for flow cytometry experiment, as mentioned in the 'Discussion' section of Chapter 4.

Overall, the assessment of hematological and immunological parameters between the five groups demonstrated similarities between LPL, ND, and NEG group (granulocyte, monocytes, CD3+, CD4+, CD8+, CD21+, WC1+), LPL and NEG group (WBC), or ND and NEG group (WBC, lymphocyte). This evidence suggests combining LPL and ND as a single category of 'LPL' may be a simpler way of representing low proviral load group. Additionally, this might be a reason why difference in milk, fat, and protein production was not observed between LPL and BLV-negative cows (Chapter 2). When BLV-infection is differentiated based on seropositivity, the BLV-seropositive fraction contains both LPL and HPL cows with LPL cows usually in a higher proportion compared to HPL cows. This might be another reason why studies fail to observe the impact of BLV-infection when BLV-seropositive and BLV-seronegative cows are compared (Tiwari et al., 2007; Sorge et al., 2011). While assessing the HPL and MPL cows, WBC, lymphocyte, and monocyte counts, as well as antibody titers were high, whereas WC1+ populations were at low value among all groups. These results indicate similarities between HPL and MPL group, which suggest a simpler category of 'HPL' combining the HPL and MPL group could be sufficient.

Our next objective was to establish a rational cut-off value to identify a HPL cow. The cut-offs used for HPL cows are mostly arbitrary as they are not backed up by clinical or pathological significance. The dynamics of proviral load in the longitudinal study in Chapter 4 demonstrated instances when the proviral load fluctuated from one group to the other. This may have happened because of using thresholds not representing the proviral load status accurately. Use of such cut-offs cause improper HPL classification as observed in a study where cows with

normal lymphocyte counts had high BLV proviral loads (Juliarena et al., 2007). A false-positive HPL detection might lead to its removal from the herd, whereas a false-negative HPL cow could stay in the herd and contribute to BLV transmission. This has a substantial economic impact. We established two optimal BLV proviral load cut-off values that can differentiate BLV-seropositive cows with high WBC (0.25) and lymphocyte counts (0.21). This approach of establishing a cut-off for HPL cow determination is backed by clinical reasoning (elevated WBC and lymphocyte count). The optimal HPL cut-offs in our study were lower than that used by John et al. (0.5 and 1.0 BLV copies/WBC). While both studies used the same qPCR system (BLV SS1 qPCR assay by CentralStar Cooperative Inc.), there were differences in DNA extraction method (Qiagen DNEasy kit vs Quick-DNA Miniprep Plus kit), qPCR instrument (ABI 7500 qPCR instrument vs CFX96 Touch Real-Time PCR system), and BLV proviral load calculation method, which might have contributed to these variations. We calculated BLV proviral load by dividing the starting number of BLV copies by the starting number of Beta-Actin copies. However, in the study by John et al. (2022), the number of Beta-Actin copies were divided by 2, to represent a single diploid WBC cell, which was further used as a denominator for the number of BLV copies. These variations likely produced higher values of proviral load in the study by John et al. (2022). Therefore, the proviral load values from these two studies need to be interpreted based on the methods used. An optimal HPL cut-off value should represent clinical or physiological effects of the proviral load as well as the transmission risk of the virus. Our study used some blood parameters, however, this information should be supplemented with more physiological and epidemiological data, such as health and production impact, risk of BLV transmission, in order to generate a good estimate for HPL vs LPL cows.

Using BLV antibody titer to differentiate BLV-seropositive cows with a high WBC and lymphocyte count produced a similar cut-off value (32), however the sensitivity, specificity, and AUC were much lower than those observed for BLV proviral load. This emphasizes that BLV proviral load cut-offs have better accuracy than BLV antibody titers cut-off to differentiate high WBC and lymphocyte count in BLV-seropositive cows. Use of BLV antibody titer has been sought as an option to monitor BLV proviral load because of a positive association (John et al., 2022). However, this needs to be validated before application in BLV control programs as negative correlation between milk ELISA titers and proviral load was also reported (Jaworski et al., 2016).

Results from our study demonstrate the negative impacts BLV infection and proviral load have on the host immune system, particularly on the hematological and immunological parameters. The HPL cut-off values results indicated a lower than previously used threshold between low and high proviral loads, which may result in a more stringent HPL removal approach requirement to achieve a BLV-free status in shorter period.

Table 5-1. Descriptive statistics for the hematological and immunological parameters of the enrolled cows from ten dairy herds.

Variables	**No. of obs.**	**Minimum**	**Mean**	**SD**	**Median**	**Q1-Q3**	**Maximum**
[a]White blood cell count ($\times 10^3$ cells/µL)	429	2.5			10.3	8.4-14.6	47.4
[a]Lymphocyte count ($\times 10^3$ cells/µL)	429	1.4			5.3	3.5-9.1	35
[a]Monocyte count ($\times 10^3$ cells/µL)	424	0.3			0.9	0.7-1.2	2.9
[a]Granulocyte count ($\times 10^3$ cells/µL)	424	0.8			4	3.2-5	9.9
[b]Bovine leukemia virus (BLV) proviral load (BLV copies/Beta-Actin copies)	348	0			0.19	0.01-0.40	0.95
High proviral load (≥0.5)	46	0.50			0.59	0.50-0.83	0.95
Moderate proviral load (≥0.25 to <0.5)	100	0.25			0.38	0.26-0.48	0.49
Low proviral load (>0 to <0.25)	146	1.0e-05			0.07	4.0e-05-0.24	0.24

Not detected (BLV-seropositive, proviral load-negative)	56				0		
[c]Antibody titer (number of dilutions)	257	1			32	16-64	512
[d]CD3+%	45	2.8			15.2	10.6-22.6	58.3
[d]CD4+%	45	8.6	30.4	13			56.2
[d]CD8+%	45	3	25.7	12.4			52.9
[d]CD21+%	45	5.9	36.7	15.9			70.6
[d]WC1+%	45	0.4			2.3	1.5-3.7	6.8

[a] = Cows were either BLV-seropositive or BLV-seronegative, and represented all ten dairy herds in the study.

[b] = BLV-seropositive cows from all ten study herds were used for this analysis.

[c] = A subset of BLV-seropositive cows from all ten study herds were the participants.

[d] = A subset of either BLV-seropositive or BLV-seronegative cows from four dairy herds were the participants.

Note: Mean and SD are presented for the variables that were normally distributed, while median and Q1-Q3 are presented for those that were not normally distributed.

Table 5-2. Number of BLV-seropositive [differentiated into four groups based on bovine leukemia virus (BLV) proviral load (BLV copies/Beta-Actin copy)] and BLV-seronegative cows demonstrating normal or high white blood cell and lymphocyte count.

Group	Normal white blood cell count ($\leq 12.6 \times 10^3$ cells/μL)	High white blood cell count ($>12.6 \times 10^3$ cells/μL)	Normal lymphocyte count ($\leq 6.3 \times 10^3$ cells/μL)	High lymphocyte count ($>6.3 \times 10^3$ cells/μL)
High proviral load (≥0.5) n = 46	3	43	0	46
Moderate proviral load (≥0.25 to <0.5) n = 100	25	75	7	93
Low proviral load (>0 to <0.25) n = 146	127	19	118	28
Not detected (BLV-seropositive and proviral load-negative) n = 56	55	1	56	0
BLV-seronegative n = 81	76	5	78	3

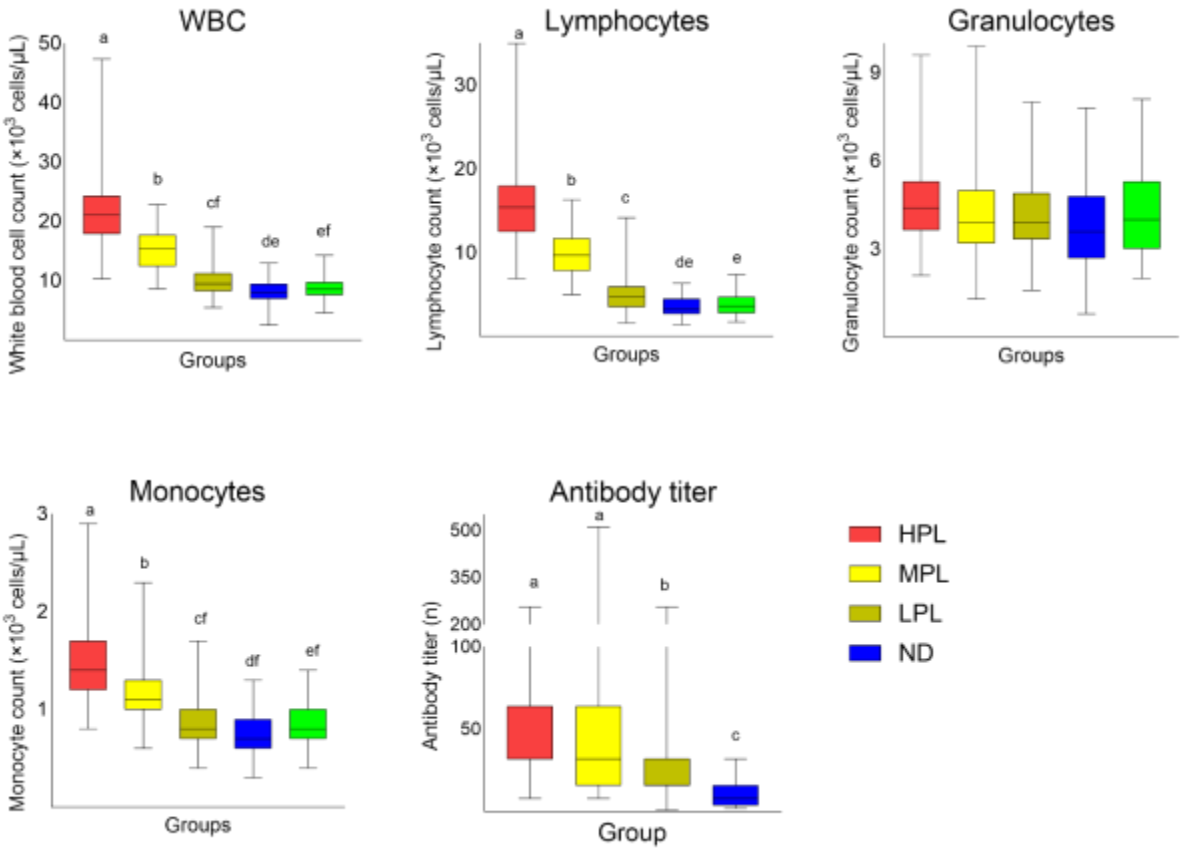

Figure 5-1. Box plots demonstrating white blood cells (WBC) and its differentials cell count ($\times 10^3$ cells/µL) and antibody titer measurement among BLV-negative and BLV-positive cows [further categorized based on proviral load (BLV copies/Beta-Actin copy) as high proviral load (HPL: ≥0.5), moderate proviral load (MPL: ≥0.25 to <0.5), low proviral load (LPL: >0 to <0.25), and ND (proviral load-negative)]. WBC differentials included granulocytes and non-granulocytes (lymphocytes and monocytes) conducted among BLV-seronegative cows (n = 81 cows), HPL (n = 46 cows), MPL (n = 100 cows), LPL (n = 146 cows), and ND (n = 56 cows) groups. Antibody titer measurements were conducted among HPL (n = 43 cows), MPL (n = 75 cows), LPL (n = 93 cows), and ND (n = 46 cows) groups. Differences between groups were assessed using a Kruskal-Wallis test followed by Dunn's pairwise comparison as a post-hoc test.

Groups with different superscript letters (a-f) are significantly different from each other ($P < 0.05$).

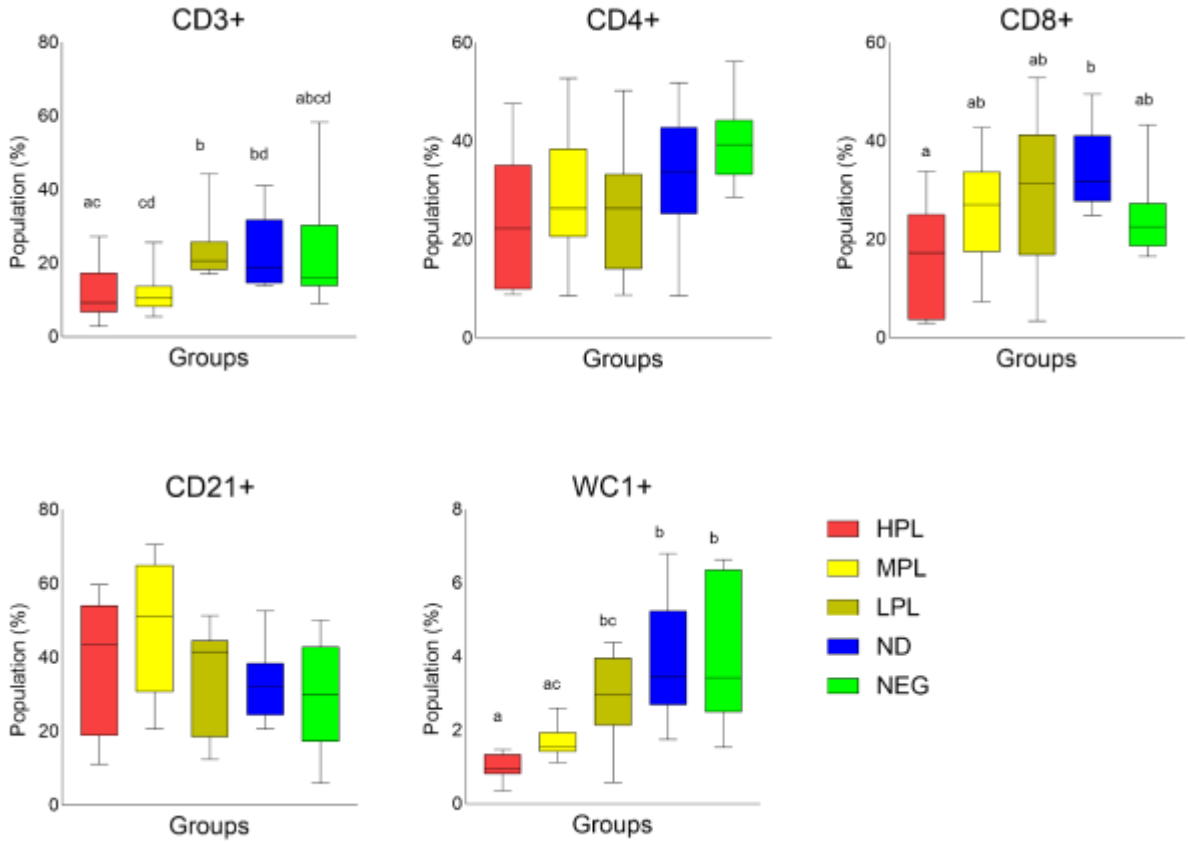

Figure 5-2. Box plots demonstrating the B- (CD+21) and T-cell (CD3+, CD4+, CD8+, and WC1+) population distributions (%) of BLV-seronegative cows (NEG) and BLV-seropositive cows [further categorized based on proviral load (BLV copies/Beta-Actin copy) as high proviral load (HPL: ≥0.5), moderate proviral load (MPL: ≥0.25 to <0.5), low proviral load (LPL: >0 to <0.25), and ND (proviral load-negative)]during flow cytometry analysis. Each group has similar sample size (n = 9). Differences between groups were assessed using a Kruskal-Wallis test followed by Dunn's pairwise comparison (CD3+ and WC1+) or ANOVA followed by Tukey-Kramer honestly significant difference post-hoc test (CD4+, CD8+, and CD21+). Groups with different superscript letters (a-d) are significantly different from each other ($P < 0.05$).

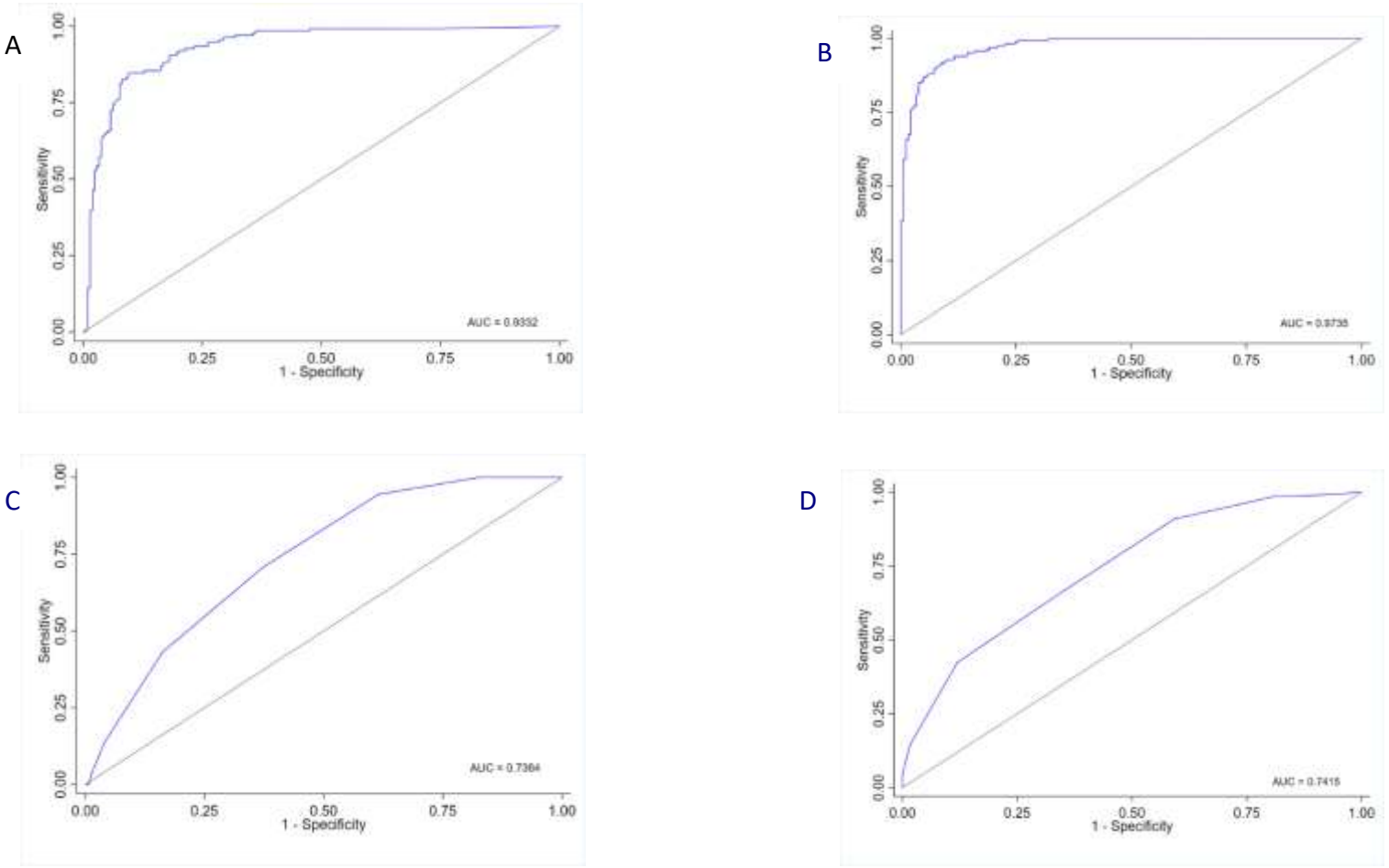

Figure 5-3. Receiver operator characteristics (ROC) curves for the detection of: A) high white blood cells (WBC; >12.6 $\times 10^3$ cells/µL) using bovine leukemia virus (BLV) proviral load, B) high lymphocyte count (>6.3 $\times 10^3$ cells/µL) using BLV proviral load (BLV

copies/Beta-Actin copy), C) high WBC count using BLV antibody titers, and D) high lymphocyte count using BLV antibody titers. AUC = area under the curve.

CHAPTER 6: General Discussion

6.1 An overview

This PhD research builds upon the prior recommendations to focus on BLV proviral load assessments to strengthen on-farm BLV control programs and understand the importance of various BLV transmission routes (Ruggiero et al., 2019; Kuczewski et al., 2021b; Taxis et al., 2023). This resulted in the evaluation of the efficacy of a BLV control program that primarily focused on the removal of BLV-seropositive cows with high proviral loads (HPL), and its effectiveness in reducing the BLV within-herd prevalence and incidence rate in an extensive field trial. Firstly, it was important to understand the implication of BLV proviral load on the milk, fat, and protein production of dairy cows (Chapter 2). The aim was to encourage participation of dairy farmers in BLV control programs focusing on HPL removal. Next, the BLV control strategy needed validation by engaging a larger number of dairy herds with varied management practices. Therefore, a three-year BLV control study, whereby high proviral load cows' removal as the primary intervention was conducted in ten dairy herds to monitor the changes in BLV within-herd prevalence and incidence rate (Chapter 3). The proviral load monitoring on BLV-seropositive cows requires a qPCR test which imposes a substantial cost to the farmers. The requirement of proviral load testing frequency needs to be communicated to the farmers and veterinarians for effective planning. Additionally, the knowledge of how BLV infection evolves overtime may be crucial in understanding its implication on animal health and production. Therefore, a longitudinal study was conducted with animal groups that differed in BLV-infection status and proviral load category evaluating various hematological and immunological parameters (Chapter 4). In order to produce a substantial impact of HPL-focused

BLV control strategy, an accurate system of identifying HPL cows cannot be underestimated. Many current systems rely on arbitrary HPL cut-offs. Therefore, a study was conducted to assess variabilities in hematological and immunological parameters among different BLV proviral load categories and to estimate BLV proviral load cut-off values using WBC and blood lymphocyte count information (Chapter 5). Based on the findings from these four studies, its implications are summarized in the discussions below.

6.2 Using BLV proviral load for improving current knowledge on BLV

6.2.1 Assessment of BLV impact on health and production

Assessment of BLV effects based on BLV seropositivity is not straightforward. This approach lumps together LPL and HPL cows into 'BLV-positive' cows, and comparison is made with 'BLV-negative' cows. The untoward effect of this approach is that LPL cows are often comparable to BLV-negative cows in terms of their production levels, and hematological and immunological parameters. For example, in one of the studies within this thesis, the milk, fat, and protein production of LPL cows were similar to BLV-negative cows (Chapter 2). Since the LPL subgroup significantly influences the overall BLV-positive group, comparisons made on the basis of seropositivity might not always unveil the differences in milk production (Tiwari et al., 2007; Sorge et al., 2011; Chapter 2). The true impact of BLV becomes apparent when comparisons are made on the basis of proviral load, changes in hematological parameters, or changes in ELISA OD values. For instance, BLV status did not dictate the differences in the longevity of beef cows within a herd, however, high proviral load cows exhibited lower survival rates than BLV-negative cows (Benitez et al., 2020). Subclinical mastitis incidence was similar when BLV-positive cows with normal WBC and lymphocyte counts were compared with BLV-

negative cows, but it was higher in BLV-seropositive cows with elevated WBC and lymphocyte count in comparison to BLV-negative cows (Sandev et al., 2004). Milk ELISA OD values were inversely associated with milk production in BLV-seropositive cows (Norby et al., 2016). Given the incidence of persistent lymphocytosis and elevated ELISA OD values being positively associated with BLV proviral loads (Juliarena et al., 2007; Jimba et al., 2010), we can anticipate the use of BLV proviral load information to be crucial in understanding the impact of BLV infection on different aspects of dairy health. For example, high proviral load cows are associated with an increased likelihood of developing severe mastitis (Watanabe et al., 2019). Future works are required to explore other areas of dairy health such as lameness, reproductive performance, and more to investigate further effect caused due to BLV infection.

6.2.2 Assessment of BLV transmission

Information about BLV proviral load could be used to investigate various transmission routes. For instance, HPL cows have higher risk of transmitting BLV infection during prenatal or peripartum period compared to LPL cows (Mekata et al., 2015; Sajiki et al., 2017). Interestingly, BLV transmission risk was low with BLV-seropositive cows that had a low proviral load (Mekata et al., 2018; Benitez et al., 2019a). Whether high proviral load bulls have a considerable risk for BLV transmission needs to be explored. Provirus of BLV has been detected in the saliva and nasal excretions of study cows in Japan (Yuan et al., 2015); however, its potential threat for BLV transmission has not been established. Therefore, there is a need for future studies that can explain the role of such routes in BLV transmission.

While transmission routes involving BLV-infected blood lymphocytes are considered high risk for BLV transmission, single use of rectal palpation sleeves or needles was not

sufficient in prohibiting BLV transmission within a dairy herd (Nekouei et al., 2015b; Ruggiero and Bartlett, 2019). Separate routes of transmission and the level of BLV proviral load could have caused these discrepancies. Routes such as colostrum are conflicted for their potential role in BLV transmission, which could be investigated through BLV proviral load (Kuczewski et al., 2021b). Veterinarians and farmers can benefit from such information to develop an effective BLV management plan.

6.2.3 Assessment on pathological and immunological influences

Nearly 2/3rd of BLV-infected animals maintains clinical health, whereas the remaining exhibit either lymphocytosis or lymphoma (Schwartz and Levy, 1994). The risk of developing the latter conditions is significantly higher in BLV-seropositive cows displaying a high proviral load (Ohno et al., 2015; Kobayashi et al., 2020). The previous notion that persistent lymphocytosis evolves over time in BLV-infected animal has been challenged by recent studies that indicated establishment of lymphocytosis as well as a specific proviral load status (high or low) shortly after infection (Hutchinson et al., 2020a). Once established, the proviral load remains relatively stable over-time (Hutchinson et al., 2020a; Chapter 4). Therefore, a single proviral load test conducted once its relative stable state has been established can provide vital information for the decision making process. According to an experimental study, BLV establishes its stable proviral load within 60 days of first detection (Hutchinson et al., 2020a).

The immunological consequences of a high proviral load are another important factor that directs the future of dairy cows on farm. Maintaining immunocompromised cows on farm could increase the risk of secondary infections and its associated treatment cost as well as decrease the production level. In one of the studies in this thesis, cows with high proviral loads (HPL and

MPL cows) demonstrated a relatively high WBC, lymphocyte, and monocyte counts, whereas HPL cows exhibited relatively low WC1+ population, when compared with BLV-negative cows (Chapter 5). Prior study demonstrated similar results with proliferation of B-cells (CD21+) and exhaustion of T-cells and its subsets (CD3+, CD4+, CD8+) among HPL cows (Farias et al., 2018). These dysregulations can influence antibody production, cytokine expression, and cytotoxic activity in response to immune challenge. Thus, BLV proviral load information can be valuable in anticipating host's response to infections.

6.3 Unraveling the conundrum of BLV proviral load categorization

6.3.1 A simple BLV categorization of HPL and LPL

Multiple ways of categorizing BLV-infected cows based on proviral load exist, with some studies categorizing into very high, high, moderate, and low proviral load (Ruggiero et al., 2019). Additionally, some BLV-seropositive cows are PCR-negative or not detectable (ND) in the proviral load tests (Jimba et al., 2012; Hutchinson et al., 2020a; Chapter 4; Chapter 5). Whether such group need to be distinguished from other proviral load group requires further inspection. Moreover, the justification behind creating multiple BLV proviral load categories is not clear.

We investigated the variations in hematological (WBC, lymphocyte, monocytes, and granulocytes count) and immunological parameters (antibody titer, CD3+, CD4+, CD8+, CD21+, and WC1+) among the four proviral load categories: HPL (≥0.5), MPL (≥0.25 to <0.5), LPL (≥0 to <0.25), and ND (Chapter 5), and their dynamics (Chapter 4). Results obtained from these studies were used to explore the relevance of creating multiple proviral load categorization. The hematological parameters (WBC, granulocytes, and monocytes) and immunological

parameters (CD3+, CD4+, CD8+, CD21+, WC1+) of LPL cows closely resembled to BLV-negative cows (Chapter 5). Similarly, ND and BLV-negative cows did not differ from each other with regards to WBC, lymphocyte, monocytes, granulocytes count, CD3+, CD4+, CD8+, CD21+, or WC1+ populations. While comparing LPL and ND cows, all parameters, except WBC count, lymphocyte count, and antibody titer values, were similar between these two groups. Based on these observations, it can be inferred that there are less variations between LPL and ND groups. Additionally, close resemblance of these two groups with BLV-negative cows with regards to immunological and hematological parameters suggests less severe impact of BLV-infection. Therefore, combining LPL and ND group as a single category of LPL should suffice to distinguish the BLV-infected subset with lower risk of BLV transmission and less severe effect of BLV infection.

On the other hand, HPL and MPL groups were similar in terms of higher values of WBC, lymphocyte, monocyte count, antibody titer, and lower values of WC1+ proportions (Chapter 5). Additionally, statistical models generated to assess the impact of BLV proviral load on milk, fat, and protein production were similar whether the HPL threshold was ≥0.5 or ≥0.25 (Chapter 2). Combining results from these two studies, it can be concluded that there is no additional advantage in categorizing MPL as a separate group. To elaborate, both HPL and MPL groups have severe impact of BLV infection, whether the consequences are suppressed immune system or production loss. Therefore, combining HPL and MPL into a single HPL category is justified. Whether the threshold value should be >0.25 or something else is up for debate. We tried to answer this question in Chapter 5 and is discussed below (6.3.2. section). Overall, a simpler categorization of BLV proviral load (HPL and LPL) lessens confusions and makes it easier for researchers, farmers, and veterinarians to adopt this technology.

6.3.2 Need for harmonization of HPL cow detection system

Current BLV control studies have prioritized removal of HPL cows in order to reduce bovine leukosis prevalence on farms (Ruggiero et al., 2019; Taxis et al., 2020, 2023; Chapter 3). The quantification of BLV proviral load relies on qPCR method. However, various research institutes and laboratories have developed their own qPCR systems, for instance, BLV-CoCoMo-qPCR, BLV SS1 qPCR, and other self-designed qPCR assays (Juliarena et al., 2007; Jimba et al., 2010; Mekata et al., 2018; Taxis et al., 2020). Without a coherent proviral load detection system, results obtained from multiple laboratories become difficult to interpret, as inter-laboratory variations may lead to discordant results even from a same DNA sample (Jaworski et al., 2018).

Each qPCR system targets a specific BLV gene, such as *Pol, Tax, Env*, for the amplification process, and therefore their relative sensitivity and specificity can impact their ability in distinguishing BLV from other genes (Jaworski et al., 2018). Additionally, the qPCR platforms have their own fluorescence detection limits which could influence detection and quantification of BLV and house-keeping genes and, ultimately, BLV proviral load values. The studies under this thesis employed BLV SS1 qPCR assay using the CFX96 Touch Real-Time PCR system (Bio-Rad, Hercules, CA) for proviral load quantification (Chapter 2, 3, 4, and 5). Interestingly, studies that used the same BLV SS1 qPCR assay in different platforms reported proviral load values exceeding 1 copy/cell (Taxis et al., 2020; John et al., 2022), in contrast to the results from Chapter 4 where the highest proviral load value quantified was 0.99. The discrepancy in proviral load values could be attributed to the detection limit in qPCR machines, as observed in the serial dilution assessment in this study, demonstrating a detection limit of 100 copies of the BLV target gene (*Pol*) and 100 copies of the endogenous gene (Beta-Actin) (results not shown). There were variations in the DNA extraction methods from this study. Our study

used Quick-DNA Miniprep Plus Kit (Zymo Research, Irvine, CA) whereas the other two studies (Taxis et al., 2020; John et al., 2022) used Qiagen DNEasy blood and tissue kit (Qiagen Inc. Canada, Montreal, Québec). The DNA yield was considerably high in our study, which was adjusted by increasing the quantity of elution buffer in the DNA extraction process. Additionally, there were discrepancies in the amount of DNA used in the qPCR assay, for instance, we used 2 µL of DNA while the other two studies used either 3 µL (John et al., 2022) or 4 µL (Taxis et al., 2020) of DNA. The concentration of DNA in the qPCR assay can impact the availability of reagents for the completion of DNA amplification cycle. Therefore, DNA extraction method and its quantity are other factors for consideration, even though previous study indicated no implications of variations in DNA extraction methods on DNA quality (Jaworski et al., 2018).

Additional variations might occur due to metrics used in BLV proviral load quantification, such as copies per 50-ng of DNA, copies/10^5 cells, relative proviral load compared with the 18s reference gene, etcetera (Kuczewski et al., 2021b). Discrepancies in proviral load calculation methods, as mentioned in the discussion section of Chapter 5, also impacts the values of BLV proviral load. Most importantly, lack of consistency in thresholds used in categorizing HPL cows creates uncertainty. An attempt was made to establish a threshold for BLV proviral load to correctly identify HPL cows using information from WBC and lymphocyte count (Chapter 5). While this may not be the ultimate method, the characterization is based of clinical conditions, and not arbitrarily.

Although every qPCR systems have their own strength and weaknesses, BLV SS1 qPCR assay is currently being used in various research institutes in North America (Taxis et al., 2020; John et al., 2022), including this study. This assay targets the BLV Polymerase gene, a conserved section essential for the reverse transcription process. Therefore, this assay can be considered a

robust system that can accurately distinguish BLV from other viruses (Heenemann et al., 2012; Rola-Łuszczak et al., 2013).

With multiple qPCR assays, metrics, and cut-offs used in determining BLV proviral load and identifying HPL cows, it creates extraneous confusion for researchers, veterinarians, and farmers while adopting this technology. Without a comparable system used in various proviral load studies, it becomes difficult to analyze and interpret the results. Therefore, we recommend an overall harmonization in the following five key aspects: i) DNA extraction method, ii) BLV qPCR assay, iii) qPCR machine selection, iv) BLV proviral load calculation method, and v) HPL cut-off.

6.4 Future directions for BLV control by HPL detection

6.4.1 Awareness of farmers and veterinarians

While research institutions are well acquainted with the concepts of BLV proviral load and importance of HPL removal, the majority of the farmers and veterinarians remain unaware. The initial step to encourage the participation of farmers and veterinarians in BLV control programs is to raise awareness regarding the importance of identifying and removing HPL cows. Human behaviour models employed in explaining farmer behaviour suggest that a farmer's involvement in disease control measures is contingent upon their level of awareness regarding the impact of the disease and whether they perceive it as a substantial threat worthwhile their engagement (Janz and Becker, 1984; Ellis-Iversen et al., 2010). Additionally, BLV control is time consuming and requires a sincere participation of the farmers and veterinarians with a dedicated collaboration from the government agencies (Irimia et al., 2021; Kuczewski et al., 2022). Veterinarians play a pivotal role in the management practices of farms as most farmers

tend to trust their veterinarians and are more likely to adopt a control program if recommended by their veterinarian (Ellis-Iversen et al., 2010). Results from this thesis highlight the importance of removing HPL cows for BLV control (Chapter 3), and culling a subset of the herd that tend to produce less milk, fat, and protein (Chapter 2) and at higher risk of being immuno-suppressed (Chapter 5). Such information need to be disseminated to the farmers, veterinarians, and the dairy industry to attract their interest in bovine leukosis control.

6.4.2 On farm BLV and HPL detection system

The first step in any disease control program is to investigate the presence of the disease and determining its prevalence within the population of interest. For BLV detection, ELISA methods are relatively convenient and cheaper compared to qPCR. However, conducting this test for all cows, present in the herd, just for the initial investigation can be expensive, as a single ELISA test may cost up to $6 USD (Hutchinson et al., 2021). As the cost of BLV testing is one of the barriers that prohibit dairy farmers and veterinarians from involving in BLV control programs, alternative cost-effective options need to be explored (Kuczewski et al., 2022).

One reasonable approach to this may be to start with bulk tank milk screening to obtain an initial approximation of the within-herd BLV prevalence (Nekouei et al., 2015a). Another option may be to test a subset of the herd in order to identify the lactation-specific within-herd BLV prevalence. This approach enables identification of the age-group with the highest prevalence and disease transmission risk (Erskine et al., 2012c). Even though from cost-effective perspective these strategies offer value, in order to implement an effective BLV control system, the importance of individual testing of milk or blood samples is second to none. Detection of

HPL cows should be conducted specifically on BLV-seropositive cows using a harmonized qPCR system and BLV proviral load cut-offs as discussed earlier.

6.4.3 Alternative methods of HPL detection

In order to correctly identify a HPL cow, using combination of ELISA and qPCR test may increase the cost of test per cow substantially, reaching up to $16 USD/animal (Hutchinson et al., 2021). Conducting qPCR test directly without the initial ELISA screening for the BLV-seropositivity might not offer value as it further increases the testing cost with qPCR for all animals and the ND BLV-seropositive subsets cannot be distinguished decreasing the sensitivity of this approach. Therefore, alternative methods such as WBC count, lymphocyte count, and antibody titers estimation have been explored as potential proviral load determination options that can offer value in terms of cost-effectiveness and feasibility.

Dairy farmers can employ a simpler diagnostic tests such as WBC count and lymphocyte count to indirectly identify a cow with a high probability of having a HPL, as these parameters have shown high sensitivity and specificity in detecting HPL cows (John et al., 2022). This is supported by the positive association of these parameters with BLV proviral load values (Juliarena et al., 2007; Alvarez et al., 2013; Chapter 5). Information from ELISA tests such as ELISA OD values, ELISA percent positivity, or antibody titers are positively associated with BLV proviral load and therefore may offer an alternative for HPL detection (Gutierrez et al., 2012; John et al., 2022; Chapter 5). ELISA OD values, in particular, seem to correlate with BLV severity as higher OD categories associates with reduced milk production and decreased longevity (Bartlett et al., 2013; Norby et al., 2016).

Future BLV control programs can be initiated by screening methods using WBC or lymphocyte count on all cows in the herd to identify the subset with elevated values, which are confirmed for their HPL status using selective proviral load test. Another strategy could be conducting milk ELISA test on all adult cows and identifying the ELISA-positive cows with high OD values to undergo selective proviral load test. However, the fact that the HPL cows-focused BLV control programs have implemented ELISA test, lymphocyte count, and proviral load test in combination, and not as one test substituting the other (Ruggiero et al., 2019; Taxis et al., 2020), the proposed control approach needs validation through field trials.

6.4.4 Plan for removal of HPL cows

Depending on the herd BLV prevalence, complete removal of all HPL cows may not be economically feasible in a single attempt. This is also evident from results from our study (Chapter 3), where some farms removed all HPL cows if the HPL numbers were low where as some farms removed only a fraction of the HPL cows if the HPL numbers were large. Therefore, a hierarchical removal system where the cows with the highest proviral loads are removed first could be a feasible approach. In the mean time, the BLV control management practices of the herd should emphasize minimizing contacts of BLV-seropositive cows that remain in the herd with the other non-infected cows. Segregation of HPL animals is an option to prevent transmission from HPL cows. However, a lack of space and additional management requirements makes this strategy less viable for dairy farmers (Roche et al., 2019; Kuczewski et al., 2022). Therefore, for an effective BLV control program, priority-based removal of HPL cows together with a stringent management practice need to be implemented.

6.4.5 Frequency of proviral load test

The results from the 1.5-year longitudinal study deduced that the BLV proviral load in BLV-seropositive cows remain relatively stable over time (Chapter 4). This is in agreement with previous studies that indicate no significant changes in proviral load over a 2.5-year study (Hutchinson et al., 2021) or that the changes observed do not lead to a shift in the proviral load category over a 2.5-year time-period (Chapter 3). Therefore, a single proviral load test should in the majority of cases suffice for making decisions regarding a BLV-positive cow over the period of at least 2.5-years. However, our results are based on milk-producing adult cows or heifers that were close to calving. An experimental infection study with 9-month old Holstein steers followed for 5 months observed similar stability in proviral load (Hutchinson et al., 2020a). Whether the same can be said for calves infected at a much younger age needs further investigation.

Experimental studies indicate that the proviral DNA can be detected earlier (median: 24 days post-infection (DPI) ranging from 18-42 DPI) than BLV antibody detection (median: 36 DPI ranging from 27-51 DPI) following a BLV infection (Hutchinson et al., 2020a). This means any proviral load test or ELISA test conducted in the window between BLV-infection and detection of proviral DNA or BLV antibody may lead to false negative results. Additionally, during the initial stage of infection, the proviral load can fluctuate before it attains a relatively stable state. A 60-days sampling interval was recommended to detect the relatively stabilized proviral load in a BLV-infected cow with the aim of understanding the infection potential (Hutchinson et al., 2020a). However, the BLV sampling strategies may vary and should reflect the objectives and resources availability of the farm.

6.4.6 Testing strategy for replacement heifers

Testing young stock was beyond the scope of this study as the primary focus was on adult cows for controlling BLV (Chapter 3). However, incoming replacement heifers might already be BLV-infected and can substantially increase the number of newly BLV-infected cows in the herd (Ruggiero et al., 2019). This situation was observed in Herd G in our BLV control study, where despite of aggressive HPL culling and a reduction in BLV within-herd prevalence, the BLV incidence rate did not decrease (Chapter 3). Therefore, a plan should be in place for screening heifers before breeding and mixing with the milking herd. The initial screening should be done with an ELISA test, and complemented with a proviral load test on the BLV-seropositive heifers, 30 days after the ELISA-positive test result. The proviral load of the seropositive heifers should be considered before breeding decisions are made. This decision would be crucial because pregnant heifer with a high proviral load are at a greater risk of transmitting BLV to its calf in-utero or during the peripartum period (Mekata et al., 2015; Sajiki et al., 2017).

6.5 Importance of BLV control management practices

Unless a relative risk assessment of various BLV transmission routes can be done on the basis of BLV proviral load, prioritizing one transmission route compared to the other is not recommended. An effective BLV control program should incorporate BLV control management practices, alongside HPL removal. Research indicates that implementation of BLV control practices can significantly reduce BLV prevalence and seroconversions on farm (Kuczewski et al., 2021a). Implementation of control practices not only help in reducing BLV transmission, but it also improves farm biosecurity and hygiene, contributing to an overall health and welfare of animals on farm. For example, pasteurizing colostrum is not only important from the perspective

of BLV control but also helps in eliminating other pathogens that deteriorates calf health (Godden et al., 2012).

6.6 Working towards a BLV free herd

Previously, two US studies had used the HPL cow removal strategy to control BLV prevalence in dairy herds. One of the studies employing three dairy herds observed a reduction in median BLV prevalence from 62% to 20.7% over a 2.5-year period (Ruggiero et al., 2019). In another study, the BLV prevalence in a dairy herd decreased from 20.8% to 0.85% over a 3-year period (Taxis et al., 2020, 2023). In our study, the median within-herd prevalence in ten herds decreased from 28.9% to 23.2% over a 2.5-year period (Chapter 3). Based on these information, estimating a precise timeframe required to eradicate BLV from a herd is difficult, because there were variations in management practices, culling strategies, and farmer motivation across these studies. However, a stringent plan and a regular follow-up on the HPL removal and management practices can produce significant results even within a three-year timeframe (Taxis et al., 2020, 2023).

Currently, there are no commercial labs offering BLV proviral load tests in Canada. Only a few research institutes provides this service to the dairy farmers participating in their study. There is a need for Canadian commercial labs and dairy organizations to work towards making this test commercially available. In the US, CentralStar Cooperative, Inc. offers this service. If samples are to be shipped to these labs, simplifying the logistics of shipping samples is necessary to ensure timely delivery and maintain sample quality for laboratory analysis.

Once dairy farmers start enrolling in BLV control programs, it is necessary to set up guidelines that define a BLV-free herd. The Canada Health Accredited Herd Enzootic Bovine

Leukosis (CHAH-EBL) program offered by Canadian Food Inspection Agency (CFIA) has these regulations in place (Canadian Food Inspection Agency, 2022). Bovine producers interested in becoming BLV-free can enrol in this voluntary program and obtain the accreditation. This not only ensures compliance with the program standards but also meet requirements to trade with the European Union (EU). Some additional incentives can be introduced, such as a higher price for milk from BLV-free herds, which can further motivate farmers and encourage increased participation in BLV control programs.

6.7 Key takeaway for the farmer communities

Putting effort into a disease (bovine leukosis) that has a low visible impact on the health or production of a cow may not sound encouraging when various other issues in the farm with more direct impact demand attention. However, recent BLV studies and results from this thesis have scrutinized the BLV-infected population and recognized a subset with a high proviral load (HPL) that tend to produce less milk, fat, and protein, and has a relatively weaker immune system. Interestingly, selective removal of these HPL cows from the herd has been effective in controlling the within-herd BLV transmission and its prevalence. This is encouraging from the farmers' perspective as the focus of BLV control can now shift from all BLV-seropositive cows to a more manageable HPL subset.

It is essential to have discussions with the veterinarians and farm personnel regarding the testing strategy, HPL identification system, management changes, and HPL removal strategy. Eradicating BLV from the herd takes time, but it is achievable. Consumers are concerned about their product and especially about animal welfare if the products are of animal origin. As BLV is significant from the perspective of animal welfare, there needs to be dialogues between farmers,

concerned organizations, and government agencies regarding concerns surrounding BLV infection.

CHAPTER 7: Complete list of references

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APPENDICES

Appendix A

Supplementary material for Chapter 1

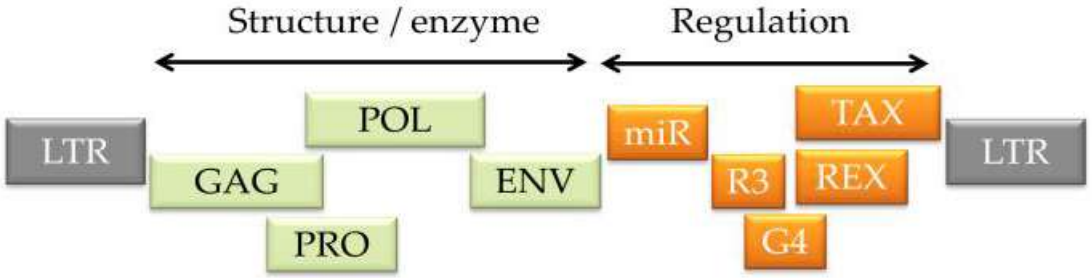

Figure S1-1. Bovine leukemia virus (BLV) genome structure. Reproduced from "Recent advances in BLV research", by Barez et al., 2015, Viruses, 7(11): 6080-6088. Copyright 2015 by the authors; licensee MDPI, Basel, Switzerland.

Appendix B

Supplementary materials for Chapter 3

Table S3-1. Schedule summary of field and laboratory works.

Sampling year	**Sampling period (Date range)**	**Days between milk and blood sampling Median (max-min)**	**Days between milk sampling and ELISA result Median (max-min)**	**Days between milk sampling and proviral load result Median (max-min)**
First year[1]	10 July 2020 to 01 March 2021	19.5 (7 to 28)	12 (5 to 27)	99 (24 to 129)
Second year[2]	03 June 2021 to 08 February 2022	21 (5 to 33)	12 (7 to 37)	37.5 (8 to 71)
Third year[2]	19 May 2022 to 19 January 2023	13 (7 to 18)	15 (10 to 28)	28.5 (15 to 73)

[1] = Only blood samples collected in Herd A and B. Herd G and J conducted BLV ELISA test through Lactanet, only blood samples collected from positive cows. Both milk and blood samples collected in remaining herds.

[2] = Herd G conducted BLV ELISA test through Lactanet, only blood samples collected from positive cows. Both milk and blood samples collected in remaining herds

Table S3-2. Individual rates of change in bovine leukemia virus (BLV) proviral load (BLV copies/Beta-Actin copy) among the five study group[1] of cows as indicated by the linear combination assessment following a mixed-effect linear regression model (number of observations = 1553).

Group	**Coef.**	**SE**	**P-value**	**95% CI**
ND	0.5	0.1	<0.001	0.3 to 0.7
LPL	0.4	0.05	<0.001	0.3 to 0.5
MPL	0.03	0.1	0.56	–0.1 to 0.1
HPL	–0.1	0.1	0.64	–0.3 to 0.2
BLV ELISA-negative	–0.03	0.1	0.79	–0.3 to 0.2

[1]HPL = High proviral load (≥0.5), MPL = Moderate proviral load (≥0.25 to <0.5), LPL = Low proviral load (≥0 to <0.25), and ND = Not detected = BLV ELISA-positive and not detected in quantitative PCR test.

Note: Each results of BLV proviral load was added with 0.001 and transformed using a natural log-transformation. The results are presented without back-transformation. $P < 0.05$ indicate significant result.

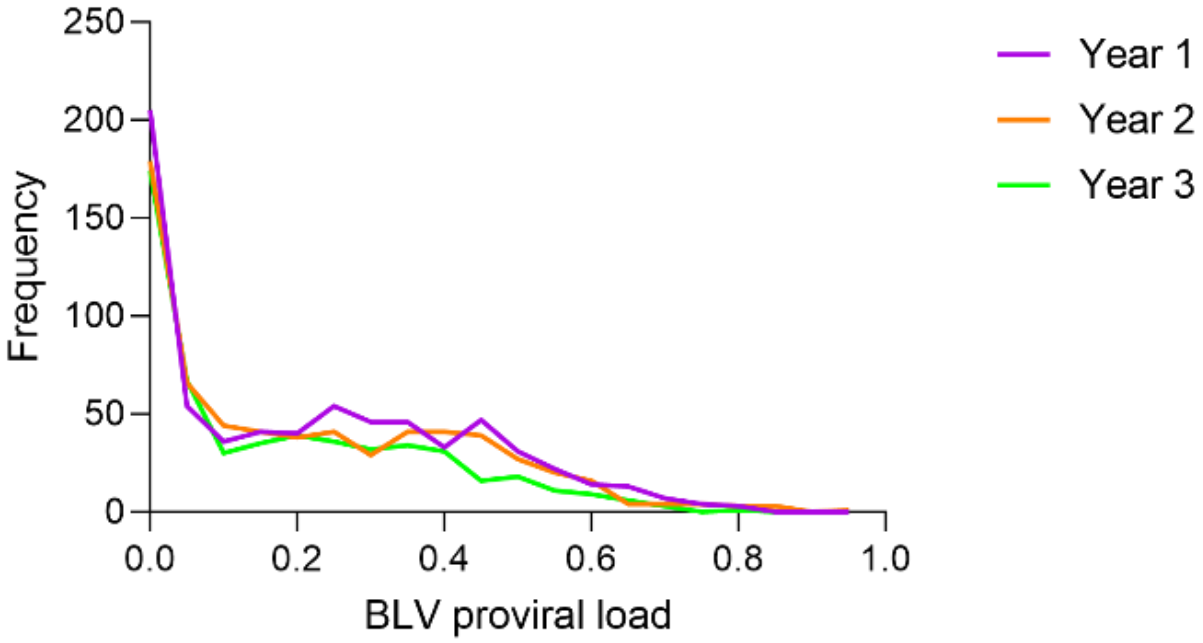

Figure S3-1. Frequency distribution of bovine leukemia virus (BLV) proviral load (BLV copies/Beta-Actin copy) of BLV-seropositive cows in the ten participating dairy herds in year 1, 2, and 3. The X-axis represents the value of BLV proviral load and Y-axis represents the number of cows with given proviral load. Each line represents year 1, 2, or 3.

Appendix C

Supplementary materials for Chapter 4

Table S4-1. Details of the antibodies and ingredients used in staining cells for the flow cytometry analysis.

Ingredients/ Antibody marker (Concentration)	**Conjugate**	**Vendor, Catalog**	**Antibody mixture group and quantity (µL)**			
			M1	M2	M3	Unstained
Rat anti human CD3 (0.05 mg/mL)	Alexa Fluor 647	Bio-Rad Laboratories, Inc., MCA1477A647	2	2	2	–
Mouse anti-bovine CD4 (1 mg/mL)	Maxlight 405	United States Biological, Salem, MA, 227418-ML405	1	1	–	–
Mouse anti bovine CD8 (0.1 mg/mL)	FITC	Bio-Rad Laboratories, Inc., MCA837F	1	1	–	–
PE/Cy5 Anti-CD21 antibody (0.055 mg/mL)	PE/Cy5	Abcam Inc, Toronto, ON, ab201303	2	2	–	–

Mouse anti-bovine WC1 (0.7 mg/mL)	Maxlight 750	United States Biological, Salem, MA, W0481-74C-ML7	2	–	–	–
Mouse anti bovine WC1 (1 mg/mL)	FITC	Bio-Rad Laboratories, Inc., MCA838F	–	–	1	–
5% Bovine Serum Albumin (BSA)	–	–	12	14	17	20

Table S4-2. Final multilevel mixed-effect analysis model for assessing the changes in white blood cells (WBC) and lymphocyte count (×1,000 cells/μL) over the seven time-points of the study among the five study groups (number of observations = 256).

	Log transformed-white blood cell count				Log transformed-lymphocyte count			
	Coef.	SE	P-value	95% CI	Coef.	SE	P-value	95% CI
Intercept	2.2	0.1	<0.001	2 ; 2.3	1.3	0.1	<0.001	1 ; 1.5
Group								
Not detected	Ref.				Ref.			
Low Proviral Load	0.2	0.1	0.12	–0.05 ; 0.4	0.5	0.2	0.003	0.2 ; 0.8
Medium Proviral Load	0.5	0.1	<0.001	0.3 ; 0.8	1	0.2	<0.001	0.7 ; 1.3
High Proviral Load	0.8	0.1	<0.001	0.6 ; 1	1.4	0.2	<0.001	1.1 ; 1.7
BLV-negative	0.04	0.1	0.68	–0.2 ; 0.2	0.2	0.1	0.22	–0.1 ; 0.5
Time-point	–0.004	0.01	0.5	–0.01 ; 0.01	–0.02	0.01	0.01	–0.03 ; –0.004
Cow	–0.04	0.01		0.02 ; 0.1	0.1	0.02		0.05 ; 0.1
Residuals	0.02	0.002		0.02 ; 0.03	0.05	0.004		0.04 ; 0.06

Note: Each time-points (T) were three-months apart, except T5 (four months interval) and T6 (two months interval). A natural log-transformation was applied to measure the outcome variables white blood cell and lymphocyte count. The results are presented without back-transformation. Cow ID was used as the random effect in all models.

Table S4-3. Final multilevel mixed-effect analysis model for assessing the changes in bovine leukemia virus (BLV) proviral load (BLV copies/Beta-Actin copy) and BLV antibody titer (number of dilutions) over the seven time-points of the study among the five study groups (number of observations = 214).

	Log transformed-BLV proviral load				Log transformed-antibody titer			
	Coef.	SE	P-value	95% CI	Coef.	SE	P-value	95% CI
Intercept	–9.3	0.6	<0.001	–10 ; –8	1.8	0.2	<0.001	1.3 ; 2.3
Group								
Not detected	Ref.				Ref.			
Low Proviral Load	6.8	0.8	<0.001	5.3 ; 8.3	1	0.3	0.002	0.3 ; 1.6
Medium Proviral Load	8.2	0.7	<0.001	6.7 ; 9.6	1.6	0.3	<0.001	1 ; 2.2
High Proviral Load	8.6	0.8	<0.001	7.1 ; 10.1	1.4	0.3	<0.001	0.8 ; 2
BLV-negative	5.9	0.8	<0.001	4.2 ; 7.5	0.5	0.3	0.14	–0.02 ; 0.1
Time-point	0.02	0.01	0.2	–0.01 ; 0.05	0.06	0.02	0.004	0.02 ; 0.1
Cow	2	0.5		1.2 ; 3.3	0.3	0.1		0.1 0.5
Residuals	0.1	0.01		0.1 ; 0.2	0.4	0.04		0.3 ; 0.4

Note: Each time-points (T) were three-months apart, except T5 (four months interval) and T6 (two months interval). A value of 0.0001 was added to all proviral load results and a natural log-transformation was applied. A natural log-transformation was also applied for antibody titer. The results are presented without back-transformation. Cow ID was used as the random effect in all models.

Table S4-4. Final multilevel mixed-effect analysis model for assessing the changes in CD3+ and CD21+ cell populations over the seven time-points of the study among the five study groups (number of observations = 194).

	Log-transformed CD3+				CD21+ %			
	Coef.	SE	P-value	95% CI	Coef.	SE	P-value	95% CI
Intercept	3.3	0.2	<0.001	2.9 ; 3.8	25	4.1	<0.001	17 ; 33.1
Group								
Not detected	Ref.				Ref.			
Low Proviral Load	–0.5	0.2	0.03	–1 ; –0.05	13.2	4.9	0.01	3.6 ; 22.8
Medium Proviral Load	–1.3	0.3	<0.001	–1.8 ; –0.7	17.5	5.2	0.001	7.3 ; 27.8
High Proviral Load	–1.6	0.3	<0.001	–2.1 ; –1	15.6	5.2	0.003	5.3 ; 25.9
BLV-negative	–0.3	0.2	0.24	–0.8 ; 0.2	7.9	4.9	0.11	–1.7 ; 17.5
Time-point	–0.2	0.03	<0.001	–0.2 ; –0.1	1.6	0.3	<0.001	1 ; 2.3
Cow	0.06	0.05		0.02 ; 0.3	48.8	18.1		23.6 ; 100.8
Residuals	0.6	0.07		0.5 ; 0.7	86.3	9.5		69.6 ; 107.1

Note: Each time-points (T) were three-months apart, except T5 (four months interval) and T6 (two months interval). A natural log-transformation was applied for CD3+ cell population analysis and the results are presented without back-transformation. Cow ID was included as the random effect in all models.

Table S4-5. Final multilevel mixed-effect analysis model assessing the changes in CD4+, CD8+, and WC1+ cell populations over the seven time-points of the study among the five study groups (number of observations = 194).

	CD4+ (%)				**CD8+ (%)**				**Log-transformed WC1+**			
	Coef.	SE	P-value	95% CI	Coef.	SE	P-value	95% CI	Coef.	SE	P-value	95% CI
Intercept	49	3.7	<0.001	41 ; 56	14	3	<0.001	8 ; 19	2	0.3	<0.001	1.5 ; 2.6
Group												
Not detected	Ref.				Ref.				Ref.			
Low Proviral Load	–5.7	3.7	0.12	–13 ; 1.6	5	3	0.13	–1 ; 11	–0.5	0.3	0.14	–1.2 ; 0.2
Medium Proviral Load	–5.2	3.9	0.19	–13 ; 2.5	0.4	3.5	0.90	–6 ; 7	–1.0	0.4	0.01	–1.7 ; –0.2
High Proviral Load	–11	3.9	0.01	–18 ; –2.8	4	4	0.22	–3 ; 11	–1.4	0.4	<0.001	–2.2 ; –0.7
BLV-negative	–1	3.7	0.76	–8 ; 6	1	3	0.77	–5 ; 7	–0.1	0.3	0.72	–0.8 ; 0.6

Time-point	–1	0.6	0.06	–2 ; 0.03	1	0.3	<0.001	1 ; 2	–0.2	0.02	<0.001	–0.23 ; –0.15
Cow ID	1.4e–09	4.4e–06		0	19	8		8 ; 44	0.3	0.1		0.1 ; 0.5
Residuals	239	25		195 ; 293	56	6		46 ; 70	0.3	0.03		0.2 ; 0.4

Note: Each time-points (T) were three-months apart, except T5 (four months interval) and T6 (two months interval). A natural log-transformation was applied for WC1+ cell population analysis and the results are presented without back-transformation. Cow ID was included as the random effect in all models.

Appendix D

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