

Evaluation of total white blood cell count as a marker for proviral load of bovine leukemia virus in dairy cattle from herds with a high seroprevalence of antibodies against bovine leukemia virus

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Objective—To determine the reference interval for WBC counts in Holstein dairy cows from herds with high seroprevalence for anti-bovine leukemia virus (BLV) antibodies, analyze the correlation of total WBC counts and blood proviral load (bPVL) in BLV-infected animals, and determine whether total WBC count can be used a hematologic marker for in vivo infection.

Animals—307 lactating cows from 16 dairy herds with high BLV seroprevalence.

Procedures—Blood samples were collected for assessment of plasma anti-BLV p24 antibody concentration (all cows), manual determination of WBC count (161 BLV-seronegative cows from 15 herds), and evaluation of bPVL (146 cows from another herd).

Results—The WBC count reference interval (ie, mean \pm 2 SD) for BLV-seronegative dairy cows was 2,153 to 11,493 cells/ μ L. Of the 146 cows used to analyze the correlation between WBC count and bPVL, 107 (73%) had WBC counts within the reference interval; of those cows, only 21 (19.6%) had high bPVL. Most cows with high WBC counts (35/39) had high bPVL. Mean WBC count for cows with high bPVL was significantly higher than values for cows with low or undetectable bPVL. White blood cell counts and bPVL were significantly ($\rho = 0.71$) correlated.

Conclusions and Clinical Relevance—These data have provided an updated reference interval for WBC counts in Holstein cows from herds with high BLV seroprevalence. In dairy cattle under natural conditions, WBC count was correlated with bPVL; thus, WBC count determination could be a potential tool for monitoring BLV infection levels in attempts to control transmission. (*Am J Vet Res* 2013;74:744–749)

Bovine leukemia virus is among the most widespread livestock pathogens in many countries, especially in dairy herds. Most infected animals (60% to 70%) are subclinically affected, having no clinical or hematologic signs, and are designated as aleukemic. A smaller proportion of infected cattle (30% to 40%) develop persistent lymphocytosis, and although they have no clinical signs, these animals develop hematologic alterations characterized by a chronic increase in the absolute number of circulating B lymphocytes.¹ A third and much smaller group of infected cattle (1% to 10%) develop lymphosarcoma or localized lymphoid tumors in various organs.² Cattle with persistent lymphocytosis have high absolute lymphocyte counts, and 25%

ABBREVIATIONS

BLV	Bovine leukemia virus
bPVL	Blood proviral load
ROC	Receiver operating characteristic

to 35% of the circulating lymphocytes have integrated proviral DNA, compared with approximately 5% of the circulating lymphocytes in aleukemic cows.³ In animals with persistent lymphocytosis, leukocytosis is the consequence of an increase in the absolute number of total WBCs. Hence, WBC count could be an indirect marker of the in vivo bPVL and a surrogate marker of the risk of BLV transmission. This assumption is supported by results of previous studies,^{4,5,a} which indicate that the development of persistent lymphocytosis is an important risk factor for the horizontal and vertical transmission of BLV.

The purpose of the study reported here was to calculate the reference interval for WBC count in lactating Holstein cows from herds with high seroprevalence for anti-BLV antibodies in Argentina and to analyze the correlation of total WBC counts and bPVL in naturally infected cattle. The intent was to determine whether

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WBC count could be used as a hematologic marker to estimate the level of in vivo infection and, indirectly, the potential of transmission in dairy herds with high BLV infection rates.

Materials and Methods

Animals and blood samples—All the cows used in the study from highly BLV-infected herds were in their first to eighth parity and lactating and were brought to the laboratory for analysis of farm BLV seroprevalence. Owner consent to use the collected blood samples for study purposes was obtained. Blood samples were collected via jugular venipuncture. For each cow, the parity and stage of lactation (in thirds: 1 [day 1 to 101 of lactation], 2 [day 102 to 203 of lactation], 3 [day 204 to 305 of lactation], or > 3 [\geq day 306 of lactation]) were recorded.

To define the reference interval for WBC counts, a blood sample (5 mL) was collected from each of 161 BLV-seronegative cows from 15 dairy herds for analysis. To determine the correlation between total WBC counts and bPVL, a blood sample (5 mL) was collected from each of 146 cows on a 16th farm for analysis.

Fresh whole blood was collected into tubes containing heparin and immediately used to assess total WBC counts in all samples ($n = 307$). Plasma and whole blood samples were kept frozen until analyzed.

Total WBC count—Fresh blood was diluted 1:20 in a 1% gentian violet solution in 2% acetic acid. For each of the 307 samples, a manual WBC count was performed with a hemocytometer.

Plasma anti-BLV antibody testing—Plasma antibodies against BLV p24 were detected with the p24 ELISA test, as has been reported.⁶ For each of the 307 samples, normalized results (reactivity) were expressed as a sample-to-positive ratio on the basis of the mean values of replicate wells, in reference to an internal weak positive control and an internal negative control. The antibody titers were assayed by means of 2-fold dilutions of sera.

BLV proviral load quantification in blood samples—Total DNA was extracted from whole blood with a commercial kit, according to the manufacturer's instructions.^b The relative quantification of the bPVL was assessed by real-time PCR assay, as previously described.⁷ The BLV MGBF and BLV MGBR primers⁸ were used to amplify a fragment of the BLV pol gene together with a 3' minor groove binder probe (6-carboxyfluorescein) by use of 50 ng of DNA as template. A fragment of the 18S cellular gene was amplified in parallel as a reference to correct for differences among samples. As an internal control and calibrator sample for both amplifications, 50 ng of DNA from fetal lamb kidney cells infected with BLV, containing 4 copies of BLV proviral DNA/cell, at a final concentration of 1% in peripheral blood mononuclear cells purified from a noninfected cow was used. The relative bPVL was expressed as the ratio of results obtained for the sample for the BLV pol gene against the 18S reference gene, according to the equation described by Pfaffl.⁹ The reaction had a detection limit of 1 BLV-infected cell in 5,000 noninfected

cells (0.02% of infected cells). With this method, the relative bPVL of the control sample was set to 1 and all samples were referenced against it. The bPVL was defined as undetectable when a cycle threshold value was not obtained from the BLV pol-specific reaction (< 0.02% infected cells), low when the ratio obtained was < 1 (0.02% to 1% infected cells), and high when the ratio obtained was ≥ 1 (> 1% infected cells).

Statistical analysis—The reference interval for WBC counts was developed with statistical methods.¹⁰ Outliers between WBC counts were detected by the Grubb test performed with software available online.^c The WBC counts were tested for normal (Gaussian) distribution via the Kolmogorov-Smirnov method, and the mean ± 2 SD was used to define the reference interval. Descriptive summary statistics were expressed by means of box-and-whisker plots. The Kruskal-Wallis test was used to evaluate differences among categories for parity, stage of lactation, and bPVL. Correlations between bPVL and WBC counts or serum anti-BLV p24 antibody titers were examined with the Spearman rank order statistic. Receiver operating characteristic curves were constructed to analyze the predictive potential of serum anti-BLV p24 antibody titers to discriminate between cows with high and low or undetectable bPVL and to select the optimal predictive variable. The ROC analyses were performed with software available online.^d The ROC curve was used to predict the WBC count that would differentiate cows with high bPVL from those with low or undetectable bPVL. In that case, sensitivity was considered as the proportion of cows with high PVL that were correctly identified and specificity was considered as the proportion of aleukemic cows that were correctly identified. Values of $P < 0.05$ were considered significant. Statistical analysis was performed with commercially available software.^e

Table 1—Population of 161 lactating Holstein cows from 15 herds with high seroprevalence for BLV from which collected blood samples were analyzed to calculate a reference interval for WBC count.

Herd	No. of animals used to determine WBC count reference interval	Farm BLV seroprevalence (%)	No. of animals tested to determine farm BLV seroprevalence
1	6	93.0	86
2	22	72.5	80
3	6	89.6	58
4	3	98.1	158
5	9	94.3	140
6	3	97.0	100
7	5	97.1	174
8	5	96.3	137
9	26	81.2	138
10	30	75.6	123
11	21	82.6	121
12	7	90.0	70
13	9	92.2	115
14	4	96.4	112
15	5	95.0	100

All cows used for WBC count reference interval determination were seronegative for BLV. Serologic testing was performed with an ELISA.⁶ Data for 5 cows were excluded from the analysis because the values were considered outliers.

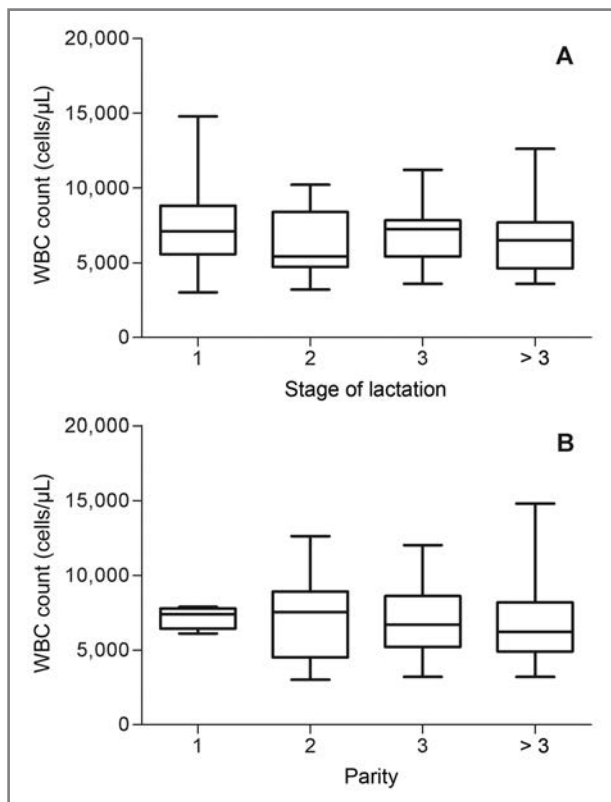


Figure 1—Box-and-whisker plots of WBC counts in 156 lactating Holstein cows (seronegative for BLV) from 15 herds with high seroprevalence for anti-BLV antibodies classified by stage of lactation (in thirds: 1 [day 1 to 101 of lactation], 2 [day 102 to 203 of lactation], 3 [day 204 to 305 of lactation], or > 3 [\geq day 306 of lactation]; A) and parity (B). Data for 5 cows were excluded from the analysis because the values were considered outliers. For each box, the horizontal line represents the median and the upper and lower boundaries represent the upper and lower quartiles, respectively. Whiskers represent the minimum and maximum values. There was no significant ($P > 0.1$) difference in mean WBC count among stage of lactation or parity.

Results

Reference interval for WBC count in lactating cows from herds with high seroprevalence for BLV—As a population to define the reference interval for WBC count in lactating cows from herds with high seroprevalence for BLV, 161 cows from 15 farms were selected (Table 1). All of those cows were negative for plasma anti-BLV p24 antibodies, as determined via ELISA. Five cows were excluded from the analysis because their WBC counts were considered outliers. The WBC counts of the remaining 156 animals had Gaussian distribution ($P > 0.1$). The mean \pm SD lactation stage and parity of the 156 cows were 2.404 ± 1.581 and 3.583 ± 1.324 , respectively. The mean WBC counts among cows in various stages of lactation or among cows with various parity were not significantly different (Figure 1). The mean WBC count for a dairy cow was calculated as $6,823 \pm 2,335$ WBCs/ μ L, giving a reference interval of 2,153 to 11,493 WBCs/ μ L. For study purposes, counts $> 11,500$ WBCs/ μ L of blood were considered high WBC counts and counts $< 2,100$ WBCs/ μ L of blood were considered low WBC counts.

Table 2—Distribution of BLV bPVLs and WBC counts in 146 lactating Holstein cows from an additional herd (farm 16) with high seroprevalence for anti-BLV antibodies.

WBC	bPVL			Total
	Undetectable	Low	High	
Normal	54	32	21	107 (73.3%)
High	0	4	35	39 (26.7%)
Total	54	36	56	146 (100%)

On the basis of the WBC count reference interval determined from data collected from 156 lactating Holstein cows (seronegative for BLV) from 15 herds with high BLV seroprevalence, WBC counts $> 11,500$ WBCs/ μ L of blood were considered high, WBC counts $< 2,100$ WBCs/ μ L of blood were considered low, and WBC counts 2,100 to 11,500 WBCs/ μ L of blood were considered normal. Blood PVLs were categorized as undetectable (ie, $< 0.02\%$ infected cells), low (ie, 0.02% to 1% infected cells), or high (ie, $> 1\%$ infected cells).

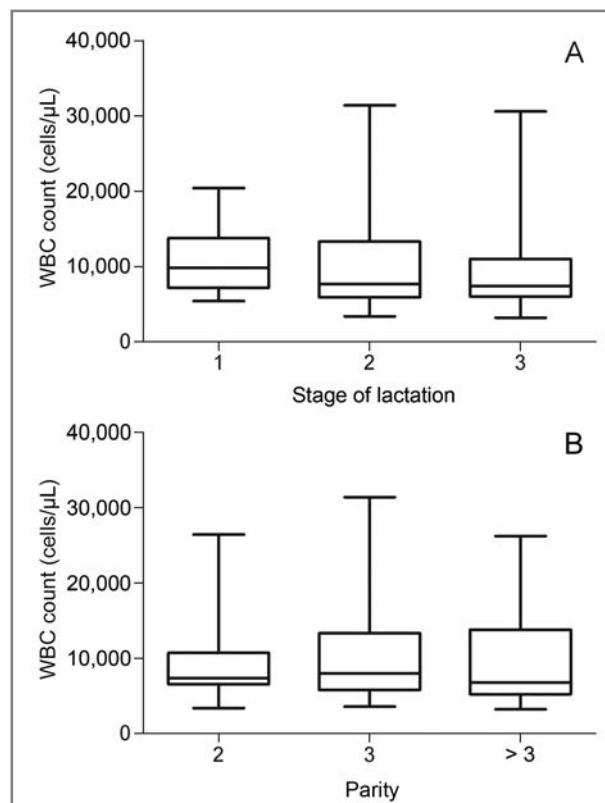


Figure 2—Box-and-whisker plots of WBC counts in 146 lactating Holstein cows from a herd (farm 16) with high seroprevalence for anti-BLV antibodies classified by stage of lactation (A) and parity (B). The mean \pm SD stage of lactation and number of lactations for the 146 cows were 2.603 ± 0.581 and 2.849 ± 1.122 , respectively. See Figure 1 for key.

Correlation of total WBC counts and bPVL in cows from a herd with high seroprevalence for BLV—In the 146 animals tested on the 16th farm, where the correlation between bPVL and WBC count was studied, the seroprevalence was 87.7%, as determined by ELISA. In these 146 cows, the mean WBC count did not differ significantly with regard to stage of lactation or parity (Figure 2). Blood proviral load distribution and total WBC counts among the 146 cows were summarized (Table 2). Of those cows, 107 had WBC counts within the calculated refer-

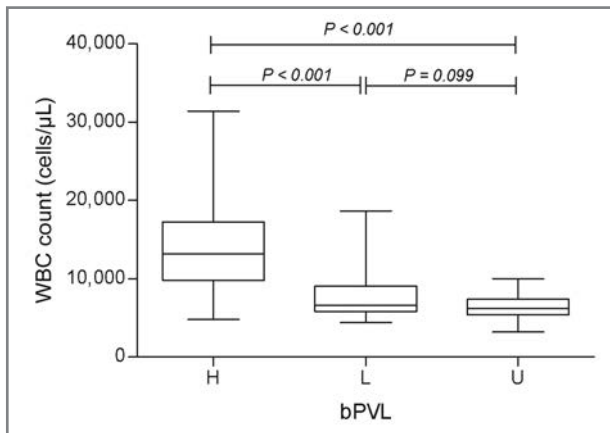


Figure 3—Box-and-whisker plots of WBC counts in 146 lactating Holstein cows from the herd of farm 16 with high seroprevalence for anti-BLV antibodies classified by bPVL category (high [ie, > 1% infected cells; H], low [ie, 0.02% to 1% infected cells; L], or undetectable [ie, < 0.02% infected cells; U]). See Figure 1 for key.

ence interval, only 21 (19.6%) of which had a high bPVL. Most (89.7%) of the cows with a high WBC count had a high bPVL, whereas all 54 cows with undetectable bPVL had WBC counts within the calculated reference interval. Also, 88.8% and 37.5% of cows with a low or high bPVL, respectively, had WBC counts within the calculated reference interval. The mean WBC count was significantly ($P < 0.001$) higher among the cows with high bPVL, compared with the value among cows with low bPVL or cows with undetectable bPVL (Figure 3). Eighteen of the 146 (12.3%) cows were found to be negative for serum anti-BLV p24 antibodies as determined via ELISA; all of those cows had WBC counts within the calculated reference interval and undetectable ($n = 13$) or low (5) bPVL.

Analysis of data from the 146 cows revealed a significant correlation between the WBC count and bPVL (Table 3). A similar correlation was identified between bPVL and serum anti-BLV p24 antibody titer ($\rho = 0.71$; $P < 0.001$) or reactivity ($\rho = 0.71$; $P < 0.001$). The correlation coefficients for bPVL and each lactation stage were similar; however, the correlation coefficients for bPVL and number of lactations ≥ 3 were higher than values for bPVL and parities of 2 or 3. The distribution of high, low, and undetectable bPVLs with regard to WBC count was assessed (Figure 4). The number of cows with undetectable or low bPVL decreased as the WBC count increased. Among the cows with WBC counts $\geq 11,500$ WBCs/ μL , none had an undetectable bPVL and only 4 had a low bPVL. An ROC analysis was performed with WBC counts and only 2 categories of bPVL: undetectable to low bPVL and high bPVL, reflecting the aleukemic and lymphocytotic stages of BLV infection, respectively (Figure 5). The ROC curves suggested that a value of 13,400 WBC/ μL of blood was the best cutoff to differentiate aleukemic from nonaleukemic animals, with 86.6% sensitivity and 80.35% specificity. The area under the curve was similar for WBC counts (0.911) and anti-BLV p24 antibody reactivity (0.855).

Table 3—Correlation of WBC count with bPVL determined from data obtained from 146 lactating Holstein cows from the additional herd with high seroprevalence for anti-BLV antibodies on the basis of stage of lactation (in thirds: 1 [day 1 to 101 of lactation], 2 [day 102 to 203 of lactation], 3 [day 204 to 305 of lactation], or > 3 [\geq day 306 of lactation]) and number of lactations.

Variable	Spearman rank order correlation coefficient (r)	P value	No. of cows
Total correlation	0.7055	< 0.001	146
Parity			
2	0.6994	< 0.001	74
3	0.7192	< 0.001	41
> 3	0.8096	< 0.001	31
Stage of lactation			
1	0.6847	0.110	7
2	0.6931	< 0.001	44
3	0.6969	< 0.001	95

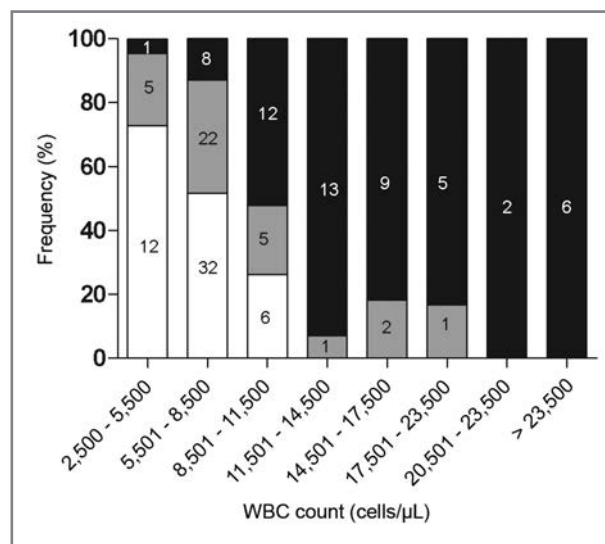


Figure 4—Cumulative frequency distribution of BLV bPVL (categorized as high [black bars], low [gray bars], or undetectable [white bars]) on the basis of WBC count in 146 lactating Holstein cows from the herd of farm 16 with high seroprevalence for anti-BLV antibodies. The respective number of cows is shown in each bar.

Discussion

In this study, we found a correlation between the total WBC count and the BLV bPVL in dairy cattle under natural conditions. This finding suggests that the WBC count could be a potential tool with which to monitor the level of BLV infection in herds highly BLV-infected and evaluate efforts toward the control of transmission.⁷

In the past, a high total lymphocyte count in blood samples was used to diagnose BLV infection in cattle by use of the key described by Bendixen.¹¹ Since 1976, with the development of an agar gel immunodiffusion test as the first serologic test specific for BLV,¹² the diagnosis of BLV infection has been based primarily on the detection of circulating antibodies because not all infected animals have persistent lymphocytosis.

Bovine leukemia virus–induced persistent lymphocytosis is the result of a relatively stable increase in the number of circulating B lymphocytes, which leads to an increase in absolute leukocyte count or leukocytosis.^{1,13}

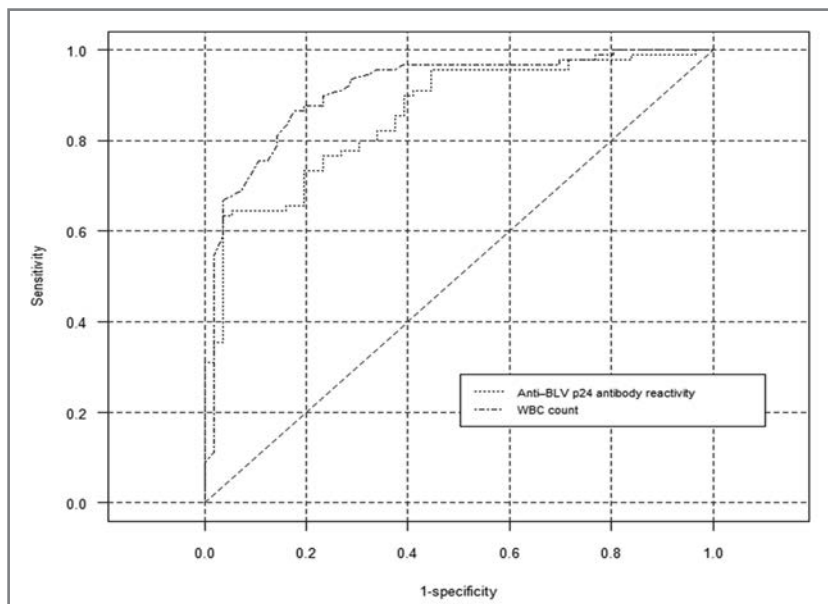


Figure 5—Receiver operating characteristic curve analysis of WBC counts and anti-BLV p24 antibody reactivity in 146 lactating Holstein cows from the herd of farm 16 with high seroprevalence for anti-BLV antibodies according to their bPVL category classification. For this analysis, cows were allocated to 2 groups: those with high bPVL and those with undetectable to low bPVL.

Apart from BLV infection, an increase in the number of WBCs can develop as a consequence of inflammation, stress, injury, leukemia, and infections.^{10,14} The increase in WBC count can be attributable to an increase in the number of lymphocytes (eg, lymphocytosis) or neutrophils (eg, neutrophilia), the 2 most abundant WBC subpopulations in cattle. Most frequently, leukocytosis is the result of transient neutrophilia as a host response to acute bacterial, viral, fungal, protozoal, or parasitic infections. Persistent neutrophilia is very rare in cattle and is associated with the bovine leukocyte adhesion deficiency syndrome. In general, lymphocytosis is also not common in ruminants, although it may develop in response to stress, chronic infections, and leukemia, the last 2 of which lead to a persistently high lymphocyte count.¹⁰ In this context, one can assume that in the absence of clinical evidence of severe infections, stress, or inflammation, a persistently high total WBC count in a bovid is mostly derived from an abnormal number of lymphocytes.

Because the WBC count was performed manually in the present study, errors could have occurred as a consequence of variance in the dilution of each blood sample and the distribution of cells in the hemocytometer chamber. Nevertheless, it is known that the accuracy of this method is very good, compared with automated cell counting, with minimal differences in results obtained via the 2 methods.¹⁰ Manual WBC counting is a simple and inexpensive hematologic analysis; given the results of the present study, it appears that manual WBC counts could be extremely helpful in attempts to control BLV infection in areas without specialized laboratory facilities or with low economic resources, as in Argentina.

In the present study, we first defined the WBC count reference interval for the study cows because it has been shown that values vary markedly as a re-

sult of BLV infection, and therefore, classical reference intervals¹⁵ should not necessarily be considered valid.¹⁰ Argentina is a country where BLV is highly endemic, with high prevalence of infection among individual cows on national dairy farms.¹⁶ Thus, to adjust the WBC count reference interval to the current situation, we based the calculation on WBC counts for healthy BLV-seronegative lactating Holstein cows from 15 dairy herds with high seroprevalence for anti-BLV antibodies in Argentina. For study purposes, the reference interval for WBC count in lactating cattle was considered to be 2,100 to 11,500 cells/ μ L of blood. Further analysis revealed that the parity or stage of lactation had no influence on the WBC count.

The percentage (1%) of BLV-infected and noninfected cells used as a calibrator and threshold to differentiate aleukemic from nonaleukemic cows via real-time PCR assay of whole blood samples was supported by published data on the basis of the *in vivo* level of infection.³ Nevertheless, the cutoff of bPVL was selected to segregate animals with low levels of infection with a security margin in the initial screening steps of an alternative control strategy based on selective segregation according to the bPVL.⁷ Among the 107 cows evaluated that had WBC counts within the calculated reference interval, 21 (19.6%) had high bPVLs; this finding was not unexpected because the aleukemic stage could have > 1% and up to 5% of BLV-infected blood cells. Moreover, this finding could be indicative of the proviral load peak after recent infection or even an improved stage of persistent lymphocytosis.

We have recently found that anti-BLV p24 antibodies reflect the bPVL and could be a good candidate for bPVL screening or monitoring.¹⁷ Surprisingly, a similar correlation coefficient was obtained between the total WBC count and bPVL and was even more elevated in animals with > 3 parities than in animals with 2 or 3 lactations. However, assessment of WBC count could be more reliable than assessment of antibody reactivity, given that the area under the curve on the ROC curve analysis was greater for the former variable in the present study, which suggests a better predictive potential.

A sustainable strategy to manage BLV infection in cattle herds is still lacking in countries where the high prevalence of infection does not allow the application of an official policy to eliminate infected animals. The data obtained in the present study provide useful information that could help in the design of an alternative control strategy based on the permanence of cattle with low levels of infection in herds.^{7,18} The main goal of this program would be the rational control of virus propagation through selection of cattle with undetectable or low bPVL to remain in the herd to finally diminish the risk of animal-to-animal transmission. In the case of the herd used to assess the correlation of total WBC count

and BLV bPVL in the present study, the farmer would eliminate approximately 40% of the cows (on the basis of high bPVL) instead of 87% of the cows (on the basis of seropositivity). Following the assumptions that bPVL is relatively constant in natural infections¹⁹ and that the development of persistent lymphocytosis is considered an important risk factor for BLV transmission,^{4,5,a} the bPVL should have a major role in the success or failure of BLV propagation.¹⁸ This is well known for human T-cell lymphotropic virus type 1 infections, where the level of bPVL is related to the risk of vertical and horizontal transmission.²⁰

Selective segregation of BLV-infected individuals based on the level of infection has been proposed by some authors, but there are no reports of studies showing the success of this strategy, to our knowledge. Thus, a field trial would have to be carefully designed to analyze the feasibility of such a strategy as a rational method of control of this highly endemic infection. According to the findings of the present study, the intervention procedure could include some screening or monitoring steps based on the total WBC count rather than on bPVL quantification. This potential program, based on assessment of WBC counts, would be more sustainable for local producers than classical measures of BLV control. In addition to the WBC count, the strategy should include serologic analysis because a significant correlation was found between the titer of anti-BLV antibodies and the WBC count or bPVL (data not shown).

Although the correlation of total WBC count and BLV bPVL was investigated in only 1 dairy herd in the present study, this is the first study to show that the WBC count is correlated with bPVL in cattle from a herd with high BLV seroprevalence. More work has to be done to validate these findings in a larger number of herds, with particular attention to assessment of parity via a well-designed categorical analysis. Whereas the quantitative evaluation of bPVL by real-time PCR assay is expensive and technically demanding, the manual WBC count is easy and inexpensive and could therefore constitute a more applicable tool in a BLV control intervention strategy. Further studies should be performed in different herds, especially in dairy farms, to define whether the WBC count could be considered a robust marker for the potential of transmission of BLV among cattle.

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