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Development of a predictive model for bovine leukemia virus proviral load

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Abstract

Background: There is currently no commercially available method in Canada to identify bovine leukemia virus (BLV)-positive cows with high proviral load (PVL).

Objectives: First, develop a model to predict PVL using common, commercially available, cost-effective diagnostic tests. Second, investigate the relationship between lymphocyte count and PVL in BLV-positive cows.

Animals: A total of 339 BLV-positive and 62 BLV-seronegative cows on 15 dairy farms.

Methods: Cross-sectional study. Blood and milk samples were collected from all lactating BLV-positive cows on each farm and 5 to 10 BLV-seronegative cows depending on herd size. Blood and milk samples were tested for anti-BLV antibodies using enzyme-linked immunosorbent assay (ELISA). Complete blood counts were performed on blood samples, and standard components analyses were obtained for milk samples. Proviral load was determined by quantitative polymerase chain reaction for each cow.

Results: The inverse of lymphocyte count, the square of the inverse of lymphocyte count, and milk ELISA percent positivity were positively associated with increasing PVL in BLV-positive cows. For BLV-positive cows, lymphocyte count >5.2 $\times$ 10^9/L predicted a high PVL (BLV:Bovine DNA of >1 in blood) with a sensitivity of 92.4% and a specificity of 79.8%. For BLV-positive cows, white blood cell count >10.8 $\times$ 10^9/L predicted a high PVL, with a sensitivity of 85.5% and a specificity of 83.6%.

Conclusions and Clinical Importance: Based on these results, producers can implement commonly available diagnostic tests to identify cows with high probability of having high PVL, which may help in designing effective disease control strategies for BLV-positive herds.

Abbreviations: AGID, agar gel immunodiffusion; AUC, area under the curve; AVC, Atlantic Veterinary College; AVCDSL, Atlantic Veterinary College Diagnostic Services Laboratory; BHB, beta-hydroxybutyrate; BLV, bovine leukemia virus; DHI, dairy herd improvement; EBL, enzootic bovine leukosis; EDTA, ethylenediaminetetraacetic acid; MQM, Maritime Quality Milk; OD, optical density; PP, percent positivity; PVL, proviral load; ROC, receiver operator characteristic; SCC, somatic cell count.

KEYWORDS
ELISA, enzootic bovine leukosis, lymphocytosis, PCR

1 | INTRODUCTION

Enzootic bovine leukosis (EBL) is a disease of cattle caused by persistent infection with bovine leukemia virus (BLV).[1] The BLV deltaretrovirus integrates into the host genome, and in many cases causes no outward signs of infection or clinical disease.[2] In approximately 30% of infected cows, the only clinical abnormality is an increase in circulating B-lymphocytes (persistent lymphocytosis). Up to 5% of infected cows may develop lymphoid tumors in a number of organs, which eventually result in death.[3] Because the number of cows with clinical disease is low, EBL historically has been considered of low economic importance in the dairy industry. However, recent research shows that BLV-infected cows are more likely to be culled earlier and have decreased lifetime production as compared to BLV-seronegative cows,[4] that the average herd milk production decreases as within-herd BLV prevalence increases,[4,5] and BLV-infected cows do not produce as robust a serological response to vaccination against coliform mastitis and are more prone to infections than BLV-negative cows.[6] There are also financial losses when BLV-infected cows are sent to slaughter, because of carcass condemnation if tumors are present.

Testing methods for BLV infection include agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA) for anti-BLV antibodies and polymerase chain reaction (PCR) for viral or proviral genetic material, either on blood or milk samples.[7] A more recent development is the measurement of BLV proviral load (PVL), or the number of copies of viral genome integrated into the host genome, using quantitative PCR.[8,9] This is of interest because it has been shown that cows with low PVL are less infective to uninfected cows via lymphocyte transfer than those with high PVL.[10] Provirus also has been identified in saliva and nasal secretions[11] as well as in colostrum from high PVL cows and colostrum replacers,[12,13] potentially acting as sources of infection or false positive results on serological testing. Proviral load qPCR is performed in a number of research laboratories, but currently is not commercially available in Canada.

Previous studies have shown that all BLV-seropositive cows with persistent lymphocytosis have high PVL, making these animals relatively easy to identify.[14] However, some BLV-seropositive cows with high PVL have normal lymphocyte counts.[14] The ability to identify these cows using current commercially available testing methods is desirable, because removal of all BLV-infected cows currently is impractical for most herds because of the high within-herd prevalence. Our primary objective was to develop a model to predict PVL using common, commercially available, cost-effective diagnostic tests as well as demographic information. The secondary objective was to explore the relationship between PVL and lymphocyte count in the dairy cow population in the Canadian Maritimes.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Inclusion criteria included participation in the ongoing regional BLV surveillance program by twice-yearly collection of bulk tank milk samples and completion of individual adult cow BLV testing, via either blood or individual milk sample ELISA testing. Herds containing at least 30 BLV-positive cows were contacted to determine their interest in participating.

For participating herds, farm visits were arranged for the collection of whole blood samples; individual milk samples were collected in milk sample cups using milk meters by the regional dairy herd improvement (DHI) organization, preserved with 1 BROTAB (Sierra Court, California, USA), and sent to the Maritime Quality Milk (MQM) laboratory after standard components testing was completed. For herds not participating in the DHI program, the farmer collected the individual milk samples in milk sample cups provided by the MQM laboratory containing 1 BROTAB per cup, and samples were shipped to MQM within 2 days of collection. Blood and milk samples were collected from all lactating, BLV-positive cows as well as from 5 to 10 BLV-seronegative cows per farm.

Blood samples were collected into 1 serum and 2 EDTA vacutainer tubes from the coccygeal vein or artery. Two blood smears were made on-farm from the EDTA tube, and all blood tubes were transported chilled to the Atlantic Veterinary College (AVC) and processed within 24 h of collection. All milk antibody testing was completed within 7 days of sample collection.

2.2 | Sample processing

One EDTA tube was submitted to the AVC Diagnostic Services Laboratory (AVCDSL) for automated cell counts using a Sysmex XT 2000i hematology analyzer (Sysmex Canada, Inc., Mississauga, Ontario) as well as a manual differential (CBC). The second EDTA tube underwent DNA extraction using the Qiagen DNEasy blood and tissue kit (Qiagen Inc. Canada, Montreal, Québec). Briefly, 40 μL of proteinase K and 219 μL of buffer AL were added to 200 μL of whole blood in a 1.5 mL Eppendorf tube, which was pulse vortexed 10 times. Tubes were incubated at 56°C for 15 min, then 219 μL of 100% ethanol was added to each tube and pulse vortexed 10 times. Samples were centrifuged at 8000*g* for 5 min after being transferred to spin columns. The collection tubes then were replaced and 500 μL of solution AW1 was added to each tube before centrifuging at 8000*g* for 5 min. Collection tubes were replaced again and 500 μL of solution AW2 was added to each tube before centrifuging at 16300*g* for 10 min. After spin

columns were moved to new 1.5 mL Eppendorf tubes, 40 μL of solution AE was added to each membrane and then centrifuged at 8000*g* for 1 min. The DNA extracted and collected in the Eppendorf tubes was stored at −80°C after quantification until qPCR was performed. The DNA concentration of each sample then was measured by NanoDrop analysis (Thermo Fisher Scientific, Mississauga, Ontario). The DNA samples were used to determine the estimated PVL with the BLV SS1 qPCR Assay protocol provided by CentralStar Cooperative Inc., using an ABI 7500 qPCR instrument (Thermo Fisher Scientific, Mississauga, Ontario) in the AVCDSL. Briefly, 3 μL of each DNA sample was combined in the well of a 96-well plate with 7.25 μL nuclease-free water, 12.5 μL of Prime time 2× master mix, 1.25 μL of BLV SS1 primer 20× master mix, and 1 μL of spike-in recombinant positive amplification control; DNA samples ranged from 12.3 ng/μL to 150.0 ng/μL. The 96-well plate then was heated to 95°C for 3 min to denature the DNA, then underwent 40 cycles of 95°C for 15 s and 60°C for 1 min, before a final 1 min of 60°C. Standard curves for both Bos Actin and BLV DNA were constructed by aliquotting 1 μL of either Bos Actin or BLV standard concentrations into wells of the 96-well plate, using 5 concentrations of each standard. The standard curves were used to estimate the number of copies of Bos Actin and BLV that were amplified for each DNA sample. The estimated PVL of each sample was determined by first dividing the copies of Bos Actin by 2 (because each cell contains 2 copies of the gene) to estimate the number of white blood cell genomes amplified, then dividing the number of copies of the amplified BLV genomes by the estimated number of white blood cell genomes.

The ELISA for anti-gp51 antibodies to BLV was performed on the serum tube and milk samples. Serum tubes were allowed to clot overnight, then centrifuged for 20 min at 1000*g*. Serum was removed and concentrations of anti-gp51 antibodies were determined using a commercial indirect ELISA kit (SVANOVIR ELISA gp51-Ab, Svanova, Uppsala, Sweden) following the manufacturer's instructions. Milk samples also were analyzed using this ELISA kit, following the manufacturer's instructions. This kit has a sensitivity of 100% and specificity of 99.8% for serum samples, and a sensitivity of 100% and specificity of 99.4% for milk samples. The results of the ELISA were reported as percent positivity (PP), which is the optical density at 450 nm of the sample divided by the OD_{450} of the positive control, multiplied by 100:

$$(OD_{corr}\ \text{sample})/(OD_{corr}\ \text{positive control}) \times 100\%.$$

For blood samples, a PP of ≥15 indicates a positive result for BLV, and a PP of <15 indicates a negative result. For individually collected milk samples, a PP of ≥10 indicates a positive result for BLV, and a PP of <10 indicates a negative result. For samples collected using a milk meter, a PP of ≥60 indicates a positive result for BLV, a PP of <10 indicates a negative result, and a PP of 10 to 59 indicates a suspect positive cow because of potential carryover of milk between cows as a result of using the milk meter.[15]

For herds participating in the regional DHI program, results of standard components analysis of the individual milk samples (fat, protein, somatic cell count [SCC], lactose, urea, and beta-hydroxybutyrate [BHB]) were obtained with the producer's permission. Demographic information (breed, age in years, and days in milk for both blood and milk collection dates) also was collected for all cows for which this information was available.

2.3 | Statistical analysis

2.3.1 | Sample size calculations

Sample size was calculated by generating simulated data for total white blood cell count, lymphocyte count, and gp51 ELISA titers based on previous research[14,16-18] as well as proviral status of the simulated cows. These simulated data were used in a logistic regression model. A range of sample sizes was investigated, and the diagnostic sensitivity and specificity for percent correctly classified as having a high or low PVL were generated. Using a sample size of 400 BLV-positive cows and all 3 simulated predictors, >92% of cows (SD, 1.4%) were correctly classified in terms of PVL status with a sensitivity of at least 95% (SD, 1.1%) and specificity of at least 88% (SD, 2.3%).

2.4 | Data analysis

All analyses were carried out using Stata version 16.1 (Statacorp, College Station, Texas, USA). The outcome measure of interest was PVL, which was defined as the relative concentration of viral DNA amplified by qPCR, in blood, per estimated bovine nucleated blood cell. Any BLV-seronegative cows were excluded from the final analysis. Because of a large number of PVL values of 0 in the BLV-positive cows, tobit regression was used for univariable and multivariable analyses, with a lower censoring limit of 0. Herd was included in all models as a random effect. The outcome variable was square-root transformed, as suggested by Box-Cox analysis, in order for the residuals to be approximately normally distributed with constant variance. Because a high amount of collinearity between blood cell parameters was expected, variables were not excluded from analysis despite high levels of correlation between them. All variables were investigated in univariable analyses, and any variables with a *P* value ≤.20 were retained for final model-building. Univariable analyses showed some relationships between predictors and the transformed outcome to be strongly nonlinear, and suitable transformations of predictors were determined by use of fractional polynomials. Those predictors that required transformation then were used for backward elimination model-building to develop a final multivariable model. Of the predictors retained for final model-building, criteria for remaining in the final multivariable model included a *P* value <.05, predictors having a fixed effect, and any predictors showing confounding or interaction effects.

The relationships between estimated PVL and lymphocyte count (determined by manual differential), and between estimated PVL and total white blood cell count (determined by automated cell counts), also were investigated independently of the multivariable

model-building. The BLV-seropositive cows were classified as having either a high or a low PVL at 2 cut-off points: 0.5 copies of viral genome per estimated white blood cell, and 1 copy of viral genome per estimated white blood cell. These cut-off points were assigned arbitrarily because no consensus on what constitutes a high or low PVL has been established in the literature. Cows also were classified as having a normal or an increased lymphocyte count based on the normal reference interval provided by the AVCDSL (high end of normal range = 7.5×10^9 cells/L), and based on a reference interval constructed from the BLV-seronegative cows collected in our study. Briefly, the lymphocyte counts of all BLV-seronegative cows were determined (excluding any cows that seroconverted between blood and milk collection), and the mean and SD of the lymphocyte counts were determined. A reference interval was constructed by determining the lymphocyte values 2 SD above and below the mean. The BLV-positive cows then were classified as having an increased lymphocyte count if their result was higher than the upper limit of the reference interval for the BLV-seronegative cows, which was defined as >5.2×10^9 cells/L for this population of cows. A similar method was used to classify BLV-positive cows as having a normal or an increased total white blood cell count based on the normal reference interval provided by the AVCDSL (high end of normal range is 12.0×10^9 cells/L), and based on a reference interval constructed from the BLV-seronegative cows collected in this study; the high end of this normal range is 10.8×10^9 cells/L. Receiver operator characteristic (ROC) analysis was performed for the 4 combinations of high vs low PVL and high vs low lymphocyte count as well as for the 4 combinations of high vs low PVL and high vs low white blood cell count.

3 | RESULTS

Fifteen dairy herds participated in the study: 8 herds in New Brunswick, 3 herds in Nova Scotia, and 4 herds in Prince Edward Island. A total of 402 cows were enrolled, and the number of cows enrolled per herd ranged from 10 to 57. Blood ELISA testing determined that 339 of the cows were BLV-seropositive and 63 were BLV-seronegative. One BLV-seronegative cow was excluded from analysis because its blood ELISA was BLV-negative but its milk ELISA was BLV-seropositive. All cows were Holstein-Friesians and ranged in age from 2 to 11 years old; age was not recorded for 40 cows from herds not participating in the local DHI program. When blood samples were collected, cows ranged from 1 to 639 recorded days in milk, and when milk samples were collected, cows ranged from 1 to 648 recorded

TABLE 1 Descriptive statistics for bovine leukemia virus-positive cows of blood and milk ELISA tests, components of the complete blood count, standard milk components testing, age, and days in milk (DIM) during blood and milk sample collection

Variable	# of obs.	Mean	Median	SD	Minimum	Interquartile range	Maximum
Blood ELISA value	339	104.40	109.61	23.37	29.64	94.34-119.93	157.29
Milk ELISA value	308	89.78	91.20	20.26	16.02	75.95-103.74	138.17
Total WBC count	339	11.45	9.70	6.08	3.90	7.40-13.60	61.90
Neutrophil count	339	3.82	3.48	1.75	0.76	2.75-4.45	12.31
Band neutrophil count	339	0.04	0.00	0.36	0.00	0.00-0.00	4.93
Eosinophil count	339	0.41	0.29	0.40	0.00	0.16-0.55	3.08
Basophil count	339	0.04	0.00	0.07	0.00	0.00-0.07	0.54
Lymphocyte count	339	6.86	5.13	5.43	1.13	3.36-8.59	56.33
Monocyte count	339	0.28	0.22	0.21	0.00	0.14-0.37	1.54
Neutrophil: lymphocyte ratio	339	0.81	0.64	0.61	0.05	0.41-1.02	4.16
Total plasma protein	179	79.30	79.00	6.39	62.00	75.00-83.00	100.00
Fibrinogen	173	3.38	3.00	1.85	1.00	2.00-4.00	13.00
Milk fat %	232	4.04	4.04	0.96	0.90	3.49-4.54	8.17
Milk protein	232	3.35	3.34	0.41	2.40	3.06-3.58	4.73
Somatic cell count	232	360.71	83.00	1046.04	6.00	32.50-245.00	9999.00
Milk urea	136	6.96	8.50	3.91	1.60	7.25-12.00	21.90
Milk lactose	232	4.50	4.52	0.23	3.41	4.38-4.64	4.97
Milk BHB	204	0.08	0.08	0.04	0.00	0.06-0.10	0.37
Age (years)	304	5.13	5.00	1.88	2.00	4.00-6.00	11.00
Days in milk, blood collection	304	163.54	146.50	136.53	0.00	42.00-262.00	639.00
Days in milk, milk collection	304	174.44	163.00	138.04	0.00	54.50-272.00	648.00

Note: For blood samples, cows with a DIM of 0 were either dry or had given birth the day of blood collection. For milk samples, cows with a DIM of 0 had given birth the day of milk collection.
Abbreviation: ELISA, enzyme-linked immunosorbent assay.

days in milk; this information was not available for 41 cows. Thirteen of the herds participated in the local DHI program; SCC (reported as cells/mL), fat (kg/100 kg milk), protein (kg/100 kg milk), and lactose (kg/100 kg milk) were recorded for 281 cows, milk urea concentration (mg/dL) was recorded for 166 cows, and BHB (mmol/L) was recorded for 247 cows. All blood samples received CBCs with manual white blood cell differentials, but total protein was only available for 239 cows and fibrinogen was only available for 232 cows. Tables 1 and 2 summarize the results for all milk and blood variables; Table 1 presents variables for BLV-seropositive cows and Table 2 variables for BLV-seronegative cows.

With the exception of 1 BLV-seronegative cow that seroconverted during the study period, PVL was determined for the remaining 401 cows. For the 339 BLV-seropositive cows, estimated PVL ranged from 0 to 3.58 viral genomes amplified per bovine white blood cell. Thirty-seven BLV-seropositive cows had a PVL of 0 (Figure 1). Sixty-one of 62 BLV-seronegative cows had a PVL of 0; the estimated PVL of the 1 BLV-seronegative cow with a non-zero PVL was 0.0023 viral genomes amplified per bovine white blood cell.

Univariable analysis of linear relationships with all blood and milk variables (Table 3) resulted in 9 variables that had a *P* value ≤.20; these were retained for multivariable model-building. Model reductions from a multivariable model with the selected predictors at their most suitable scale resulted in only 2 predictors being retained: milk ELISA PP, and lymphocyte count represented by its inverse and

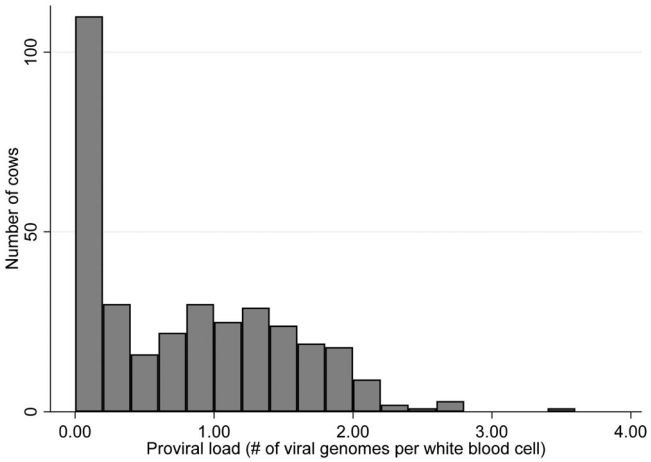

FIGURE 1 Histogram of proviral load values of 339 bovine leukemia virus-positive cows determined by real-time polymerase chain reaction.

TABLE 2 Descriptive statistics for bovine leukemia virus-negative cows of blood and milk ELISA tests, components of the complete blood count, standard milk components testing, age, and days in milk during blood and milk sample collection

Variable	# of obs.	Mean	Median	SD	Minimum	Interquartile range	Maximum
Blood ELISA value	62	0.99	0.28	1.97	−2.16	0.00-0.91	8.20
Milk ELISA value	60	5.92	2.42	8.04	0.00	0.53-5.45	29.73
Total WBC count	62	7.16	7.12	1.83	4.70	5.94-7.89	16.44
Neutrophil count	62	3.15	2.94	1.49	0.11	2.26-3.65	10.03
Band neutrophil count	62	0.01	0.00	0.07	0.00	0.00-0.00	0.57
Eosinophil count	62	0.36	0.29	0.28	0.00	0.15-0.52	1.24
Basophil count	62	0.02	0.00	0.04	0.00	0.00-0.00	0.15
Lymphocyte count	62	3.24	3.06	0.99	1.55	2.52-3.78	6.78
Monocyte count	62	0.32	0.29	0.17	0.00	0.23-0.44	0.74
Neutrophil: lymphocyte ratio	62	1.06	0.98	0.57	0.02	0.62-1.41	2.96
Total plasma protein	60	78.30	79.00	4.56	66.00	75.5-81.00	87.00
Fibrinogen	59	3.10	3.00	1.42	0.00	2.00-4.00	7.00
Milk fat %	49	3.89	3.68	0.86	2.28	3.27-4.56	5.51
Milk protein	49	3.35	3.37	0.42	2.52	3.03-3.63	4.52
Somatic cell count	49	146.69	58.00	283.88	8.00	21.00-131.00	1379.00
Milk urea	30	11.13	9.50	4.69	4.30	7.60-13.20	23.50
Milk lactose	49	4.59	4.59	0.19	3.86	4.53-4.71	4.94
Milk BHB	43	0.07	0.08	0.03	0.01	0.05-0.10	0.14
Age (years)	57	4.14	4.00	1.47	2.00	3.00-5.00	7.00
Days in milk, blood collection	56	173.20	151.00	123.57	0.00	73.50-264.50	512.00
Days in milk, milk collection	56	187.46	157.50	124.10	1.00	83.50-283.00	521.00

Note: For blood samples, cows with a days in milk of 0 were either dry or had given birth the day of blood collection. For milk samples, cows with a days in milk of 0 had given birth the day of milk collection.
Abbreviation: ELISA, enzyme-linked immunosorbent assay.

TABLE 3 Variables after unconditional tobit regression (lower limit of 0), where the outcome variable of estimated proviral load was square-root transformed and where herd was included as a random effect

Variable	# of obs.	Coefficient	95% CI	*P*-value
Blood ELISA value	339	0.002	0.004 to 0.011	<.01
Milk ELISA value	308	0.002	0.010 to 0.016	<.01
Total white blood cell count	339	0.004	0.049 to 0.063	<.01
Neutrophil count	339	0.017	0.013 to 0.080	.01
Band neutrophil count	339	−0.091	−0.248 to 0.066	.26
Eosinophil count	339	0.095	−0.050 to 0.239	.20
Basophil count	339	0.552	−0.246 to 1.349	.18
Lymphocyte count	339	0.063	0.055 to 0.072	<.01
Monocyte count	339	0.451	0.180 to 0.722	<.01
Neutrophil: lymphocyte ratio	339	−0.497	−0.576 to −0.417	<.01
Total plasma protein	179	−0.001	−0.014 to 0.012	.92
Fibrinogen	173	0.012	−0.036 to 0.059	.63
Milk fat %	232	−0.016	−0.089 to 0.057	.67
Milk protein	232	−0.048	−0.214 to 0.118	.57
Somatic cell count	232	−0.00004	−0.0001 to 0.00003	.28
Milk urea	136	−0.014	−0.037 to 0.009	.23
Milk lactose	232	−0.008	−0.304 to 0.287	.96
Milk β-hydroxybutyrate	204	−0.663	−2.551 to 1.225	.49
Age (years)	304	0.015	−0.019 to 0.048	.39
Days in milk, blood collection	304	−0.0005	−0.0005 to 0.0004	.82
Days in milk, milk collection	304	−0.0005	−0.0005 to 0.0004	.81

Note: All variables were untransformed for initial unconditional tobit regression. Variables with an unconditional *P*-value of ≤.2 were retained for multivariable model-building.
Abbreviation: ELISA, enzyme-linked immunosorbent assay.

TABLE 4 Results of multivariable mixed-effects tobit regression (lower limit of 0), with herd included as a random effect

Variable	Coefficient	95% CI	*P*-value
Milk ELISA value[a]	4.946	3.280 to 6.612	<.01
Inverse lymphocyte count	−6.168	−6.812 to −5.524	<.01
Square of inverse lymphocyte count	5.634	4.623 to 6.643	<.01
Intercept	1.264	1.061 to 1.467	
Herd-level variance	0.013	0.005 to 0.034	
Cow-level variance	0.061	0.051 to 0.072	

Note: The outcome variable of proviral load (number of bovine leukemia virus genomes amplified per nucleated white blood cell) was square-root transformed for analysis. All coefficients and confidence intervals are presented without back-transformation.
Abbreviation: ELISA, enzyme-linked immunosorbent assay.
[a]Numerical values for the milk ELISA coefficient and 95% confidence interval have been multiplied by 1000 to allow for display of more significant digits.

squared inverse terms (Table 4). The major assumptions of independence, heteroscedasticity, normality, and linearity were statistically examined and found to be met. The Pearson correlation coefficient between observed and predicted PVL values was 0.86, indicating good predictive ability.

The estimates in Table 4 show a positive association between estimated PVL and the milk ELISA PP, as well as a more complex relationship with lymphocyte counts, which is illustrated in the predicted values of PVL based on the multivariable model in Figure 2. Overall, values for estimated PVL increase as the lymphocyte count increases. The exception is that the estimated PVL decreases as lymphocyte count increases when the lymphocyte count is $<2.1 \times 10^9$/L.

Figure 3 presents the lymphocyte counts of BLV-positive cows plotted against estimated PVL, and Table 5 the sensitivity and specificity of using different cut-offs for lymphocyte count to classify BLV-seropositive cows as having high or low PVL, at 2 different definitions of high PVL. Using a cut-off of 1.0 viral genome amplified per bovine white blood cell to define a cow with high PVL and a lymphocyte count of $>5.2 \times 10^9$ cells/L to define a high lymphocyte count had the highest sensitivity (92.4%) and the lowest specificity (79.8%)

whereas using cut-offs of 0.5 viral genomes amplified per bovine white blood cell for high PVL and >7.5 × 10^9 cells/L for lymphocyte count had the lowest sensitivity (57.4%) and highest specificity (99.3%).

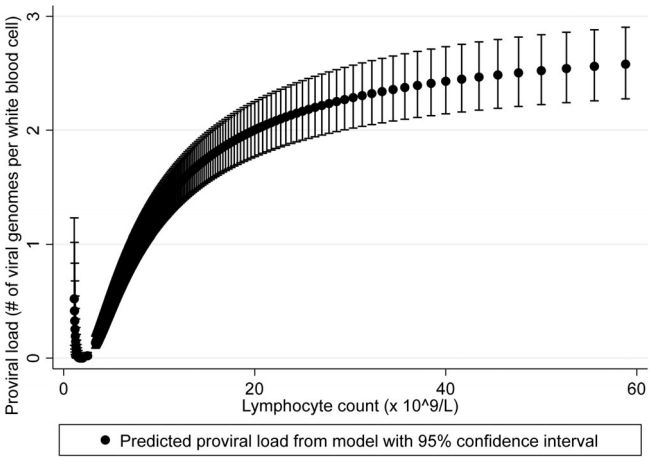

FIGURE 2 Predictions of proviral load from multivariable mixed-effects tobit regression model using the predictor variables of milk enzyme-linked immunosorbent assay (ELISA) value and blood lymphocyte count, where lymphocyte count was transformed by taking its inverse and including its quadratic term and where proviral load was square-root transformed for analysis. Graph shows predictions after back-transforming all variables. A fixed value of percent positivity of 90 was used for the milk ELISA variable.

Figure 4 presents total white blood cell count of BLV-positive cows plotted against estimated PVL, and Table 6 the sensitivity and specificity of using different cut-offs for white blood cell count to classify BLV-seropositive cows as having high or low PVL, at 2 different definitions of high PVL. Using a cut-off of 1.0 viral genome amplified per bovine white blood cell to define a cow with high PVL and a white blood cell count of >10.8 × 10^9 cells/L to define a high white blood cell count had the highest sensitivity (85.5%) and lowest specificity (83.6%) whereas using cut-offs of 0.5 viral genomes amplified per bovine white blood cell for high PVL and >12.0 × 10^9 cells/L for white blood cell count had the lowest sensitivity (58.4%) and highest specificity (96.0%).

Receiver operator characteristic analysis showed that for both cut-points for high vs low PVL, using lymphocyte count resulted in a higher area under the curve (AUC) compared to using the white blood cell count, indicating that lymphocyte count has higher predictive ability for whether a cow has high or a low PVL. When using a cut-point of 0.5 viral genomes amplified per bovine white blood cell to classify a cow as having high PVL, the AUC for lymphocyte count was 0.956 whereas it was 0.913 for white blood cell count. Similarly, when using a cut-point of 1.0 viral genome amplified per bovine white blood cell to classify a cow as having high PVL, the AUC for lymphocyte count was 0.939 whereas it was 0.914 for white blood cell count.

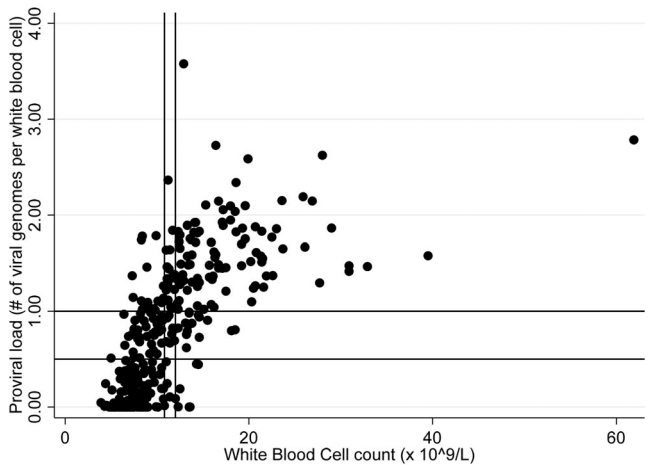

FIGURE 3 Proviral load (PVL) of bovine leukemia virus-positive cows determined by quantitative polymerase chain reaction plotted against blood lymphocyte count. Horizontal reference lines represent 2 cut-offs for cows with high vs low PVL (cut-offs of 0.5 and 1.0). Vertical reference lines represent 2 cut-offs for cows with increased vs normal lymphocyte counts (cut-offs of 5.2 × 10^9/L and 7.5 × 10^9/L).

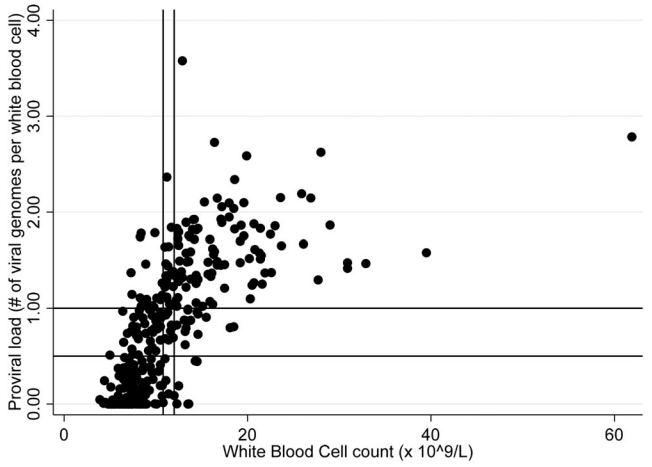

FIGURE 4 Proviral load (PVL) of bovine leukemia virus-positive cows determined by quantitative polymerase chain reaction plotted against white blood cell count. Horizontal reference lines represent 2 cut-offs for cows with high vs low PVL (cut-offs of 0.5 and 1.0). Vertical reference lines represent 2 cut-offs for cows with increased vs normal white blood cell counts (cut-offs of 10.8 × 10^9/L and 12.0 × 10^9/L).

TABLE 5 Sensitivity and specificity of using 2 different cut-offs for defining an increased blood lymphocyte count to predict whether a cow has a high or low proviral load, also using 2 different cut-offs

	Lymphocytes >5.2 × 10^9/L		Lymphocytes >7.5 × 10^9/L	
	Sensitivity	Specificity	Sensitivity	Specificity
High PVL = PVL >0.5	80.5%	93.3%	57.4%	99.3%
High PVL = PVL >1.0	92.4%	79.8%	71.8%	92.3%

	WBC >10.8 × 10^9/L		WBC >12.0 × 10^9/L	
	Sensitivity	Specificity	Sensitivity	Specificity
High PVL = PVL >0.5	71.6%	93.3%	58.4%	96.0%
High PVL = PVL >1.0	85.5%	83.6%	72.5%	89.4%

TABLE 6 Sensitivity and specificity of using 2 different cut-offs for defining an increased total white blood cell (WBC) count to predict whether a cow has a high or low proviral load, also using 2 different cut-offs

4 | DISCUSSION

The final multivariable model identified 1 parameter measured on a CBC and 1 parameter measured on a milk sample that were associated with increasing estimated PVL in BLV-positive cows. We expected that increasing lymphocyte count would be positively associated with higher PVL, because previous literature has found up to 30% of BLV-infected cows develop an increased lymphocyte count over the course of their lifetime.[1] Additionally, another study[14] found that the majority of cows with high PVL had lymphocytosis. Indeed, a higher proportion of BLV-infected cows may develop an increased lymphocyte count than previously thought, because many of the older lymphocyte reference intervals were constructed without knowing the BLV status of the contributing animals.[19-21]

Interestingly, however, the estimated PVL initially decreased with low values for lymphocyte count before increasing again. A possible explanation for this finding is that, when cows are initially infected with BLV, the virus preferentially integrates into the host lymphocyte genome near transcriptionally active sites.[22] Within a relatively short period of time after infection, the lymphocytes with BLV integrated near transcriptionally active sites are preferentially removed by the immune system.[2,22] This typically occurs much earlier in the disease process than the development of persistent lymphocytosis.[1] It is possible that these cows with low-normal lymphocyte counts are in an early stage of BLV infection where the immune system is in the process of removing the lymphocytes with BLV integrated near transcriptionally active genetic material, and so they have higher PVL in relation to lymphocyte count than cows more advanced in the disease process. However, because only 5% of sampled cows are included in the population of cows with low lymphocyte counts (ie, <2.1 × 10^9/L) it is possible these results represent random fluctuation and are not representative of the population as a whole.

No other parameters measured on the CBC were significantly associated with increasing PVL in BLV-positive cows. This observation could be a result of BLV-induced alterations to the immune system having little effect on the kinetics of these cell populations. Additionally, the results that were obtained in our study for all of the other parameters measured on the CBC were mostly consistent with healthy animals and so likely would not have much impact in predicting BLV status in an otherwise healthy cow.

The milk ELISA PP was also positively associated with increasing estimated PVL. This observation is consistent with findings in other studies performed in different populations of Holstein cattle.[12,14] Although it did not remain significant in the final multivariable model, blood ELISA PP was also positively associated with increasing estimated PVL in univariable analyses and would likely be a reasonable alternative for estimating PVL if a milk sample was not available (eg, a non-lactating cow).

Our secondary objective was to investigate the relationship solely between lymphocyte count and estimated PVL in BLV-seropositive cows. This relationship has been reported in other studies,[14,16,23,24] but has not been investigated in Canada to date. Depending on the cut-off used to define a cow as having high estimated PVL, and the cut-off used to determine a normal lymphocyte count in dairy cows, lymphocyte count alone can be a sensitive parameter to identify BLV-seropositive cows with a high PVL. We obtained the best sensitivity (92.4%) with a lymphocyte cut-off of >5.2 × 10^9/L and a PVL of >1 viral genome amplified per bovine white blood cell. The specificity of this combination was also robust (79.8%). In the case of PVL, it is arguably more important to maximize sensitivity. Doing so would decrease the number of false negative cows with high PVL that remain in the herd, which might prevent effective disease-control strategies and also decrease further screening with a more expensive test, such as quantitative PCR.

The relationship solely between total white blood cell count and estimated PVL also was investigated, because in certain situations this parameter may be more available than lymphocyte count. Depending on the cut-off used to define a cow as having a high estimated PVL, and the cut-off used to determine a normal white blood cell count in dairy cows, white blood cell count alone also can be a sensitive parameter to identify BLV-seropositive cows with high PVL, although it did not perform as well as lymphocyte count. We obtained the best sensitivity (85.5%) with a white blood cell count cut-off of >10.8 × 10^9/L and PVL of >1 viral genome amplified per bovine white blood cell. The specificity of this combination was also relatively robust (71.6%), although both sensitivity and specificity were lower than when using lymphocyte count rather than total white blood cell count. This finding is unsurprising, because the total white blood cell count contains lymphocytes as well as up to 5 additional cell populations (segmented neutrophils, band neutrophils, eosinophils, basophils, and monocytes) that do not contain substantial amounts of BLV genetic material.

Currently, beause no consensus exists on how to define or report "high PVL" for BLV-infected dairy cows, 2 cut-offs were decided on to define high PVL cows: either a ratio of BLV:Bovine DNA of 0.5 or a ratio of BLV:Bovine DNA of 1. Both of these numbers represent high amounts of viral DNA circulating in the bloodstream. Unfortunately, no published studies suggest the cut-off at which a BLV-infected cow becomes an infection risk to BLV-uninfected cows. Thus, the infective potential of a "high PVL" cow is only conjecture. One study used a simple ranking system of highest to lowest PVL when communicating results to producers to help prioritize removal of the highest-risk

cows.[25] However, in situations where within-herd prevalence is low (eg, 5%-10%) and the producer has the ability to remove multiple cows at once, a cut-off for infectivity risk may be more helpful than a ranked list.

We also used 2 cut-offs to determine the upper limit of the reference interval for lymphocyte and total white blood cell counts. The cut-off of 7.5×10^9 cells/L was used for lymphocyte count because it is the current upper limit of the reference interval used by the AVCDSL and is reported on CBC results from the laboratory. However, this limit is based on a reference interval established in 1961 from dairy cows in California[20] and thus does not reflect the current genetics of dairy cows in Canada. The cut-off of 5.2×10^9 cells/L for lymphocyte count is the upper end of a reference interval constructed from the BLV-seronegative cows collected in our study, and thus directly reflects the current dairy cow population in the Canadian Maritimes, and specifically the farms enrolled in our study. A previous study[18] found a similar lymphocyte count in BLV-negative dairy cows in Michigan as we found in our study. Similarly, the cut-offs of 12.0×10^9 cells/L and 10.8×10^9 cells/L for total white blood cell count came from the current reference interval used by the ACVDSL and from a reference interval constructed from the BLV-seronegative cows collected in our study, respectively. Ideally, at least 120 animals are used to construct a reference interval for a hematological parameter, but when modifying an existing reference interval, a smaller number is acceptable.[26] The 62 BLV-seronegative cows used in our study therefore represent an acceptable number to use to modify the existing AVCDSL reference intervals for lymphocyte and total white blood cell counts.

Our study had some limitations. Although the total number of cows recruited was higher than the calculated sample size, only 339 of the 401 cows were BLV-positive. This may have had an impact on the ability of the statistical analysis to identify all variables associated with increasing PVL. Additionally, only Holstein cows were included in our study. Although Holsteins account for approximately 94% of dairy cows in Canada and thus the results of our study can be applied to the majority of dairy cows in the region, it is unknown if these results would apply to other common dairy breeds.[27]

Another limitation was the number of cows sampled on each farm. Initially, herds that had >30 BLV-positive cows were contacted for participation in the study. However, for some herds up to 2 years had elapsed between the initial individual cow testing and the sample collection for our study. This situation resulted in a significant change in the population of cows in some herds, and in some cases a marked decrease in the number of BLV-positive cows sampled compared to the anticipated number present on each farm (eg, 1 farm culled 15 of 42 BLV-positive cows between individual cow testing and PVL sample collection, and an additional 6 were not lactating at the time of sample collection). Also, when the randomly sampled BLV-seronegative cows were re-tested by blood ELISA, a number of these cows had seroconverted during the time interval between the initial and PVL samplings. This situation increased the number of BLV-positive cows sampled per farm in some cases. Anecdotally, some of the producers noted that a higher proportion of BLV-positive cows had been culled than BLV-seronegative cows.

It was not always possible to coordinate blood and milk sample collections, and in some cases up to 49 days elapsed between blood collection and milk collection. This delay could have affected the relationship between the blood and milk ELISA results. In 1 case, a cow had to be excluded from analysis because it was BLV-seronegative at the time of blood collection, but BLV ELISA-positive at the time of milk collection 49 days later. A relationship between milk components and PVL may have been present if the blood and milk samples had been collected on the same day.

The individual cows' BLV status was determined by blood ELISA for anti-gp51 antibodies. This assay is 1 of the recommended methods for determining BLV status by World Organization for Animal Health guidelines,[28] and the test has excellent sensitivity and specificity when compared to AGID.[29,30] Although misclassification of BLV status is possible, the accuracy of the test makes it unlikely that cows were misclassified as BLV-seronegative if they were truly BLV-positive, and vice versa.

Either the multivariable model or the use of lymphocyte count alone can provide valuable information to a producer, while also remaining cost-effective. The results of the multivariable model can be used to estimate the PVL of an individual cow as well as to rank cows based on their PVL. Doing so may be helpful for producers with high within-herd prevalence who want to minimize new BLV infections, but are not able to cull all BLV-infected cows at once. They can create a ranked list of cows based on estimated PVL and remove those with the suspected highest infection risk.

For herds with low within-herd prevalence, or those producers who do not wish to perform blood or milk ELISA testing to determine BLV status, using a lymphocyte count of $>5.2 \times 10^9$ cells/L will identify >90% of the BLV-seropositive cows with high PVL. They will not be able to discriminate cows with the highest PVL from those with lower PVL, but if only a small percentage of cows are BLV-infected, the producer may be able to cull all of the predicted high-PVL cows at once, with little overall financial impact on the farm. Ideally, the PVL PCR eventually will become commercially available in Canada, but our results show that a good prediction of PVL can be obtained from common diagnostic tests, namely CBC and milk ELISA for anti-BLV antibodies.

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CONFLICT OF INTEREST DECLARATION

Casey Droscha developed the BLV SS1 qPCR Assay used in this study to measure BLV proviral DNA and is employed by CentralStar Cooperative, Inc. who offers the BLV SS1 qPCR Assay as a commercial test. No other authors declare a conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All procedures used for blood collection and animal handling were reviewed and approved by the University of Prince Edward Island Animal Care Committee (protocol 18-029).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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