INTEGRATED METHODS TO REDUCE BOVINE LEUKEMIA VIRUS PREVALENCE WITHIN A U.S. DAIRY HERD

By

Tawnie Nicole DeJong

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PUBLIC ABSTRACT

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Enzootic bovine leukosis is an infectious cattle disease caused by the retrovirus bovine leukemia virus (BLV). Bovine leukemia virus creates a persistent infection associated with a disruption in immune response and has consistently been associated with reduced milk production, shortened lifespan, predisposition to lymphoma, and an impaired response to some vaccines. While over 21 countries have managed to eradicate BLV, approximately 45% of all United States (U.S.) dairy cattle are infected. Bovine leukemia virus research must be disseminated to and understood by producers, who may then develop and implement BLV control and eradication programs to maintain the sustainability of the U.S. dairy industry. The objective of this intervention study was to develop an integrated method to reduce BLV prevalence within a large commercial dairy herd. Blood samples were collected from milking cows to determine lymphocyte count (LC), antibodies against BLV, and proviral load (PVL). Test results were used to inform herd management decisions targeting cows most likely to transmit BLV or develop disease by reducing contact with herdmates and culling. Significant decreases in the percentage of cows with high LC and high PVL were observed for all lactations throughout the year of intervention management strategies. Additionally, it was found that LC and PVL were associated with clinical lameness but not with clinical mastitis. Methods utilized in this pilot study showed promise for reducing and importantly, maintaining control over BLV infection within the herd.

ABSTRACT

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Cattle infected with bovine leukemia virus (BLV) have disrupted immune systems, associated with reduced milk production, shortened lifespan, and predisposition to lymphoma. The objective of this intervention study was to develop an integrated method to reduce BLV prevalence within a large commercial dairy herd. Blood samples were collected from milking cows to determine lymphocyte count, antibodies against BLV, and proviral load (PVL) using complete blood cell counts, enzyme-linked immunosorbent assay, and quantitative polymerase chain reaction methods, respectively. Anticoagulated whole blood samples were collected to measure LC and harvest plasma for antibody detection. The PVL was quantified from cows with positive antibody results. Test results were used to inform herd management decisions targeting those cows most likely to transmit BLV or develop disease by reducing contact with herdmates and culling. The risk value for lymphocytosis ($P<0.001$) and the mean PVL ($P<0.001$) was significantly reduced during the four quarters of intervention. Additionally, it was found that PVL was associated with clinical lameness ($P<0.001$) but not with clinical mastitis ($P=0.557$), and there was no association found between LC and clinical lameness ($P=0.074$) or clinical mastitis ($P=0.966$). This novel, multifaceted pilot study effectively reduced BLV prevalence within the herd.

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KEY TO ABBREVIATIONS

AGID: agar gel immunodiffusion

BLV: bovine leukemia virus

BoLA: bovine leukocyte antigen

CBC: complete blood count

CI: confidence interval

ELISA: enzyme-linked immunosorbent assay

EU: European Union

LACT: lactation

LC: lymphocyte count

miRNA: microRNA

OD: optical density

OIE: World Organization for Animal Health

PHA: passive hemagglutination assay

PVL: proviral load

qPCR: quantitative polymerase chain reaction

RIA: radio immunoassay

US: United States

USDA: United States Department of Agriculture

WBC: white blood cell

CHAPTER 1

LITERATURE REVIEW

Introduction

Enzootic bovine leukosis is an infectious cattle disease caused by a retrovirus called bovine leukemia virus (BLV).[20] Bovine leukemia virus creates a persistent infection associated with a disruption in immune response.[32,59,74] Approximately 30-50% of cattle infected with BLV will develop a condition known as persistent lymphocytosis, which is an exponential expansion of blood lymphocytes.[11,32,74] Further, 1-5% of BLV-positive cattle develop malignant lymphomas (or tumors) in their lymph nodes and other organs, which is ultimately fatal to the host animal.[20,32,74,115] Bovine leukemia virus has consistently been associated with reduced milk production, shortened lifespan, predisposition to lymphoma, and an impaired response to some vaccines.[7,20,30,39,74,92,98,115,121,135] While over 21 countries have managed to eradicate BLV, approximately 45% of all United States (U.S.) dairy cattle are infected.[7,65,96] Bovine leukemia virus research must be disseminated to and understood by producers, who may then develop and implement BLV control and eradication programs to maintain the sustainability of the U.S. dairy industry.

History of BLV

In 1871, the first reported case of bovine leukemia was discovered by Leisering in the Klaipeda area of Lithuania, who described a cow with yellowish nodules in her enlarged spleen.[72] Following this discovery, other cases were reported, characterized by the growth of lymphomas in various organs, ultimately leading to organ dysfunction and emaciation.[32,115] Research focused on bovine leukemia slowly followed the initial discovery, with significant advancements being made

decades apart. Some of the most notable discoveries started with Kenneth in 1917, where the disease was determined to be caused by a contagious agent, then identified as bovine leukemia virus.[20] Then in 1969, Miller et. al. discovered that lymphocytes of cows with persistent lymphocytosis produced viral particles visible by electron microscopy after *in vitro* culture.[81] In 1976, Kettmann et. al. found that BLV particles are exogenous RNA viruses and carry an RNA reverse transcriptase complex, which classified BLV among the oncogenic retroviruses.[56] Commercial exchanges of cattle eventually lead to the spread of the disease around the world.[115]

Prevalence of BLV Infections in the World

Following the initial report of clinical bovine leukemia in Lithuania, other cases continued to be reported.[20,115] Following World War II, lymphoma was reported in cattle on all continents.[26,112,116,132] The World Organization for Animal Health (OIE) reports updated animal disease status for individual countries worldwide.[96] Figure 1 shows the distribution of bovine leukemia in domestic cattle at the national and sub-national level from January to June 2019.[96]

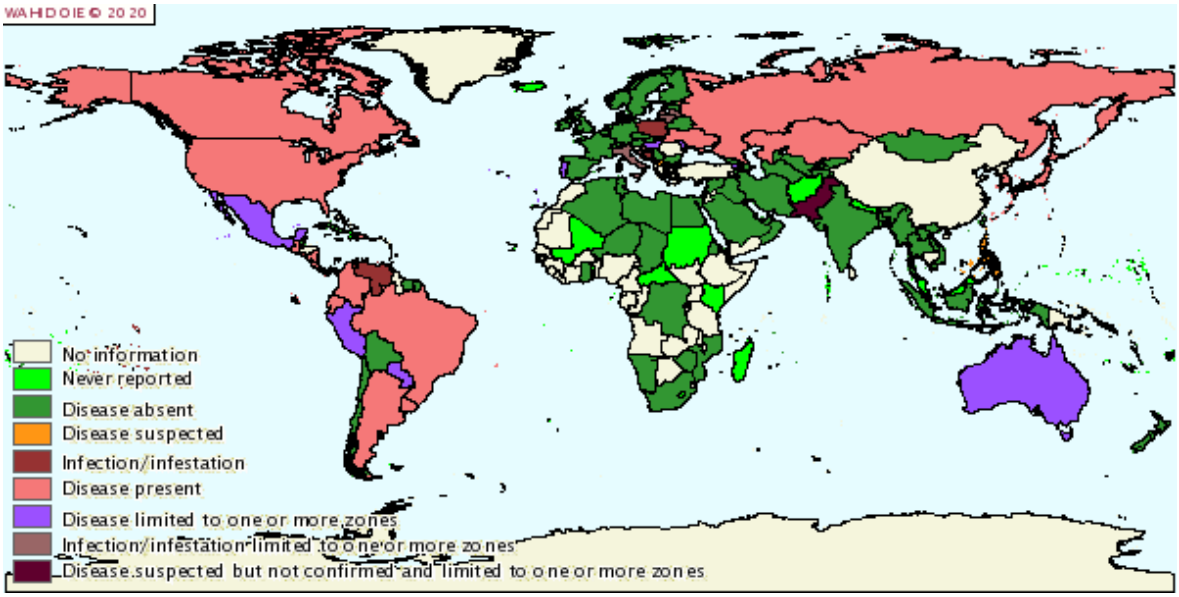

Figure 1.1. Worldwide bovine leukemia distribution reported to OIE for the time period of January-June 2019.[96]

The estimation of bovine leukemia prevalence in domestic cattle varies significantly from continent to continent.[96] While over 21 countries have eradicated the disease, prevalence remains unknown in dozens of countries.[7,96] Though a country or region may not have bovine leukemia cases, BLV may still be present in livestock. Therefore, prevalence estimates may not reflect true prevalence worldwide. Several attempts at BLV testing and eradication have been published, and results are summarized by continent below.[7,65,115]

Europe

Efforts to implement control measures and eradication plans in many European countries have been widely successful, including Andorra, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, Ireland, Luxembourg, the Netherlands, Norway, Poland, Spain, Switzerland, Sweden, Slovenia, and the United Kingdom.[2,38,61,92,95] By contrast, the disease remains present with unknown prevalence rates in several countries in eastern Europe, including Croatia, Latvia, Romania, and Ukraine.[96]

Africa

Though South Africa, Tunisia, and Egypt have successfully eradicated BLV, fewer than a dozen African countries have estimated BLV infection rate.[96] The countries that have completed such studies have determined prevalence to be between 7-50%, including Kenya, Zimbabwe, West Africa, and Malawi.[54,115,142]

South America

Bovine leukemia has been recognized in South America since 1943.[28] Prevalence was reported between 34-50% in Colombia, Venezuela, Chile, and Uruguay.[4,49,76,85] Previous studies have indicated individual and herd prevalence levels in Argentina to be 32.8% and 84%, respectively.[137] In Brazil, prevalence varies considerably between states and ranges from 17.1-60.8%.[24,28,83,114]

Asia

Individual BLV infection rates of dairy cattle in China approach 49.1%, while approximately 1.6% of beef cattle were BLV-positive.[150] Serological tests have also revealed that 20.1% of yaks in China were BLV-positive.[75] Prevalence in Japan was found to be 28.6% and 68.1% at individual and herd levels, respectively.[86] An average individual prevalence of BLV in Thailand was determined to be 58.7%.[71] In Korea, 54.2% of dairy cattle and 86.8% of dairy herds were BLV-positive, whereas only 0.14% of beef cattle were infected with BLV.[71] Three countries were found to have a less than 6% individual BLV prevalence: Mongolia – 3.9%, Cambodia – 5.3%, and Taiwan - 5.8%.[78,93,143]

Oceania

Nationwide BLV control and eradication programs began in 1983 in Australia and 1996 in New Zealand. Since then nearly all (99.7%) of the Australian dairy herds have been declared BLV-free, and New Zealand successfully eradicated the disease in 2008.[25,32]

North America

In Canada, studies of BLV prevalence found up to 37.2% individual prevalence and 89% herd prevalence.[141] In Mexico, BLV is also present in dairy and beef cattle.[119] However, the disease is considered limited to a restricted region of the country.[96] In 1996, the first rigorous survey to provide a baseline prevalence of BLV in the U.S. showed that 89% of U.S. dairy operations had cattle that were seropositive for BLV, while 74.8% of these operations had an estimated within-herd prevalence of 25% or higher.[138] In 2007, the United States Department of Agriculture (USDA) completed a follow up study that showed 83.9% of U.S. dairy herds were positive for BLV.[138] Studies completed in 2018 found individual and herd BLV prevalence at 46.5% and 94.2%, respectively.[65] Fewer prevalence estimates have been reported for beef cattle within the U.S. The 2007 USDA survey found an individual prevalence of 10.3% in beef cattle.[138] A decade later, it was found that 33.6% of all tested cull cows brought into U.S. slaughterhouses were positive for BLV.[9] In early 2020, a prevalence of 39.1% was estimated for U.S. beef cattle.[12]

The continued prevalence of BLV within the U.S. has economic impacts. United States dairy producers are estimated to lose $285 million annually due to BLV, and consumers lose an estimated $240 million annually due to losses associated with BLV.[97] Additionally, the cost of carcass condemnation due to BLV-associated disease has been estimated at over $400 per case.[106,107] With these economic losses associated with BLV, actionable methods for producers to reduce BLV prevalence within their own herds are more crucial than ever.

Pathology

Morbidity associated with BLV infection is related to clinical BLV infection, caused by the growth of lymphomas in various organs, ultimately leading to organ dysfunction and

emaciation.[32] Following initial infection, four stages in BLV infection progression can be identified as primary infection, persistent infection, persistent lymphocytosis, and lymphosarcoma.[32]

Bovine leukemia virus creates a persistent and chronic infection that affects B-lymphocytes.[32,59] Primary infection is when a BLV-infected cell with a copy of BLV provirus integrated into the host genome is transmitted to a susceptible animal.[32] When infected cells containing an integrated BLV virus are transmitted to a new host, the BLV provirus is expressed into viral particles that infect other B-lymphocytes.[32,59] During primary infection, BLV provirus is expressed into viral particles, further infecting the host's B-lymphocytes.[32] Genes for regulatory proteins Tax and Rex contained in the BLV proviral genome can activate cellular oncogenes, which causes dysregulation of the host the immune system.[108] Within 2-8 weeks after infection, cattle will develop a serological response to both viral capsid and envelope proteins.[20] The presence of these antibodies is lifelong.[20]

The second stage, persistent infection, is when provirus-carrying cells expand mainly by mitosis due to proliferation of B-lymphocytes.[32] Within a few weeks, the host's developing immune response strongly limits infection of new target cells, causing the provirus cells to proliferate by clonal expansion by mitosis.[32] This persistent infection phase is characterized by immune dysregulation and can last several months to years.[32,74] Approximately 50-70% of BLV-positive animals remain in this phase as asymptomatic carriers throughout their lifetime and can only be identified by testing for the presence of BLV antibodies and/or BLV DNA.[32]

Following a latent period, 30-50% of BLV-infected cattle develop exponential expansion of B-lymphocytes, classified as persistent lymphocytosis.[11,32,74] Persistent lymphocytosis is when the number of lymphocytes in blood severely increases beyond normal levels.[32] Morbidity is

characterized by weakness and opportunistic infections during this stage.[32] Though persistent lymphocytosis is subclinical itself, animals in this stage may suffer immune disruption with impaired defense to pathogens and other infections.[32] This phase is typically stable for several years, but it can lead to the development of tumors in the lymphoma phase.[32,74]

The fourth and rarest expression of BLV-infection is lymphosarcoma, affecting 1-5% of all BLV-infected cattle.[32] Lymphosarcoma is when lymphoma is formed inside and outside of the lymph nodes when an infected cell undergoes genetic mutations.[32] Lymphosarcoma can occur in animals with or without persistent lymphocytosis.[32] Very few BLV-positive animals progress to develop lymphosarcoma, or the development of malignant lymphomas (tumors) in the lymph nodes and other organs of the host.[20,32,74,115] Lymphosarcoma occurs in 1-5% of BLV-positive animals roughly 1-8 years after initial infection in adult cattle over 2 years of age, with most tumors occurring in cattle 5-8 years of age.[20,32,74,115] Two-thirds of animals in the lymphosarcoma phase have exhibited persistent lymphocytosis; However, aleukemic animals may also develop lymphosarcoma.[20,32,115]

The lymphosarcoma phase is characterized by rapid, progressive loss of body condition and tumor development, which often occur in lymph nodes, heart, abomasum, uterus, spleen, caudal spine, liver, kidneys, and behind the eye.[11,32,77] Clinical signs of heart lymphosarcoma include increased heart rate, difficulty breathing, jugular pulsing, abnormal heart rhythm, or heart failure, resembling chronic heart disease.[11] Tumors located in the abomasum can cause pain, loss of appetite, diarrhea, and constipation.[11] Tumors in the spleen can cause it to rupture which leads to sudden death from internal bleeding, while spinal tumors can compress the spinal cord or nerves and cause hind limb weakness or paralysis.[11] Uterine tumors may result in reproductive failure,

and clinical signs of tumors behind the eye are bulging of the eyeball from the socket and visible irritation.[11.32.77] Clinical manifestation of lymphosarcoma leads ultimately to death.[11,32]

Diagnostic Methods

Worldwide, a variety of diagnostic methods have been developed to identify BLV infections and can be categorized into two groups: (1) antibody-based serological tests, and (2) detection of the proviral genome by nucleic acid-based polymerase chain reaction (PCR) assays.[32,102]

Serological Tests

Antibodies against viral capsid and envelope proteins are produced shortly after BLV infection. These antibodies can be detected 2-3 weeks post-infection and remain detectable for the life of the host.[52] Several conventional serological techniques can be used to target antibodies against these proteins such as agar gel immunodiffusion (AGID), passive hemagglutination assay (PHA), enzyme-linked immunosorbent assay (ELISA), and radio immunoassay (RIA).[32,102] Most of these methods are used to detect antibodies in bovine serum, milk, and the supernatants of BLV-infected cell cultures.[32,102]

Though it is inexpensive and can be used to screen multiple serum samples simultaneously, AGID is not sufficiently sensitive and is not suitable for analysis of milk samples.[32,87,102] In comparison, ELISA is highly sensitive, easily implemented, and can be used to analyze both serum and milk samples.[32,102] However, ELISA requires several controls to assess antibody presence.[23,32,87,102] In addition, ELISA can produce both false-negative results in serum samples from cattle in early stages of infection, and false-positive results in calves with maternally derived

antibodies.[23,32,87,102] Although PHA aims to detect BLV glycoproteins, test efficiency is sensitive to pH, temperature, and trypsin.[102] Diagnosing BLV-infection soon after animals have been exposed can be achieved through RIA, but the test is not suitable for the purpose of mass screening.[88,102] To avoid false-positive results due to the presence of maternal antibodies, these antibody-based detection methods should not be used to test calves less than six months old.[32,94,102]

PCR Assays

Bovine leukemia virus appears to be transcriptionally silent *in vivo* after integration into dispersed sites within the host genome, even in the absence of detectable BLV antibodies. Absence of antibodies would allow for undetectable spread of the virus if only serologically-based tests were utilized for BLV diagnosis.[57,58,102,124,126] However, throughout the course of the disease, a copy of the full-length proviral genome can be detected in BLV-infected cattle.[102,121] Another study demonstrated that BLV-induced tumors and BLV-infected cells contain provirus.[21,101] These findings suggest proviral DNA detection as an alternative method for identifying BLV infection. Therefore, nucleic acid-based PCR methods can greatly accelerate the detection of BLV infection, in addition to using conventional serological techniques.

A variety of PCR methods have been extensively used worldwide to sequence and quantify BLV for detection, including standard PCR, nested PCR, quantitative real-time PCR (qPCR), and direct blood-based PCR.[15,51,52,63,73,84,90,102,117,122,124,127,128,129,152] Polymerase chain reaction-based diagnostic tests broaden the range of samples that can be used to detect BLV infection compared to serological tests.[102] Standard PCR, nested PCR, and qPCR can all use DNA extracted from blood, tumor, milk somatic cells, semen, saliva, and nasal secretion samples to detect BLV

provirus.[15,51,52,63,73,84,102,117,121,124,127,130,152] However, direct blood-based PCR can only be completed using blood samples.[90,128]

In addition, these PCR-based genome screening methods for BLV diagnosis increase testing sensitivity, specificity, efficiency, and are less time consuming, when compared to serological diagnostic methods.[102] Bovine leukemia virus infection is also detectable with PCR-based provirus screening in cattle several weeks before it is possible to detect antibodies with a serologically based screen.[55,102] However, PCR-based provirus screening involves labor-intensive sample preparation, which can lead to false-positive sample results in the event of cross contamination.[15,51,52,73,102,117,127,130] In addition, these diagnostic methods are typically higher cost, require specific laboratory facilities, including thermocyclers, and oligonucleotides must also be designed to utilize this diagnostic method.[15,51,52,73,102,117,127,130]

Provirus concentration can be measured and expressed as a ratio of BLV provirus copy number compared to host gene copy number, which is commonly referred to as proviral load.[55,102,111] Compared to the genes of a BLV-infected host, the BLV provirus copy number is typically lower.[102] Nested assays have a sensitivity of 98.1%.[100,101,102,122] Therefore, the majority of PCR-based BLV detection methods use a nested design since nested assays are more sensitive than standard PCR.[100,101,102,122] However, this method requires real-time PCR thermocyclers and reagents, involves tedious sample preparation protocols, and can produce false-positive results due to DNA contamination.[63,84,100,102,122,124,152] Direct blood-based PCR amplifies target DNA regions without needing to isolate and purify DNA.[90,128] This assay can detect BLV provirus with high specificity at a low cost, but the sensitivity level of this test at 75.51% is lower than that of nested PCR.[90,102,128]

Several qPCR protocols for the detection of BLV provirus have been published.[95] These qPCR protocols are suitable for detecting BLV provirus in infected cattle with low, transient, or undetectable antibody levels during the early phase of infection.[32,44,62,73,102] Compared to conventional nested PCR assays, qPCR has been shown to detect 7.8% more sero-positive cattle.[109] These qPCR-based diagnostic methods also provide accurate disease status results of cattle with inconclusive results from a serological test.[32,102,109]

Other Methods

Other BLV diagnostic techniques include detection of viral proteins by western blotting, a syncytium formation assay, and detection of BLV antigens by indirect immunofluorescent assay.[3,47,48,102,123,124]

Transmission

Horizontal transmission through infected blood lymphocytes is believed to be the most common route of transmission for BLV.[29,45,69,70] In herds, blood-related cattle management procedures such as dehorning, ear tattooing, rectal palpation, injections, and vaccinations have been hypothesized and associated with BLV transmission.[29,45,69] Prolonged, close contact with BLV-positive animals and uninfected animals has also been shown to be a horizontal transmission route for BLV.[70] Though BLV is not a vector borne disease, transfer of infected blood by hematophagous insects (i.e. biting flies and mosquitoes) may contribute to the spread of BLV.[43,144]

Vertical transmission of BLV can also occur via dam to calf in utero with an estimated transmission rate of 4-18%.[5,34,70,99] However, calves born from cows with persistent lymphocytosis

have the highest risk of contracting BLV.[5,34,70,99] Vertical transmission has also been hypothesized to occur from cows to calves via colostrum.[35,36,79]

Through the identification of BLV transmission routes, herd management practices can be adapted to reduce transmission of BLV within a herd.[8,30,92,108] Simple herd management modifications such as cleaning and disinfecting equipment after blood-related procedures, utilization of disposable equipment, implementing biting insect control programs, and segregating BLV-positive animals from BLV-negative animals have all shown to reduce BLV transmission.[8,29,43,45,69,70,144]

Impact of BLV

Economic loss resulting from BLV includes reduced milk production, cow longevity, international trade value, and cow condemnation at slaughter for cattle with lymphosarcoma.[97] United States dairy producers lose an estimated $285 million annually due to BLV, and consumers lose an estimated $240 million annually due to losses associated with BLV, though these estimates do not include the subclinical impact of BLV on cow longevity.[97] In addition to the U.S. economic impact of BLV, there are also potential immunology, animal welfare, and public health concerns.

Milk Production

In 1996, the USDA determined that an increase in within-herd BLV prevalence resulted in an approximate annual loss of 1,014 lbs. of milk per cow.[97] Similar herd-level production losses associated with BLV were reported in a later Michigan-based study.[31] Typically, older cows produced more milk than younger cows, and older animals are more likely to be infected with BLV.[7,31,64,103] This association complicates attempts to determine the true impact of BLV on milk

production. In addition, some studies have found that BLV-infected cows produced as much or even more milk than their uninfected herdmates prior to reaching the point of severe immune disruption.[31,103] Therefore, BLV-infected cows are often culled before their 305-day mature equivalent milk production decreases.[31,103]

Cow Longevity

A Michigan-based study showed that herds with a lower proportion of cows in their third or greater lactation had an increased within-herd BLV prevalence.[31] Cows with antibodies against BLV were also 23% more likely to die or be culled within the observation period than their uninfected herdmates.[7] Cows with the highest BLV antibody titers (BLV milk ELISA optical density results >0.50) were at a 40% greater risk of dying or being culled than cows without any BLV antibodies.[7] Since BLV reduces cow longevity, dairy herds with a high BLV prevalence trend toward a low mean cow age due to increased culling within these herds.[7,31]

Like associations between BLV infection and milk production, associations of cow longevity with BLV infections are also complex in nature. A study reported that BLV-infected cows tend to have reduced longevity when compared to uninfected cows.[135] While several studies have indicated that BLV infection had an adverse effect on cow longevity, this association was not consistently observed.[7,13,27,46,103,104,107,108,132,136] An additional study determined the cull rate for cattle with sero-positive status was 27% higher than the cull rate for BLV-negative cattle.[50] However, this effect was only observed in the older cow population (lactation 3+).[50] Consistently, the association between BLV status and decreased longevity is stronger for older cows than first lactation cows.[7,50]

Immunology

B-lymphocytes in the blood are a critical cell of the immune system that synthesize antibodies to protect against infection.[39,121] The B-lymphocytes play a functional role in disease protection by stimulation through vaccination.[39,121] Research has suggested that BLV-infected dairy cattle have impaired antibody production following vaccination, which presumably also impaired immune protection against disease.[30,39,105] This immune disruption may be responsible for the observation of BLV-infected cattle being culled earlier in their lifetime and for reported increased rates of mastitis and lameness.[7,31,91]

Slaughter Condemnation

According to the USDA, BLV-induced lymphosarcoma is the largest single reason cattle are condemned during postmortem slaughter inspection within the U.S.[140] Lymphosarcoma accounted for 13.5% of condemnations in beef cattle and 26.9% of condemnations in dairy cattle within U.S. slaughter plants.[140,145] Thurmond et. al. reported that 81% of rejected cattle carcasses condemned at slaughter had lymphosarcoma.[134] Annual lymphosarcoma losses to the U.S. dairy industry have been estimated at $16 million, and individual U.S. herds with a 50% or higher BLV prevalence are estimated to lose $412 per case of lymphosarcoma.[97,107,108]

International Trade

International exports of U.S. dairy cattle and products may become difficult as more countries attempt to implement BLV control and eradication programs. Current guidelines for embryo transfer from the U.S. to the European Union (EU) require embryos to be collected from donors that have spent the previous six months within no more than two herds, and each of these

two herds must have been free from clinical signs of bovine leukosis during the previous three years.[139] In addition, semen exported from the U.S. to the EU must be supplied from a USDA certified BLV-free herd.[139] As additional countries acquire and maintain BLV-free status, the U.S. may face additional export restrictions from other countries.

Animal Welfare

In 2013, Bartlett et. al. hypothesized that immune suppressed BLV-positive cattle have decreased longevity, as cows may slowly debilitate with a multitude of infections and clinical problems.[7] The development of BLV-associated lymphomas is accompanied by chronic illness, progressive loss of body condition, weakness, anemia, anorexia, and is attributable to tumor development in various internal organs.[77] These known effects of BLV on a host's immune system and associated clinical and subclinical signs of infection have significant animal welfare implications. Animal welfare consequences may vary according to the tumor location and magnitude of spread.[33,80] Overall, animals are likely to suffer when lymphomas have progressed beyond the early stages of infection for the following reasons:[80]

- Lesions in the heart seem to resemble chronic heart disease with signs such as asthenia, tachycardia, dyspnea, and increased jugular venous pressure. Lesions in the right atrium cause arrhythmias, murmurs, or heart failure.
- Infiltration into bronchial, mediastinal, and cervical lymph nodes contributes to hyperpnoea or dyspnea and tracheal constriction.

- Lesions in the abomasum lead to abdominal pain and may cause anorexia, diarrhea, and constipation. Extradural spinal lesions lead to compression of the spinal cord or nerves resulting in pelvic limb paresis.
- Lesions in the spleen lead to rupture and exsanguination into the peritoneal cavity.
- Uterine lesions cause reproductive failure and abortion.
- Lesions in the liver cause jaundice and liver failure.
- Lesions in the kidney and ureter cause severe abdominal pain and renal failure.
- Retrobulbar lesions cause protrusion of the eyeballs resulting in keratitis and eventually proptosis.

Due to these factors, it is likely BLV-infected cattle that develop lymphomas suffer considerably during the last months of their lives, especially in the later stages of lymphosarcoma development.[33,80]

Public Health

The sustainability of the U.S. cattle industry is extremely vulnerable to public perceptions of food safety. Possible public health implications of BLV have been studied and debated within the scientific community. Based on available epidemiological evidence, it has been widely accepted that BLV poses no risk to human health.[22,132] However, this issue has come under question following several recent studies.[6,16] Research within the last two decades has indicated BLV grows in human cell tissue culture, and humans exposed to BLV produce antibodies against the virus.[17,41,89] Bovine leukemia virus-associated viral DNA sequences have been identified in human mammary cells.[89] However, whether the sequences found are associated with cancerous or

noncancerous tissues has yet to be determined.[18,41,89] Despite studies indicating a strong association between BLV and human breast cancer, a lower rate of breast cancer in women was reported in North America, where BLV prevalence is higher, than in western European countries.[18,151] The association between BLV and human health implications should continue to be evaluated.

Controlling BLV in the U.S.

Since over 45% of all U.S. dairy cattle are BLV-positive with over 90% of dairy herds infected, culling all BLV-positive animals is economically infeasible.[65,138] Management practices to reduce and prevent BLV transmission within and between herds would allow the U.S. to move forward on the path to BLV eradication.[2,8,38,61,92] Certification programs for herds that have become BLV-free are currently offered by the U.S. Animal Health Association, states including New York and Missouri, and by several European countries.[14,82,139] However, the worldwide standard for BLV-free certification is maintained by the OIE.[95] This organization offers three certifications outlined below:

BLV-Free Country or Zone (or a part of a country defined by the Veterinary Authority)

- Qualification – the following requirements need to be satisfied for a minimum of 3 years:
    - All tumors, suspected to be lymphosarcoma, are reported to the Veterinary Authority, and are examined at a laboratory by appropriate diagnostic techniques.
    - All cattle with tumors that are BLV-positive are traced back to their respective herds. All cattle in those herds over the age of 24 months are individually tested for BLV.
    - At least 99.8% of the herds in the country or zone are qualified as BLV-free.

- Maintenance of free status
    - Annual serological survey of a random sample of the cattle population sufficient to provide a 99% confidence to detect BLV at a rate exceeding 0.2% of herds.
    - All imported cattle comply with the following policy:
        - Cattle are sourced from a BLV-free country, zone, compartment, or herd.
        - Cattle are sourced from a herd with no clinical evidence of BLV within the previous two years AND all cattle over 24 months have two consecutive BLV-negative blood tests within the previous year.
        - Cattle have a BLV-negative blood test within 30 days.
    - All imported cattle semen and embryos comply with the following policy:
        - At the time of semen collection, the donor bull was a resident of a BLV-free herd.
        - The donor bull had two consecutive BLV-negative blood tests within 90 days prior to semen collection.
        - Semen and embryo delivery require an accompanying international veterinary certificate attesting they have been appropriately collected, processed, and stored.

BLV-Free Compartment (or a part of a zone defined by the Veterinary Authority)

- Qualification
    - All cattle introduced into the compartment are sourced from a BLV-free herd.
    - All semen and embryos meet the country import policy.

    - The compartment is managed under a common biosecurity plan to prevent common vertical and horizontal means of BLV transmission.
    - The compartment has been approved by the Veterinary Authority.
- Maintenance of Free Status
    - All herds must remain BLV-free in accordance with the country maintenance policy.
    - Periodic surveillance implemented in accordance with the country qualification policy has not detected the virus.
- Revocation and Re-Approval of Free Status
    - If any cattle test BLV-positive, the BLV-free status of the compartment is revoked until all herds within the compartment have recovered their BLV-free status in accordance with the qualification policy.

BLV-Free Herd

- Qualification
    - There has been no clinical evidence of BLV within the previous two years.
    - All cattle over the age of 24 months have two consecutive BLV-negative results within two years.
    - Cattle imported into the herd satisfy all country import policies.
    - Semen and embryos imported into the herd satisfy all country import policies.
- Maintenance of Free Status
    - Cattle in a herd remain BLV-free in accordance with the country maintenance policy.

- Revocation and Re-Approval of Free Status
    - If any cattle test BLV-positive, the BLV-free status of the compartment is revoked until the following measures are taken:
        - The BLV-positive cattle and their progeny under 24 months of age should be removed from the herd. However, progeny that test negative by PCR may be retained.
        - Remaining cattle are tested until all receive two consecutive BLV-negative results within two years.

Currently, three main management protocols influencing BLV have been proposed: (1) test and manage; (2) test and segregate; and (3) test and cull.[8] Many U.S. dairy producers dismiss BLV management protocols as part of their operation. Therefore, the U.S. has yet to move toward a BLV-free status.[65,139] For a farm to consider BLV management protocols, first an initial whole-herd BLV scan must be completed to determine the initial herd BLV-prevalence.[8] If all cattle included in an initial whole-herd scan test negative for BLV, a producer may elect to pursue a BLV-free certification.[8] If initial herd prevalence is low enough to immediately segregate and/or cull all BLV-positive cows, a BLV-free certification may be more readily attainable than a farm that lacks the ability to segregate and/or cull all BLV-positive cows.[8] If herd prevalence is too high for a farm to economically pursue eradication following a whole-herd scan, the producer may elect to implement a comprehensive management plan to minimize BLV transmission in order to lower BLV prevalence within the herd.[8] Once a producer is able to achieve a manageable BLV-prevalence for their farm, the remaining BLV-positive animals can be segregated and then culled from the herd.[8]

Management practices can be developed and implemented based on known methods of horizontal BLV transmission. For example, equipment should be cleaned and disinfected between animals for blood-related cattle management procedures such as dehorning, ear tattooing, rectal palpation, injections, and vaccinations.[8,29,45,69] In addition, disposable equipment such as needles and examination sleeves should be made single use.[8,29,45,69] Reducing the transfer of infected blood by hematophagous insects by implementing a biting insect control program may also reduce the spread of BLV within a herd.[8,43,144] By segregating BLV-positive and BLV-negative cattle, the spread of BLV caused by prolonged, close contact between infected and uninfected animals can be reduced.[8,70] Furthermore, since the main route of BLV transmission is through infected blood lymphocytes, culling or segregating cattle with lymphocytosis and/or a high proviral load may be an option to eliminate the largest reservoir of disease within a herd.[8,11,32,74,111]

Addressing potential vertical BLV transmission may also decrease the prevalence of BLV incidences within a herd. Minimizing contact between newborn calves and BLV-positive cattle may limit vertical transmission of BLV. In addition, colostrum from BLV-positive cows should be frozen or heat-treated prior to feeding to calves.[8,10,35,36,53,68,70,110] Opting to cull BLV-positive cattle instead of rebreeding or culling offspring from BLV-positive cattle may also reduce vertical transmission within a herd by limiting spread from cows to calves *in utero*.[5,8,34,70,99]

According to emerging scientific literature, utilization of genetic selection and vaccination are potential methods to prevent BLV progression and infection.[27,42,108] Bovine leukocyte antigens (BoLAs) polymorphisms in the *DRB3* gene, have been evaluated for association with clinical and subclinical indicators of BLV.[27,37,129] Bovine leukocyte antigens have been associated with susceptibility to persistent lymphocytosis and high milk production potential.[27,37] In brief, the *BoLA-DRB3**0902 and *BoLA-A* alleles have been associated with resistance to persistent

lymphocytosis, while the *BoLA-A* allele has been additionally associated with cow longevity and realization of milking potential.[27,37] Conversely, the *BoLA-DRB3**1501 allele has been associated with persistent lymphocytosis, with cattle consistently progressing to high proviral load status.[37]

Cattle that are both BLV-positive and have a high genetic potential for milk and fat yields have been reported to be more susceptible to lymphocytosis than BLV-positive cows with genetically lower milk and fat yield potential.[27,149] Anecdotally, producers implementing or maintaining BLV reduction programs find it difficult to cull such productive cows from the herd.[118] However, research suggests that these cows may serve as a reservoir of infection for the herd, as alleles associated with disease-resistance and disease susceptibility have been identified.[27,37,111,118,129]

Retroviruses like BLV have been shown to be associated with microRNAs (miRNAs), including host- and viral-miRNAs, to prolong the life of each cell, escape host immune response, and contribute to pathogenesis of the virus.[60,117] BLV-derived viral-miRNAs are highly expressed and comprise up to 40% of the total miRNA expression in BLV-infected cells, while research to identify host-miRNAs associated with BLV has been limited.[60,65,111,127] One study identified seven circulating host-miRNAs as differentially expressed between BLV-infected and BLV-negative dairy cattle.[127] Since there is a risk of secondary disease infection associated with the presence of BLV-viral-miRNAs, the role of these miRNAs in pathogenesis has important consequences for the safety of developing a BLV vaccine.[41,43]

Vaccination against retroviruses is a challenge because of their ability to stably integrate into the host genome, undergo long-term latency in a proportion of infected cells and thereby escape immune response.[19,40,42] Vaccination for BLV is being investigated through field trials in

Argentina, though previous attempts have been unsuccessful.[19,42,60,108,146,147,148] Additionally, commercial dairy herds in the EU utilize a patented vaccine against BLV.[1]

Conclusions

While many countries outside of the U.S. have implemented nationwide BLV control and eradication programs, the U.S. has allowed cattle producers autonomy. This autonomy has led to significantly measurable economic losses within the dairy and beef industries and potential public health concerns. With the sustainability of the U.S. dairy and beef industries in question, producers may consider reducing and/or eradicating BLV within their own herds. Integrated management practices and emerging research studies will be essential in aiding producers in meeting this goal. Though other nations have successfully eradicated BLV using the test and cull management protocol, implementing similar culling programs within the U.S. would be economically infeasible due to the high BLV prevalence. Therefore, intervention studies are needed to identify management protocols that are both effective in reducing BLV prevalence and that integrate into current farm protocols with minimal disruption. Additionally, investigations into associations between BLV infection and herd health should continue in order accurately measure the economic implications of BLV within the U.S.

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CHAPTER 2

Reducing bovine leukemia virus prevalence and assessing its association with lameness and mastitis on a large midwestern dairy farm by using lymphocyte counts, antibody presence, and proviral load

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Abstract

Cattle infected with bovine leukemia virus (BLV) have disrupted immune systems, associated with reduced milk production, shortened lifespan, and predisposition to lymphoma. The objective of this intervention study was to develop an integrated method to reduce BLV prevalence within a large commercial dairy herd. Blood samples were collected from milking cows to determine lymphocyte count (LC), antibodies against BLV, and proviral load (PVL) using complete blood cell counts (CBC), enzyme-linked immunosorbent assay (ELISA), and quantitative polymerase chain reaction (qPCR) methods, respectively. Anticoagulated whole blood samples were collected to measure LC and harvest plasma for antibody detection. The PVL was quantified from cows that had positive antibody results. Test results were used to inform herd management decisions targeting those cows most likely to transmit BLV or develop disease by reducing contact with herdmates and culling. The risk value for lymphocytosis ($P<0.001$) and the mean PVL ($P<0.001$) was significantly reduced during the four quarters of intervention. Additionally, it was found that PVL was associated with clinical lameness ($P<0.001$) but not with clinical mastitis ($P=0.557$), and there was no association found between LC and clinical lameness ($P=0.074$) or clinical mastitis ($P=0.966$). This novel, multifaceted pilot study effectively reduced BLV prevalence within the herd.

Keywords

BLV, ELISA, lameness, lymphocyte count, mastitis, PVL

Introduction

Bovine leukemia virus (BLV) is an oncogenic retrovirus that affects over 40% of all dairy cattle within the United States (U.S.).[13] Cattle infected with BLV have disrupted immune systems, associated with a reduction in milk production, shortened lifespan, predisposition to lymphoma, and impaired response to some vaccines.[2,10,15,16] Additionally, an association between BLV and common dairy diseases such as mastitis and lameness has been reported.[3,8,22,28] These BLV-associated disorders have significant negative impacts on profitability for dairy farmers.

Twenty-one other nations eradicated BLV by removing all animals serologically-positive for BLV antibodies using an enzyme-linked immunosorbent assay (ELISA) test.[1,24] However, the average dairy herd in the U.S. has a 46.5% prevalence of BLV and culling this large percentage of the herd is economically infeasible.[13] Therefore, it is crucial to identify ways to progressively reduce BLV prevalence to the point that culling residual antibody-positive cows becomes affordable for dairy producers in order to mitigate the associated adverse economic impacts.

Cows with persistent lymphocytosis have an ongoing progressively increased lymphocyte count (LC) that may be measured by performing a complete blood count (CBC). Once considered a benign condition, cattle with persistent lymphocytosis recently have been shown to have decreased milk production as well as increased culling and lymphoma rates.[8] Cows infected with BLV are virus reservoirs for their herdmates, and those with lymphocytosis may be at greater risk of transmitting the infection horizontally to their calves *in utero*.[21] Since first identified, BLV has been known to cause lymphocytosis in cattle.[4] Lymphocytotic cattle can be identified and segregated or isolated from herdmates until culled. Approximately 5% of BLV-positive cattle ultimately develop lymphoma, preceded by a lymphocytosis in two-thirds of these animals.[5,9,23] The single largest cause of condemnation of dairy cattle at postmortem slaughter inspection is

BLV-induced lymphoma (26.9%), according to the United States Department of Agriculture (USDA).[27,29] More recently, quantitative polymerase chain reaction (qPCR) tests to detect the presence and amount of proviral BLV DNA integrated into circulating white blood cells (WBC) or proviral load (PVL), have been developed and may be a sensitive indicator of infectivity.[21]

Automated LC, antibody detection, and PVL measurement are tools that may be used in concert to identify, reduce, and potentially eradicate BLV in dairy herds. An increased lymphocyte concentration reportedly develops in approximately 30% of BLV-infected cattle.[4] Antibodies directed against BLV are present life-long in cattle after BLV infection, indicating prior, presumably persistent infection. Measurement of PVL is now commercially available as an indicator of potential infectivity.[21] Efficacious, economic strategies for seamless integration of BLV testing without disrupting routine herd management practices would be advantageous for the dairy industry to reduce and control BLV infection. A means to determine which BLV-infected cows are likely to be most infectious to other cattle and develop BLV-associated disease would facilitate informed herd management decisions.

A CBC is the most common routine baseline laboratory test to confirm health and assess for or monitor disease in human medicine and companion animal veterinary medicine. A CBC is comprised of total and differential WBC counts including LC as well as other WBC types, red blood cell indices, and platelet counts.[11] However, the high cost and logistics of blood sample transportation from a farm to a clinical pathology laboratory historically reduced utilization of CBCs in food animal medicine. The recent availability of on-site hematology devices has tremendously reduced CBC costs while providing results in a matter of minutes rather than days, allowing for convenient, timely management changes before a cow released with herdmates. The

utility of these instruments for controlling BLV and other diseases is only now being investigated.[21]

Detection of anti-BLV antibodies in milk, plasma, or serum is the most widely employed method to identify and manage BLV infections.[2,9] Although a positive antibody result indicates infection, it is not predictive of the relative infectiousness of an individual cow. When the anticipated herd prevalence is very low, costs can be reduced by testing pooled plasma, serum, or milk samples, then testing individual samples from cows contributing to a positive pool.

A minority of cows are referred to as "super-shedders" because they have high concentrations of provirus and are thought to be responsible for the majority of BLV transmission within a herd[12,14,21] Identifying and removing cows with high PVL may result in reduced BLV transmission.[21] In addition, the LC and PVL are reportedly highly correlated and used by Ruggiero et. al. to identify the most infectious cows for segregation or culling. [21,26] However, such studies have not been completed on larger farms thus far.

The principal purpose of this intervention study was to develop an integrated approach using LC, antibody status, and PVL to reduce the prevalence of BLV infection within a large commercial dairy herd and identify possible associations between BLV diagnostic measures and herd health concerns, such as clinical mastitis and clinical lameness.

Materials & Methods

Herd Background

This intervention field trial was completed over one year on an approximately 3,000-head milking Holstein dairy farm located in northeast Wisconsin. Herd managers routinely entered all medical treatments including those for mastitis and lameness into a computerized record system

that maintained milk production, diagnostic test, breeding and pregnancies, vaccination, and culling data for all cows. The BLV-antibody status of cows in the herd was not available at the start of the study.

Blood Collection

Anticoagulated tail blood samples were drawn from milking cows into EDTA tubes and used to sequentially perform one, two, or three of the below described diagnostic tests at each collection timepoint. Following determination of baseline LC measurements for the entire milking herd, blood was collected weekly to obtain samples from cows at parturition during the first quarter-year (three-month period) as well as during mid-lactation throughout the rest of the year. Procedures for this study were reviewed and approved by the Michigan State University Institutional Animal Care and Use Committee.

Diagnostic Tests

Lymphocyte Count – A CBC was completed on anticoagulated whole blood samples on-site (farm) using the GENESIS™ Hematology System (Oxford Science Inc., Oxford, Connecticut, U.S.). This system uses impedance and laser technologies to measure total WBC counts ($\times 10^3/\mu L$) as well as percentages of cell types in order to calculate LC and other cell counts reported as $\times 10^3/\mu L$.[18] Additional routine CBC analytes (red blood cell indices, platelet, and other white blood cell types) were measured by the analyzer, but not utilized in this study.

BLV Antibodies – An ELISA test to detect antibodies against BLV was completed (CentralStar laboratory, Grand Ledge, Michigan, U.S.) using plasma harvested from submitted anticoagulated whole blood samples. In brief, sample aliquots were diluted in sample buffer and

pipetted into 96-well plates coated with BLV-antigen. Horseradish-peroxidase-labeled bovine anti-immunoglobulin antibodies were added and incubated. Plates were washed after each incubation and before adding an enzyme substrate. Reaction times were standardized using color development of positive controls and stopped by adding 0.5 N H_2SO_4. Results were reported as corrected 450 nm optical density (OD) measurements with a corrected OD >0.5 considered antibody positive.[21]

Quantification of Proviral Load – The DNA was extracted from whole blood samples via the Qiagen DNeasy blood and tissue kit (Valencia, California, U.S.) or King Fisher MagMAX Core magnetic bead-based automated nucleic acid system (Thermo Fisher, Austin, Texas, U.S.) to consistently isolate DNA for use in the qPCR proviral load assay. The SS1 qPCR assay detected presence or absence of BLV PVL. The SS1 qPCR assay, developed by CentralStar Cooperative Inc., is a multiplex probe-based qPCR assay that targets the BLV proviral polymerase gene, bovine *Beta Actin* gene, and internal amplification spike-in control ultramer to quantify proviral load. Briefly, 4 µL extracted DNA, 12.5 µL of 2X PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Coralville, Iowa, U.S.), 1.25 µL of a 20X primer mix, 1 µL of an internal spike-in control (10,000 copies/µL), and 7.25 µL of DNA-free water were combined for each qPCR reaction. All qPCR was performed on Applied Biosystems 7500 Fast Real-Time PCR system (Foster City, California, U.S.) with qPCR conditions as follows: 95°C for 10 min., 40x (95°C for 15 sec., 60°C for 1 min.). Bovine leukemia virus and *Beta Actin* (measure of bovine genomes) copy numbers were estimated using a standard curve consisting of linearized plasmids containing respective target sequences previously quantified and normalized by digital droplet PCR. Amplification efficiency and manual thresholds were established from initial qPCR

thermocycler calibration and used for the duration of the study. Proviral load was calculated and expressed as the ratio between proviral BLV copies and bovine *Beta Actin* copies.

Protocol Timeline

A CBC was completed on all primiparous and multiparous milking cows in the herd to obtain an initial baseline LC for each cow and results were categorized as low ($\leq 4.5\times10^3$/μL); acceptable (4.6-7.0$\times10^3$/μL); moderate (7.1-9.9$\times10^3$/μL); or high ($\geq$10.0$\times10^3$/μL). At this time, aliquots of blood samples from cows with high LC were tested for antibody to assign a negative or positive BLV status, with positive samples tested for PVL. Baseline data were summarized and reported to farm management; thereafter, results were summarized and reported quarterly. Following distribution of each report, a conference call was held between farm personnel and the research team to discuss and recommend changes in management and testing protocols to optimize control of BLV infection as the project evolved. During the team meeting to discuss baseline data, the farm and research teams established culling thresholds of either: (1) LC $\geq$ 10.0$\times10^3$/μL or (2) PVL $\geq$ 0.5, or approximately one BLV-infected leukocyte out of every two cells.

At the start of the first quarter, blood was collected from cows at parturition to perform a CBC. At this point, additional blood samples from up to four randomly selected cows with LCs in the 6.0-6.9$\times10^3$/μL; 7.0-7.9$\times10^3$/μL, 8.0-8.9$\times10^3$/μL, and 9.0-9.9$\times10^3$/μL ranges also were tested for antibodies and PVL to establish a PVL baseline for cows positive for antibodies with LCs below the high LC range. Using this approach, a substantial number of cows were found to have a PVL>0.5 with less than 10.0x10^3/μL lymphocytes. Therefore, one month into the sample collection for quarter two, the protocol transitioned to submission of an aliquot of all collected anticoagulated whole blood samples at each blood collection to harvest plasma and screen for

antibodies. In addition, PVL was measured on DNA extracted from an aliquot of anticoagulated whole blood of animals positive for antibodies. Starting quarter three sample collection, the testing regimen was expanded to include cows positive for antibodies at both parturition and mid-lactation in order to detect new infections earlier during the lactation cycle. Approximately one-third of the milking herd was tested during each quarter. Therefore, about one-third of the data were from different animals for each reporting period.

Data was compiled and summarized on a quarterly basis throughout the remainder of the study to monitor BLV reduction progress. These five reports (baseline plus 4 quarters) included a full year of data after baseline measurements. Following completion of the fourth quarter report, antibody status was determined on plasma samples from the entire milking herd to calculate BLV prevalence. Proviral load was measured on aliquots of anticoagulated blood samples from the subset of cows positive for antibodies.

Statistical Analysis

Chi-squared test for linear trend was completed using OpenEpi 3.01 to determine Mantel-Haenszel odds ratios and risk values for lymphocytosis based on LC over time.[7] The confidence intervals for the whole-herd antibody (ELISA results) point prevalence was calculated in OpenEpi 3.01 with use of the Clopper-Pearson method.[7] Incidence of clinical lameness and mastitis recorded in the computerized record system was evaluated for an association with LC and PVL. To adjust for lactation (LACT) number, a multiple logistic regression equation was used:

$$Incidence = \beta_0 + \beta_1(x_1) + \beta_2(LACT)$$

where β = coefficient and x_1 = LC or PVL.[20]

Lymphocyte count and PVL were evaluated as continuous variables and lactation number was evaluated as categorical (1, 2, or 3+ lactations), while mastitis and lameness were binomial variables.

Results

The LC data indicated a reduction in the number of animals with high LC ($\geq$ 10.0 $\times 10^3/\mu L$) over the course of the study (Figure 2.1). Throughout the one-year intervention, the overall high LC risk value was reduced from 4.22% to 1.04% (Table 2.1). The Mantel-Haenszel extended chi-square summarizing linear trend was 86.79 with a p-value < 0.001. At the conclusion of the study, the average LC was 4.72 ± 0.13 $\times 10^3/\mu L$ and 5.33 ± 0.22 $\times 10^3/\mu L$ for cows negative and positive for antibodies, respectively with a 95% CI (P<0.001).

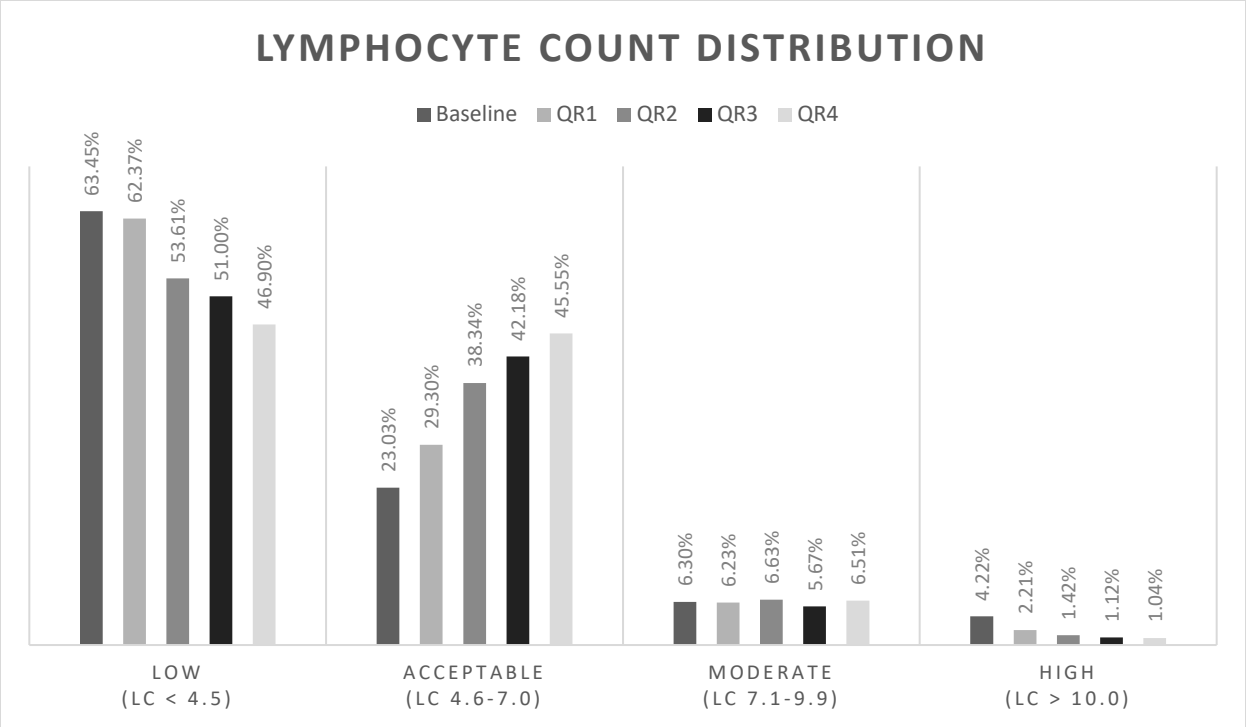

Figure 2.1. Lymphocyte count (LC) in units $\times 10^3/\mu L$ over time shown as the percentage of milking cows tested in each LC category. QR1 = Quarterly Report 1; QR2 = Quarterly Report 2; QR3 = Quarterly Report 3; QR4 = Quarterly Report 4.

Table 2.1. Chi-squared test for linear trend expressed as an odds ratio and risk value. The odds and risk of lymphocytosis decreased over the course of the study compared to the initial whole herd data at baseline. LC = Lymphocyte Count; QR1 = Quarterly Report 1; QR2 = Quarterly Report 2; QR3 = Quarterly Report 3; QR4 = Quarterly Report.

Exposure Level	LC>10.0×10^3/µL Prevalence (Lymphocytosis Risk Value)	Lymphocytosis Risk Value Confidence Limits (95%)	Lymphocytosis Odds Ratio
Baseline	4.22%	3.55, 5.01	1
QR1	2.21%	1.76, 2.76	0.51
QR2	1.42%	1.05, 1.91	0.33
QR3	1.12%	0.81, 1.55	0.26
QR4	1.04%	0.74, 1.46	0.24

The final whole herd antibody test results showed 662 BLV-antibody-positive cows out of 3,178 total cows tested. Therefore, the herd exhibited a point prevalence of 20.83% [95% CI 19.43-22.28%]. The proportion of BLV-antibody-positive tests to BLV-antibody-negative tests decreased throughout the study as a larger portion of the herd was tested (Table 2.2). Additionally, LC was higher on average for BLV-antibody-positive cows than BLV-antibody-negative cows across all lactations, and the mean PVL significantly decreased over the course of the study for cows in all lactations ($P<0.001$; Table 2.3).

Depending on their LC and PVL status, BLV-antibody-positive cows were managed differently. Starting in the first quarter, cows with lymphocytosis (LC $\geq$ $10.0x10^3/\mu L$) as well as cows with PVL $\geq$ 0.5 were marked "Do Not Breed" and were segregated into a sick pen for culling after milk production dropped below the herd's production cull threshold (Figure 2.2).

Table 2.2. Percentage of milking cows that were negative and positive for BLV-antibodies each quarter and final whole herd point prevalence. QR1 = Quarterly Report 1; QR2 = Quarterly Report 2; QR3 = Quarterly Report 3; QR4 = Quarterly Report 4.

	Proportion BLV-Antibody-Negative	**Proportion BLV-Antibody-Positive**
QR1 (n = 300)	46.67%	53.33%
QR2 (n = 1,580)	73.80%	26.20%
QR3 (n = 2,742)	79.03%	20.97%
QR4 (n= 3,179)	81.69%	18.31%
Final Whole Herd (n = 3,178)	79.17%	20.83%

Table 2.3. Mean Lymphocyte Count (LC) in units $\times 10^3/\mu L$ and Proviral Load (PVL) per lactation over the course of the study. L1 = Lactation 1; L2 = Lactation 2; L3+ = Lactation $\geq$3.

	All Cows			BLV-Antibody-Negative			BLV-Antibody-Positive		
	L1	**L2**	**L3+**	**L1**	**L2**	**L3+**	**L1**	**L2**	**L3+**
Baseline									
Mean LC	4.63±0.04	4.65±0.08	4.66±0.11	4.38±0.05	3.85±0.08	3.35±0.08	5.14±0.31	5.72±0.37	5.97±0.31
Mean PVL							1.74±0.17	1.96±0.13	2.42±0.11
Quarterly Report 1									
Mean LC	4.64±0.04	4.59±0.07	4.35±0.07	4.87±0.04	4.65±0.05	3.09±0.05	6.30±0.17	8.61±0.24	8.51±0.22
Mean PVL							0.45±0.05	0.99±0.08	1.32±0.10
Quarterly Report 2									
Mean LC	4.94±0.04	4.66±0.06	4.40±0.07	5.13±0.05	4.45±0.06	4.08±0.07	5.82±0.27	6.13±0.20	5.48±0.19
Mean PVL							0.25±0.04	0.47±0.05	0.44±0.06
Quarterly Report 3									
Mean LC	5.00±0.03	4.67±0.05	4.28±0.06	4.99±0.03	4.22±0.04	4.01±0.05	5.77±0.20	5.60±0.15	4.96±0.13
Mean PVL							0.18±0.03	0.30±0.03	0.28±0.28
Quarterly Report 4									
Mean LC	5.14±0.03	4.67±0.05	4.38±0.06	5.10±0.03	4.44±0.04	3.96±0.05	5.99±0.20	5.56±0.16	4.98±0.13
Mean PVL							0.14±0.02	0.27±0.04	0.210±0.02
Final Whole Herd Scan									
Mean LC	5.14±0.03	4.67±0.04	4.38±0.06	5.09±0.03	4.33±0.04	3.96±0.05	6.01±0.20	5.59±0.16	4.98±0.13
Mean PVL							0.14±0.02	0.27±0.04	0.21±0.02

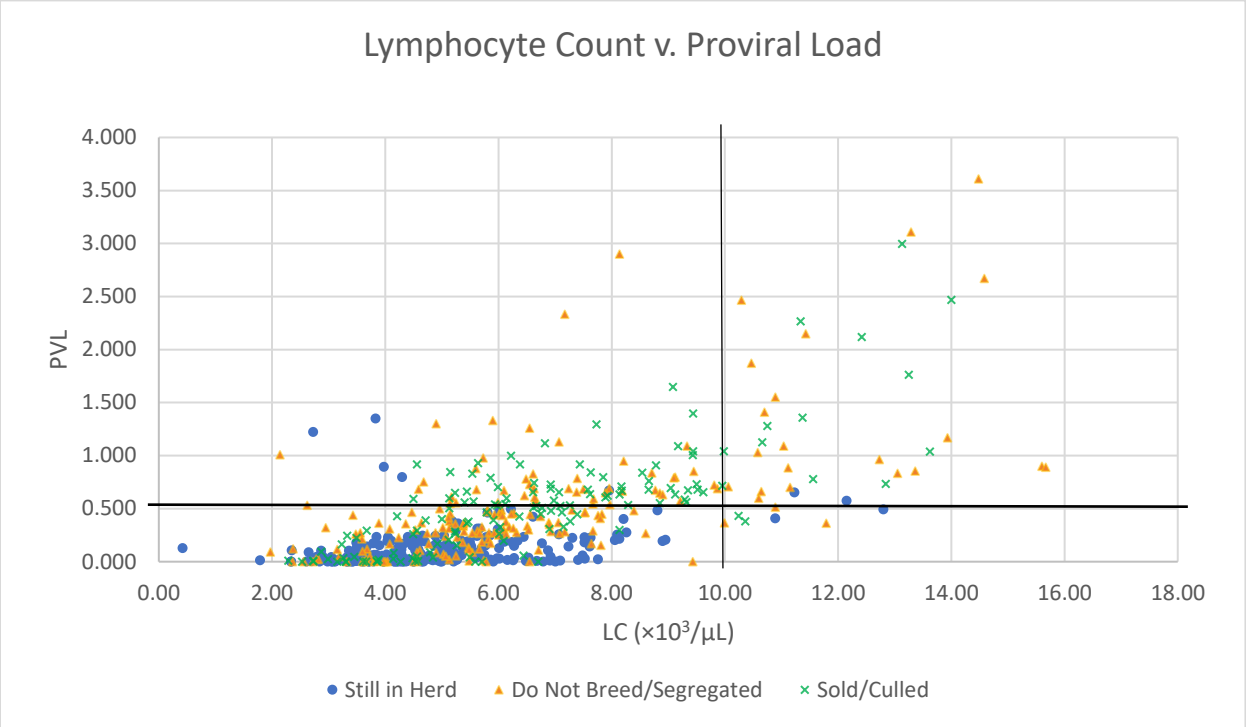

Figure 2.2. Lymphocyte Count (LC) in units $\times 10^3/\mu L$ vs. Proviral load (PVL) expressed as concentration of BLV to the host DNA of antibody-positive cows at the end of the study (n=433). Blue circles signify cows that remained in the herd. Orange triangles signify cows that had been marked "Do Not Breed" and were segregated. Green X's signify cows that had been culled from the herd within the last three months of the study. The vertical and horizontal bars represent the management cutoff thresholds on the farm during the study.

Mastitis and Lameness

At the conclusion of the study, 3,178 milking cows remained in the herd; 224 (7.05%) and 658 (20.70%) had been treated for mastitis and lameness respectively during the current lactation. Tables 2.4 and 2.5 documented that lameness incidence is strongly associated with PVL, but not LC. In addition, mastitis incidence did not have a significant association with LC or PVL.

Table 2.4. Impacts of Lymphocyte Count (LC) and Proviral Load (PVL) on mastitis incidence within the herd analyzed with a multiple logistic regression.[1]

Multiple Logistic Regression Models						
	Mean Value (Cows w/o Mastitis)	**Mean Value (Cows with Mastitis)**	**Estimate**	**Std. Error**	**Z Value**	**P-Value**
LC	4.85	4.60	<0.01	<0.01	-0.04	0.966
PVL	0.22	0.24	<0.01	<0.01	0.56	0.577

[1]LC and PVL were not analyzed together because they are known to be highly correlated (>0.90).[26]

Table 2.5. Impacts of Lymphocyte Count (LC) and Proviral Load (PVL) on lameness incidence within the herd analyzed with a multiple logistic regression.[1]

Multiple Logistic Regression Models						
	Mean Value (Cows w/o Lameness)	**Mean Value (Cows with Lameness)**	**Estimate**	**Std. Error**	**Z Value**	**P-Value**
LC	4.89	4.66	<0.01	<0.01	1.79	0.074
PVL	0.16	0.28	<0.01	<0.01	3.30	<0.001

[1]LC and PVL were not analyzed together because they are known to be highly correlated (>0.90).[26]

Discussion

This exploratory intervention focused on combining LC, ELISA, and PVL diagnostic methods to identify and reduce BLV-infection within a ~3,000 cow U.S. dairy herd over a year-long period. The study protocol evolved as herd managers and researchers learned more about the relationship between the three diagnostic tests and attempted to integrate the BLV testing protocol into the herd's existing management protocols. Combined testing resulted in a 3.18% reduction in lymphocytosis within the herd, a final BLV-ELISA-positive prevalence over 25% below the national average, and a significant reduction in PVL of BLV-positive cows across all lactations within the herd.

Identifying cows with lymphocytosis was an important starting point because approximately one-third of cows with antibodies to BLV eventually develop this condition that in turn causes increased comorbidities and culling resulting in decreased production and ultimately significant profit losses.[2,8] Using the on-site automated CBC device to identify and remove animals with lymphocytosis proved to be an effective first step. Whole herd lymphocytosis decreased from 4.22% to 1.04%. At the conclusion of the study, the average LC was 4.72 ± 0.13 x10^3/µL for BLV-antibody-negative cows and 5.33 ± 0.22 x10^3/µL for BLV-antibody-positive cows, similar to previous published research.[25]

Most recent studies of BLV prevalence rates in the U.S. estimate individual and herd BLV infection rates at 46.5% and 94.2%, respectively.[13] Point prevalence of whole herd BLV infection determined at the end of this study was 20.83% which is considerably less than the U.S. national average.[13] Because whole herd BLV-antibody-positive point prevalence was not determined at the start of the study, the precise percent reduction in BLV infection resulting from these interventions

could not be calculated. However, the proportion of the subset of cows represented in each quarter that were positive for antibodies steadily decreased over the course of this intervention study.

Besides reducing lymphocytosis, the three combined diagnostic tests also decreased PVL within the herd. Mean PVL was reduced from 1.74 ± 0.17 to 0.14 ± 0.02 for first lactation cows ($P<0.001$), 1.96 ± 0.13 to 0.27 ± 0.04 for second lactation cows ($P<0.001$), and 2.42 ± 0.11 to 0.21 ± 0.02 for third and higher lactation cows ($P<0.001$) among cows positive for BLV antibodies. Overall, the combined management strategy served to effectively reduce LC, antibody prevalence, and PVL within the herd.

Cattle with BLV are known to suffer immune disruption and therefore likely have an impaired defense to pathogens and other opportunistic infections.[9] Two important health concerns on dairy farms are mastitis and lameness, with clinical mastitis costing an average of $444 per cow within the first 30 treatment days and different types of lameness costing an average range of $120-$217 per incident.[6,19] While higher incidence of mastitis in BLV-antibody-positive than antibody negative cattle has been reported, research has been more limited on the relationship between lameness incidence in BLV-antibody-positive versus BLV-antibody-negative cattle.[3,8,22,28] In this study, PVL was associated with an increased incidence of lameness, but not mastitis. Lymphocyte was not associated with lameness or mastitis. It is unknown whether BLV-infection caused increased lameness. However, increased lameness may partly account for the decreased milk production and longevity reported in BLV-positive cows.[2,8,17]

By the conclusion of this study, management practices had evolved to more aggressively control BLV infection in the herd. The LCs were discontinued following the fourth quarter because the associated labor and expense no longer outweighed the benefits given the decrease in lymphocytosis achieved in this herd. Also, approximately 90% of BLV-antibody-positive cows

were not detected by LC screening (Figure 2.2). However, initial elimination of cows with a high LC (all were BLV-antibody-positive) was a key driver behind rapid removal of cows at risk for infectivity, clinical illness, decreased production, and increased culling.

Conclusions

The dairy farm enrolled in this exploratory intervention study used three diagnostic testing methods to develop a BLV control program that integrated efficiently into their existing management protocols. Screening animals via CBC was effective for identifying advanced lymphocytotic animals, whereas plasma or milk antibody test results determined which subset of animal samples were consequently tested for PVL. Following removal of advanced cases of BLV, PVL testing provided the most sensitive and systematic approach for identifying and removing BLV from the herd. Segregating and eventually culling cattle with the highest PVL resulted in a marked reduction in measures of BLV infection. Methods utilized in this pilot study showed promise for reducing and importantly, maintaining control over BLV infection within the herd.

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