PEER REVIEWED

Reducing bovine leukemia virus prevalence on a large midwestern dairy farm by using lymphocyte counts, ELISA antibody testing, and proviral load

Tasia M. Taxis,[1] MS, PhD; **Tawnie N. DeJong,**[2] **Cheryl L. Swenson,**[3] DVM, PhD, ACVPM; **Kelly R.B. Sporer,**[4] PhD; **Casey Droscha,**[4] PhD; **Don Niles,**[5] DVM; **Paul C. Bartlett,**[3] MPH, DVM, PhD, DACVPM

[1] Department of Animal Science, College of Agriculture and Natural Resources, Michigan State University, East Lansing, MI 48824

[2] Department of Animal Science, College of Agriculture, Food and Natural Resources, University of Missouri, Columbia, MO 65211

[3] Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824

[4] CentralStar Cooperative, Inc., East Lansing, MI 48910

[5] Pagel Family Business, Kewaunee, WI 54216

Corresponding author: Dr. Tasia Taxis; taxistas@msu.edu

Abstract

Cattle infected with BLV have disrupted immune systems, associated with reduced milk production, shortened lifespan, and predisposition to lymphoma. The objective of this exploratory case study was to develop a disease control testing and management protocol to reduce the prevalence of bovine leukemia virus (BLV) within a large commercial dairy herd. Three diagnostic tests were available: lymphocyte count (LC), ELISA for BLV antibodies, and proviral load (PVL) as determined by quantitative polymerase chain reaction (qPCR). Both testing and management protocols evolved over time as the BLV prevalence decreased and we learned how to reduce labor and unnecessary or redundant diagnostic testing. Test results were used to inform culling and pen assignments for cows most likely to transmit BLV or develop disease. Significant decreases in the percentage of cows with LC≥10.0x10^3/µL (4.22% to 1.04%) and PVL>0.5 were observed for all lactations during the 4 quarters of intervention. By October 30, 2020, 6 of ~3,000 cows remained with a detectable PVL. Additionally, it was found that LC and PVL were associated with clinical lameness, but not with clinical mastitis.

Key words: BLV, ELISA, lameness, lymphocyte count, mastitis, PVL

Introduction

Bovine leukemia virus (BLV) is an oncogenic retrovirus that affects over 40% of dairy cattle within the United States (US).[13] Cattle infected with BLV have disrupted immune systems associated with reduced milk production, shortened lifespan, predisposition to lymphoma, and impaired response to some vaccines.[2,10,15,16] Approximately 5% of BLV-positive cattle ultimately develop lymphoma, preceded by 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]

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 positive for BLV antibodies.[1,24] These nations started their control programs with low herd prevalence rates, generally considerably below 10%, and removed all cattle with BLV antibodies. However, the average US dairy herd has a 46.5% prevalence of BLV, and simultaneously culling this large percentage of the herd is economically infeasible.[13] Furthermore, because the majority of ELISA-positive cows likely pose a relatively low risk of transmitting BLV to their herdmates due to lower levels of infectivity, immediate culling of all ELISA-positive cattle may be unnecessarily costly.[12] Our approach was to progressively reduce BLV transmission by removing the most infectious cattle. Once the incidence of new cases was greatly reduced, the herd prevalence of ELISA-positive cattle would also decrease over time to the point that culling residual ELISA-positive cows would become affordable in order to achieve eradication.

Cows with persistent lymphocytosis have an ongoing progressively increased lymphocyte count (LC) that is usually measured as part of a complete blood count (CBC). An

increased lymphocyte concentration reportedly develops in approximately 30% of BLV-infected cattle.[4] Once considered a benign condition, cattle with persistent lymphocytosis 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 vertically to their calves *in utero*.[21]

Proviral Load: Recently, a quantitative polymerase chain reaction (qPCR) assay has been developed to detect the concentration of BLV proviral DNA within circulating white blood cells (WBC) referred to as proviral load (PVL). Because the integrated provirus is the infectious particle for BLV, PVL is thought to be a good indicator of infectivity. This qPCR test for PVL is now commercially available in the US.[21]

Lymphocyte Count: 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 is freed to mix with herdmates. The utility of these instruments for controlling BLV and other diseases is only now being investigated.[21]

Since first identified, BLV has been known to cause lymphocytosis in cattle. In fact, a LC was the first diagnostic test for BLV infection. These new on-farm and perhaps cow-side hematology machines may provide a useful diagnostic approach for BLV.

BLV ELISA: Antibodies directed against BLV are considered lifelong in cattle, indicating prior and presumably persistent infection. Detection of anti-BLV antibodies in milk, plasma, or serum is the most widely employed method to identify and manage BLV infections and was used to eradicate BLV from all herds in over 20 nations.[2,9] Although a positive antibody result indicates infection, it is not predictive of the relative infectiousness of an individual cow. In fact, many ELISA-positive cattle may present a relatively low risk of BLV transmission to their herdmates.[14]

A minority of cows are referred to as "super-shedders" because they have high concentrations of lymphocytes and provirus and are thought to be responsible for the majority of BLV transmission within a herd.[12,14,21] For example, 1 infected cow's PVL can measure thousands of times higher than the PVL for another infected cow, and therefore be much more infectious to her herdmates. It was thought that identifying and removing such cows may be an efficient way to reduce BLV transmission.[21] This was demonstrated by Ruggiero et al in a field study on 3 small herds that reduced BLV incidence and prevalence by semi-annual testing and removal of those cows with highest LC and/or PVL.[21,26]

The principal purpose of this exploratory intervention case study was to develop an integrated approach using LC, ELISA antibodies, and PVL to reduce the prevalence of BLV infection within a large commercial dairy herd. This was an exploratory case study and not a formal intervention study. These 3 BLV diagnostic tests measured different attributes, required different amounts of labor to collect diagnostic specimens, required different labor investments for running on-farm testing or mailing samples to the laboratory, had different costs, and had different degrees of correlation with each other. Our mission was to find a testing program that best fit the farm's management protocol, avoided redundancy, and appeared to correctly identify cattle with the highest risk of infecting their herdmates so that they could be separated or culled in order to reduce transmission and overall BLV prevalence on the farm.

Materials and Methods

Herd Background

This BLV testing trial was conducted on an approximately 3,000-head milking Holstein dairy farm located in northeast Wisconsin. Herd managers routinely entered all animal events and medical treatments, including those for mastitis and lameness, into a computerized record system that also maintained data regarding milk production, diagnostic test results, and culling data.

Herd management protocol remained consistent throughout the study. Barns were either sprayed or tunnel ventilated to reduce fly density. Horizontal transfer via blood was minimized through use of pulse guns for injections, single-use palpation sleeves, paste for dehorning, and sterilization of foot-trimming equipment at the end of each day. All cows were bred via artificial insemination and calves were fed only their own dam's colostrum.

Specimen Collection

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 of the study as well as during mid-lactation for the second through fourth quarters of the study. All blood was obtained from the tail vein or artery. 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 performed on EDTA whole blood samples using on-farm testing.[a] This machine 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 the LC and other cell counts.[18]

BLV Antibodies: An ELISA test to detect antibodies against BLV[b] was performed using plasma harvested from

submitted EDTA 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 NH_2SO_4. Results were reported as corrected 450 nm optical density (OD) measurements with a corrected OD>0.5 considered antibody positive.[21]

Proviral Load: DNA was extracted from whole blood samples via the Qiagen DNeasy blood and tissue kit[c] to consistently isolate DNA >30ng/uL for use in the qPCR proviral load assay. Nine months into the study, CentralStar Cooperative Inc.,[d] the company performing diagnostics for the study, had completed comparison validations between DNA extraction protocols. From that point on, DNA was extracted from whole blood via the King Fisher MagMAX Core[e] magnetic bead-based automated nucleic acid system. The SS1 qPCR assay, developed by CentralStar Cooperative Inc.,[d] is a multiplex probe-based quantitative PCR assay that targets the BLV proviral polymerase gene, bovine *Beta Actin* gene, and an 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,[f] 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[g] with qPCR conditions as follows: 203°F (95°C) for 10 min, 40x (203°F [95°C] for 15 sec, 140°F [60°C] for 1 min). Bovine leukemia virus and *Beta Actin* (measure of bovine genomes) copy numbers were derived 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 machine 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

Lymphocyte count was measured on all milking cows to obtain an initial baseline for each cow. Results were categorized as low (≤ 4.5×10^3/µL); acceptable ($4.6\text{-}7.0\times10^3$/µL); moderate ($7.1\text{-}9.9\times10^3$/µL); or high (≥10.0×10^3/µL). Aliquots of blood samples from cows with high LC were tested for ELISA antibody and PVL. Results were entered into the farm's computer recording system. A quarterly report of the BLV testing program was distributed and discussed on a conference call with farm personnel and the research team. Progress and proposed changes in management and testing protocols to optimize control of BLV infection were discussed. Initially, the farm set culling or segregation thresholds of either: 1) LC≥10.0×10^3/µL or 2) PVL≥0.5, which is approximately 1 BLV-infected leukocyte out of every 2 cells. The plan was that these thresholds would be tightened as the BLV control program progressed.

At the start of the first quarter, additional blood samples from up to 4 randomly selected cows with LCs in the $6.0\text{-}6.9\times10^3$/µL; $7.0\text{-}7.9\times10^3$/µL, $8.0\text{-}8.9\times10^3$/µL, and $9.0\text{-}9.9\times10^3$/µL ranges also were tested for ELISA antibodies and PVL to establish a baseline for ELISA-positive cows with a LC below 10.0×10^3/µL. It was found that many cows had a PVL>0.5 with less than $10.0\text{x}10^3$/µL lymphocytes, as seen in the upper left quadrant of Figure 1. Therefore, 1 month into sample collection of quarter 2 of the study, the protocol was revised in that blood collected for LC determination was also tested for ELISA antibodies. Animals with a positive ELISA antibody test were then analyzed for PVL. Starting quarter 3 of sample collection, PVL testing was expanded to include all milking cows at both parturition and mid-lactation in order to detect new infections throughout the lactation cycle.

BLV-antibody-positive cows were managed differently depending on their LC and PVL status. Starting in the first quarter, cows with lymphocytosis (LC≥$10.0\text{x}10^3$/µL) and cows with PVL≥0.5 were marked "Do Not Breed" and segregated into a sick pen for culling after milk production dropped below the herd's production cull threshold. The color code in Figure 1 shows the disposition (do not breed pen, cull or no action) for tested cows.

Data was compiled and summarized on a quarterly basis throughout the remainder of the study to monitor BLV reduction progress. Following completion of the fourth quarter report, ELISA 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 ELISA 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 over time.[7] The confidence intervals for the whole-herd antibody 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 using bivariate and multiple logistic regression to adjust for lactation number in RStudio.[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 significant reduction in the number of animals with high LC (≥ 10.0×10^3/µL) over the

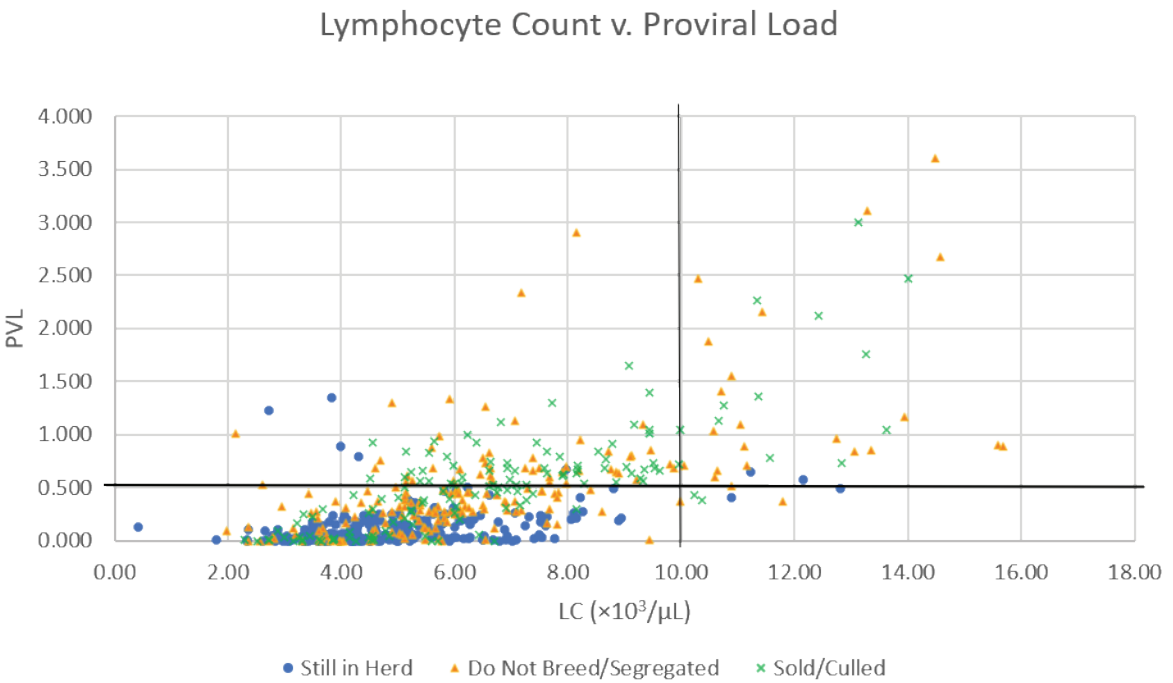

Figure 1. Lymphocyte Count (LC) in units ×10³/µL vs Proviral Load (PVL) expressed as concentration of BLV to the host DNA of ELISA-positive cows at the end of the study (n=433). Blue circles signify cows that remained in the herd. Orange triangles signify cows that had been marked "Do Not Breed" and were segregated. Green X's signify cows that had been culled from the herd within the last 3 months of the study. The vertical and horizontal bars represent the management cutoff thresholds during the study.

course of the study from 4.22% to 1.04% (Table 1, Figure 2). The Mantel-Haenszel extended chi-square summarizing linear trend was 86.79 (P<0.001). At the conclusion of the study, the average LC was 4.72 ± 0.13 ×10³/µL and 5.33 ± 0.22 ×10³/µL for cows negative and positive for ELISA antibodies, respectively with a 95% CI (P<0.001).

At the conclusion of the study, the whole-herd antibody test results showed 662 BLV antibody-positive cows out of 3,178 total cows tested (Table 2). Therefore, the herd exhibited a point prevalence of 20.83% with a 95% CI (19.43, 22.28) using the Clopper-Pearson method. Additionally, data summarized in Table 3 shows 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).

As of October 30, 2020, 204 cows positive for BLV antibody remained in the herd, of which only 6 were PVL positive. Farm management intends to continue the program until BLV is eradicated. Introduction of new BLV infection from incoming youngstock will be minimized as youngstock continue to be tested prior to breeding, and animals with PVL>0 are culled, never entering the milking herd.

Mastitis and Lameness

At the conclusion of the study, the herd's record system showed that 224 cows (7.05%) and 658 cows (20.70%) had been treated for mastitis and lameness, respectively, during

Table 1. Chi-squared test for linear trend expressed as risk values and odds ratios. 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 4.

Exposure Level	LC>10.0×103/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

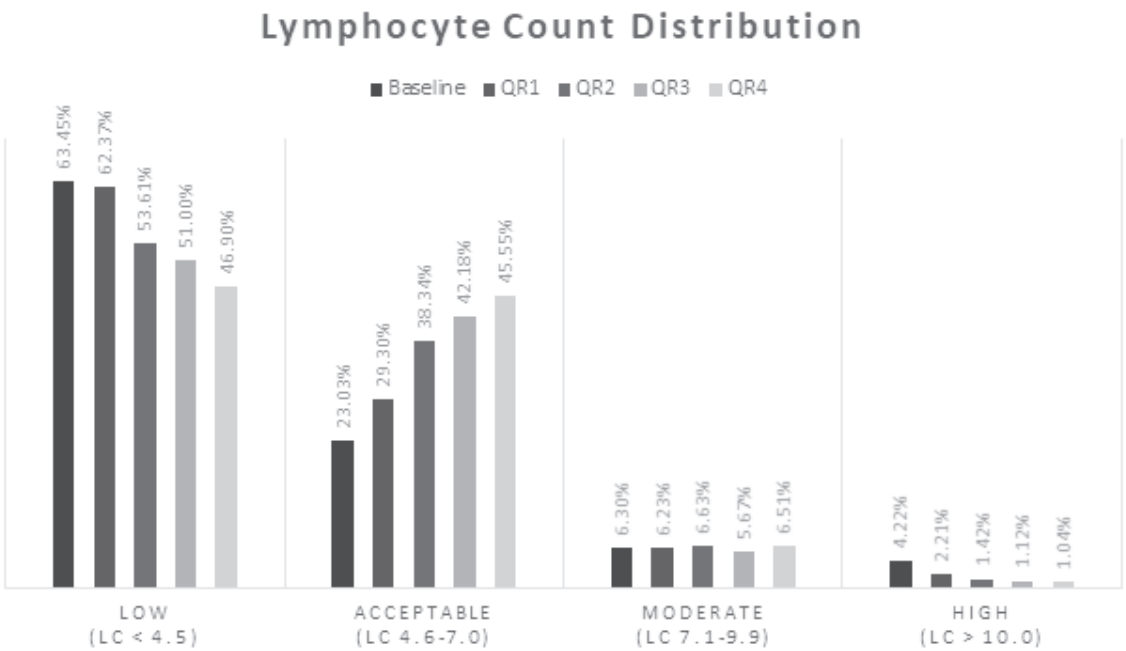

Figure 2. Lymphocyte count (LC) in units $\times 10^3/\mu L$ over time shown as the percentage of milking cows tested in each LC category with all at baseline and approximately one-third each quarter. Therefore, about one-third of the data were from different animals for each reporting period. QR1 = Quarterly Report 1; QR2 = Quarterly Report 2; QR3 = Quarterly Report 3; QR4 = Quarterly Report 4.

Table 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%

the current lactation. Tables 4, 5, and 6 show that lameness was strongly associated with LC and PVL. However, mastitis did not show a significant association with LC or PVL after adjustment for lactation number.

Discussion

This year-long exploratory intervention case study focused on integrating LC, ELISA, and PVL diagnostic methods to identify and reduce BLV prevalence and infection within a US ~3,000 cow dairy herd. The study protocol evolved as herd managers and researchers became more knowledgeable of the relationships between the 3 diagnostic tests. The team attempted to best integrate the BLV testing protocols into the existing herd management protocols. For example, initially cows with LC $\geq 10.0\times10^3/\mu L$ were selectively removed. However, LC screening became increasingly ineffective when only a few cows were identified over this threshold. Similarly, action level thresholds were discussed and sometimes lowered after the team's quarterly meeting, often based on how many cows the managers were able to cull. There is no assurance that the best protocol was achieved at any step of the process or that the testing protocol used would be optimal on other farms.

Table 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 ≥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

*Proviral load (PVL) was not performed on BLV-antibody-negative samples as indicated by the gray shaded regions.

Table 4. Association of Lymphocyte Count (LC), Proviral Load (PVL), and Lactation (LACT) on mastitis and lameness incidence within the herd analyzed by bivariate logistic regression.

	Bivariate Logistic Regression Models					
	Mean Value (Cows w/o Mastitis)	**Mean Value (Cows with Mastitis)**	**Estimate**	**Std. Error**	**Z Value**	**P-Value**
Lymphocyte Count (LC)	4.85	4.60	<0.01	<0.01	-2.39	0.017
Proviral Load (PVL)	0.22	0.24	<0.01	<0.01	0.59	0.555
Lactation (LACT)	1.79	2.59	0.96	0.09	10.68	<0.001
	Mean Value (Cows w/o Lameness)	**Mean Value (Cows with Lameness)**	**Estimate**	**Std. Error**	**Z Value**	**P-Value**
Lymphocyte Count (LC)	4.88	4.66	<0.01	<0.01	-3.33	<0.001
Proviral Load (PVL)	0.16	0.28	<0.01	<0.01	3.37	<0.001
Lactation (LACT)	1.64	2.64	1.39	0.06	22.06	<0.001

The LC test was eventually judged to be a poor screening test, and all LC testing was terminated before the end of the project. The data displayed in Figure 1 compares LC and PVL results, showing that LC testing failed to identify many cows with substantially high PVL results. Results in the upper left quadrant (Figure 1) could be viewed as LC false-negatives, and a low rate of false-negatives is a desirable feature of any screening test.

At the end of the study, ELISA screening followed by PVL assay of ELISA-positive samples was employed as it appeared to be the best testing regime for the herd at that particular time.

The herd rate of lymphocytosis decreased from 4.22% to 1.04%. At the conclusion of the study, the average LC was 4.72 ± 0.13 $x10^3/\mu L$ for BLV antibody-negative cows and 5.33 ± 0.22 $x10^3/\mu L$ for BLV antibody-positive cows, similar to previous published research.[25] Using the on-site automated CBC device to identify and remove animals with lymphocytosis may have been an effective first step in removing lymphocytotic cows. Once the ~$25,000 machine was purchased, LC testing cost approximately $4.00/sample. In contrast, the market price for BLV ELISA antibody testing was $6.50/sample and $10.00/sample for PVL. However, obtaining the blood samples also required considerable

Table 5. Association of Lymphocyte Count (LC), Proviral Load (PVL), and Lactation (LACT) on mastitis incidence within the herd analyzed by multivariate logistic regression.*

	Multivariate 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
LACT	1.79	2.59	0.10	0.01	10.50	<0.001
PVL	0.22	0.24	<0.01	<0.01	0.56	0.577
LACT	1.79	2.59	0.32	0.16	1.92	0.055

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

Table 6. Association of Lymphocyte Count (LC), Proviral Load (PVL), and Lactation (LACT) on lameness incidence within the herd analyzed by multivariate logistic regression.*

	Multivariate 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
LACT	1.64	2.64	1.41	0.06	21.93	<0.001
PVL	0.16	0.28	<0.01	<0.01	3.30	<0.001
LACT	1.64	2.64	0.59	0.12	4.94	<0.001

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

labor and blood collection supplies. For example, 20 to 25 blood samples/hour could be run through the CBC device, resulting in approximately 75 cows/person/hour being tested, while blood collection alone took approximately 45 cows/person/hour.

Our most recent studies of BLV prevalence rates in the US dairy industry put individual cow 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 US 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.

In addition to reducing lymphocytosis, the testing and management protocols 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). Overall, the combined management strategy served to effectively reduce LC, ELISA 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 opportunistic infections.[9] We looked at the association with mastitis, which reportedly has an estimated clinical mastitis cost averaging $444 per cow within the first 30 treatment days, and lameness estimated to cost in the range of $120 to $217 per case.[6,19] While there have been several reports of higher incidence of mastitis in BLV antibody-positive cattle than in BLV antibody-negative cattle, research has been more limited surrounding the potential relationship of BLV with lameness.[3,8,22,28] It is important to know that what was recorded in the herd's computer record system was mastitis and lameness treatments, not diagnoses. Therefore, the records only indicated cases that were severe enough to warrant treatment. In this study, LC and PVL were associated with an increased incidence of lameness. The association between LC and clinical mastitis became non-significant after adjustment for lactation number. Such associations are not necessarily causal. For example, cows with lameness might receive more foot trims at which blood is transferred among cows. If so, it may be just as reasonable to consider the hypothesis that lameness causes BLV as the hypothesis that BLV predisposes to lameness. Clearly, further work is needed.[2,8,17]

By the conclusion of this study, BLV testing practices had evolved to more aggressively control BLV. The measures of LC were discontinued following the fourth quarter of the study, because the associated labor and expense no longer outweighed the benefits when each week of testing only identified about 3 cows with LC ≥10.0×10^3/μL. Also, inspection of our data led us to conclude that approximately

90% of BLV antibody-positive cows were not being detected by LC screening. However, early in the control program, elimination of cows with a high LC (all were BLV antibody-positive) may have been helpful for rapid removal of cows at the greatest risk for infectivity, clinical illness, decreased production, and increased culling. In the quarter following the conclusion of this study, the farm further reduced their BLV antibody-positive point prevalence from 20.83% to 18.74% (P<0.001), at which time 51% of the BLV antibody-positive cows were segregated and on the list of cows to be culled. Moreover, because all cows with PVL≥0.5 had been eliminated, the PVL threshold for segregation and culling was further reduced to PVL≥0.25 following the conclusion of the study and subsequently to PVL>0 3 months post-study. Five months after the study, there were 139 cows remaining with a positive PVL value and only 24 of those cows had a PVL ≥ 0.1. By October 30, 2020, there were only 6 cows present with a PVL>0. Moving forward, after the marked reduction in BLV prevalence, the farm planned to continue using antibody detection as a screening method by testing milk samples on all cows at parturition and completing PVL tests on BLV antibody-positive cows. Additionally, the farm has not had any reported condemned animals at slaughter within the past year.

Conclusions

The dairy farm enrolled in this exploratory intervention case study used 3 diagnostic testing methods to develop a BLV control program that would integrate into their existing management protocols. Screening animals via LC appeared to be effective initially for identifying advanced lymphocytotic animals. As the number of lymphocytotic animals decreased, and LC was no longer beneficial, the farm relied on ELISA antibody testing with follow-up PVL testing. Combined, this method of segregating and eventually culling cattle with the highest LC at the beginning of the study and the highest PVL at the end of the study resulted in a marked reduction of measures of BLV infection. However, the procedures followed in this study were not optimized with regard to sensitivity, efficiency, or return on investment. Future BLV testing protocols will need to be tailored to the needs and capabilities of each individual farm.

Endnotes

[a] GENESIS™ Hematology System, Oxford Science Inc., Oxford, CT
[b] CentralStar laboratory, Grand Ledge, MI
[c] Qiagen DNeasy, Valencia, CA
[d] CentralStar Cooperative, Inc., East Lansing, MI
[e] ThermoFisher, Austin, TX
[f] Integrated DNA Technologies, Coralville, IA
[g] FAST Real-Time PCR, Foster City, IA

Acknowledgements

This work was supported by the USDA National Institute of Food and Agriculture award numbers 2014-67015-21632 and 2014-68004. The authors thank the farm ownership and management team (Don and Ann Niles, Steve Lambrecht), the Genesis team (Ed and Tonya Carver, Howard Jones), Michigan State University graduate students Holden Hutchinson and Katy Kessler, CentralStar Cooperative Inc. research assistant Kelsey Brigham and other laboratory technicians, and *GENESIS™* Hematology System (by Oxford Science Inc.). The authors declare no conflict of interest.

References

1. Acaite J, Tamosiunas V, Lukauskas K, Milius J, Pieskus J. The eradication experience of enzootic bovine leukosis from Lithuania. *Prev Vet Med* 2007; 82:83-89.
2. Bartlett PC, Norby B, Byrem TM, Parmelee A, Ledergerber JT, Erskine RJ. Bovine leukemia virus and cow longevity in Michigan dairy herds. *J Dairy Sci* 2013; 96:1591-1597.
3. Benitez OJ, Norby B, Bartlett PC, Maeroff JE, Grooms DL. Impact of bovine leukemia virus infection on beef cow longevity. *Prev Vet Med* 2020. Available at: https://doi.org/10.1016/j.prevetmed.2020.105055.
4. Berg C, Bøtner A, Browman H, De Koeijer A, Depner K, Domingo M, Ducrot C, Edwards S, Fourichon C, Koenen F, More S, Raj M, Sihvonen L, Spoolder H, Arend Stegeman J, Thulke HH, Vågsholm I, Velarde A, Willeberg P. Enzootic bovine leukosis. *Euro Food Safe Auth J* 2013; 13.
5. Burny A, Bruck C, Chantrenne H. *Bovine leukemia virus: Molecular biology and epidemiology.* New York: Raven Press, 1980.
6. Cha E, Hertl JA, Bar D, Gröhn YT. The cost of different types of lameness in dairy cows calculated by dynamic programming. *Prev Vet Med* 2010; 97:1-8.
7. Dean A, Sullivan K, Soe M. OpenEpi: Open source epidemiologic statistics for public health, version 3.01, 2013. Available at: www.openepi.com. Accessed Dec 22, 2019.
8. Erskine RJ, Bartlett PC, Byrem TM, Render CL, Febvay C, Houseman JT. Association between bovine leukemia virus, production, and population age in Michigan dairy herds. *J Dairy Sci* 2012; 95:727-734.
9. European Panel on Animal Health and Welfare (EPAHW) Scientific opinion on enzootic bovine leukosis. *EFSA J* 2015; 13:63.
10. Frie MC, Sporer KRB, Wallace JC, Maes RK, Sordillo LM, Bartlett PC, Coussens PM. Reduced humoral immunity and atypical cell-mediated immunity in response to vaccination in cows naturally infected with bovine leukemia virus. *Vet Immun and Immunopath* 2016; 182:125-135.
11. Harvey JW. Introduction to veterinary hematology. In: Harvey JW, ed. *Veterinary hematology: A diagnostic guide and color atlas*. Elsevier, 2012; 1-10.
12. Juliarena MA, Barrios CN, Carolina Ceriani M, Esteban EN. Hot topic: Bovine leukemia virus (BLV)-infected cows with low proviral load are not a source of infection for BLV-free cattle. *J Dairy Sci* 2016; 99:4586-4589.
13. LaDronka RM, Ainsworth S, Wilkins MJ, Norby B, Byrem TM, Bartlett PC. Prevalence of bovine leukemia virus antibodies in US dairy cattle. *Vet Med Int* 2018; 2018:5831278.
14. Mekata H, Yamamoto M, Hayashi T, Kirino Y, Sekiguchi S, Konnai S, Horii Y, Norimine J. Cattle with a low bovine leukemia virus proviral load are rarely an infectious source. *Jap J Vet Res* 2018; 66:157-163.
15. Nekouei O, VanLeeuwen J, Stryhn H, Kelton D, Keefe G. Lifetime effects of infection with bovine leukemia virus on longevity and milk production of dairy cows. *Prev Vet Med* 2016; 133:1-9.
16. Norby B, Bartlett PC, Byrem TM, Erskine RJ. Effect of infection with bovine leukemia virus on milk production in Michigan dairy cows. *J Dairy Sci* 2016; 99:2043-2052.
17. Ott SL, Johnson R, Wells SJ. Association between bovine-leukosis virus seroprevalence and herd-level productivity on US dairy farms. *Prev Vet Med* 2003; 61:249-262.

18. Oxford Science, Inc., Connecticut, U.S. 2020. Available at: www.oxfordscienceinc.com. Accessed Apr 2, 2020.
19. Rollin E, Dhuyvetter KC, Overton MW. The cost of clinical mastitis in the first 30 days of lactation: An economic modeling tool. *Prev Vet Med* 2015; 122:257-264.
20. RStudio Team. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA. 2020. Available at: http://www.rstudio.com/. Accessed May 30, 2020.
21. Ruggiero VJ, Norby B, Benitez OJ, Hutchinson H, Sporer KRB, Droscha C, Swenson CL, Bartlett PC. Controlling bovine leukemia virus in dairy herds by identifying and removing cows with the highest proviral load and lymphocyte counts. *J Dairy Sci* 2019; 102:9165-9175.
22. Sandev N, Koleva M, Binev R, Ilieva D. Influence of enzootic bovine leukosis virus upon the incidence of subclinical mastitis in cows at a different stage of infection. *Vet Arhiv* 2004; 6:411-416.
23. Schwartz I, Lévy D. Pathobiology of bovine leukemia virus. *Vet Res BioMed Cen* 1994; 25:521-536.
24. Shaghayegh A. Detection and identification of enzootic bovine leukosis (EBL) in calves in Iran. *Arch Razi Inst* 2019; 74:321-325.
25. Swenson CL, Erskine RJ, Bartlett PC. Impact of bovine leukemia virus infection on neutrophil and lymphocyte concentrations in dairy cattle. *J Am Vet Med Assoc* 2013; 243:131-135.
26. Takeshima SN, Kitamura-Muramatsu Y, Yuan Y, Polat M, Saito S, Aida Y. BLV-CoCoMo-qPCR-2: improvements to the BLV-CoCoMo-qPCR assay for bovine leukemia virus by reducing primer degeneracy and constructing an optimal standard curve. *Arch Virol* 2015; 160:1325-1332.
27. USDA FSIS. Cattle condemned postmortem in USDA inspected establishments. *Anim Dispos Report Syst* 2002. Available at: www.fsis.usda.gov/OPHS/adrsdata/2002/02rm2.htm. Accessed May 15, 2020.
28. Watanabe A, Murakami H, Kakinuma S, Murao K, Ohmae K, Isobe N, Akamatsu H, Seto T, Hashimura S, Konda K, Shinozuka Y, Kawai K. Association between bovine leukemia virus proviral load and severity of clinical mastitis. *J Vet Med Sci* 2019; 81:1431-1437.
29. White TL, Moore D. Reasons for whole carcass condemnations of cattle in the United States and implications for producer education and veterinary intervention. *J Am Vet Med Assoc* 2009; 235:937-941.

