Impact of bovine leukemia virus infection on neutrophil and lymphocyte concentrations in dairy cattle

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Objective—To determine the effect of bovine leukemia virus (BLV) infection on absolute neutrophil and lymphocyte concentrations in healthy lactating Holstein dairy cattle.

Design—Observational cross-sectional survey.

Animals—311 healthy lactating Holstein dairy cattle from herds in Michigan (n = 2), Wisconsin (1), Iowa (1), and Pennsylvania (1).

Procedures—Whole and anticoagulated (EDTA) blood samples were collected. Serum samples were tested for antibody against BLV by use of an ELISA. Absolute neutrophil and lymphocyte concentrations were measured in EDTA blood samples with an automated hematology analyzer and manual differential cell counts.

Results—208 cows tested positive and 103 cows tested negative for anti-BLV antibodies. Neutrophil concentration was not significantly different between BLV-positive versus BLV-negative cattle. The distribution of lymphocyte concentration was positively skewed for the entire cow population (n = 311) and the BLV-positive subset (208). In contrast, lymphocyte concentration distribution was approximately normal for BLV-negative cows (n = 103). Consequently, the presence or absence of BLV infection strongly influenced the calculated neutrophil-to-lymphocyte concentration ratio.

Conclusions and Clinical Relevance—Results indicated that absolute lymphocyte concentration is significantly affected by BLV infection in dairy cattle. Accordingly, hematologic reference intervals should be derived from healthy animals that are not infected with BLV and patient BLV status must be considered for meaningful interpretation of lymphocyte concentration. We recommend that the calculated neutrophil-to-lymphocyte ratio be abandoned because it does not provide more information than direct comparison of patient absolute leukocyte concentration with updated reference intervals from healthy BLV-negative cattle. (J Am Vet Med Assoc 2013;243:131–135)
2010. Samples were collected from 311 adult lactating (2 to 975 days since calving; mean and 95% CI, 168.7 ± 3.3 days) Holstein cows in their first through eighth lactation (mean and 95% CI, 2.69 ± 0.04 lactations). Cows were determined by one of the investigators (RJE) to be healthy on the basis of physical examination (appetite, attitude, and hydration) at the time of blood sample collection from the coccygeal vein. Whole blood (15-mL red stopper vacutainer tubea) was allowed to clot overnight at 4°C and centrifuged at 2,400 × g for 30 minutes at 4°C, and serum was harvested. In addition, anticoagulated blood samples (5-mL EDTA evacuated tubea) were stored at 4°C until hematologic analysis. The study protocol was approved by the Michigan State University Animal Care and Use Committee.

Anti-BLV antibody assay—Serum samples were tested for anti-BLV antibodies at the Virology Laboratory, Diagnostic Center for Population and Animal Health, Michigan State University, with an ELISA.b

Hematologic data—Total and differential leukocyte concentrations were measured in anticoagulated blood samples with an automated hematology analyzerc,d that uses cytochemistry in conjunction with flow cytometry within 24 hours after collection at the Clinical Pathology Laboratory, Diagnostic Center for Population and Animal Health, Michigan State University. Automated bovine differential cell counts were previously validated in our laboratory by use of this methodologye,f; correlation studies with resultant minor reference intervals were performed when an updated analyzer was installed. A manual leukocyte differential cell count was performed on modified Wright-stained blood smears whenever the automated analyzer—flagged samples indicated problems with the differential count. Absolute concentrations (× 10³/µL) were calculated by multiplying the percentage by the total leukocyte (WBC) concentration.

Statistical analysis—Descriptive statistics regarding the frequency distributions as well as all other tests were performed with commercially available statistical software.g Absolute neutrophil and lymphocyte concentration distributions were analyzed in a mixed linear modelh with farm (5 values) included as a random effect variable. Days in milk and lactation number were evaluated as confounders and contributors to all possible 2-way interactions. In a separate analysis, the nonparametric Kruskal-Wallis testi was also used to compare lymphocyte concentration between BLV-negative and BLV-positive cattle. Histograms were used to display the frequency distribution of lymphocyte concentration for BLV-negative, BLV-positive, and all animals combined. Normality of frequency distributions was assessed by the Shapiro-Wilk W statisticj that ranges from 0 for a nonnormal distribution to 1.0 for a perfectly normal distribution. Significance was set at P < 0.05.

Results

BLV status—Two hundred eight cows were positive and 103 cows were negative for anti-BLV antibodies.

Hematologic data—Automated differential cell counts were performed on 200 samples. Manual differential cell counts were required (because of analyzer flags) and performed on 111 of 311 (36%) samples, including samples from 28 of 103 (27%) BLV-negative and 83 of 208 (40%) BLV-positive cows.

Absolute neutrophil concentration—Statistical analyses of the distribution of neutrophil concentrations for all cows (n = 311), BLV-positive cows (208), and BLV-negative cows (103) were summarized (Table 1). Shapiro-Wilk W values approached normal distribution.

Absolute lymphocyte concentration—Statistical analyses of the distribution of lymphocyte concentrations for all cows (n = 311), BLV-positive cows (208), and BLV-negative cows (103) were summarized (Table 1). The frequency distribution of lymphocyte concentration for all animals was decidedly nonnormal (W statistic = 0.52). However, when BLV-positive animals were excluded, a nearly normal distribution was revealed (W statistic = 0.92) for BLV-negative cows (Figure 1).

Table 1—Absolute neutrophil concentration data for all cows, BLV-positive cows, and BLV-negative cows in 5 dairy herds from Michigan (n = 2), Wisconsin (1), Iowa (1), and Pennsylvania (1) during 2008 through 2010.

<table>
<thead>
<tr>
<th>Variable</th>
<th>All cows (n = 311)</th>
<th>BLV positive (n = 208)</th>
<th>BLV negative (n = 103)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD (× 10³ neutrophils/µL)</td>
<td>3.86 ± 1.96</td>
<td>3.72 ± 1.71</td>
<td>4.15 ± 2.37</td>
</tr>
<tr>
<td>25th percentile (× 10³ neutrophils/µL)</td>
<td>2.71</td>
<td>2.70</td>
<td>2.71</td>
</tr>
<tr>
<td>Median (× 10³ neutrophils/µL)</td>
<td>3.48</td>
<td>3.45</td>
<td>3.52</td>
</tr>
<tr>
<td>75th percentile (× 10³ neutrophils/µL)</td>
<td>4.66</td>
<td>4.39</td>
<td>4.76</td>
</tr>
<tr>
<td>Shapiro-Wilk W (normality)</td>
<td>0.86</td>
<td>0.91</td>
<td>0.81</td>
</tr>
<tr>
<td>Skewness</td>
<td>1.85</td>
<td>1.47</td>
<td>1.92</td>
</tr>
</tbody>
</table>

Table 2—Absolute lymphocyte concentration data for all cows, BLV-positive cows, and BLV-negative cows in Table 1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>All cows (n = 311)</th>
<th>BLV positive (n = 208)</th>
<th>BLV negative (n = 103)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD (× 10³ lymphocytes/µL)</td>
<td>6.12 ± 6.79</td>
<td>7.52 ± 7.90</td>
<td>3.30 ± 1.14</td>
</tr>
<tr>
<td>25th percentile (× 10³ lymphocytes/µL)</td>
<td>3.03</td>
<td>3.44</td>
<td>2.58</td>
</tr>
<tr>
<td>Median (× 10³ lymphocytes/µL)</td>
<td>4.03</td>
<td>5.20</td>
<td>3.19</td>
</tr>
<tr>
<td>75th percentile (× 10³ lymphocytes/µL)</td>
<td>6.70</td>
<td>8.87</td>
<td>3.98</td>
</tr>
<tr>
<td>Shapiro-Wilk W (normality)</td>
<td>0.52</td>
<td>0.57</td>
<td>0.92</td>
</tr>
<tr>
<td>Skewness</td>
<td>5.90</td>
<td>5.11</td>
<td>1.44</td>
</tr>
</tbody>
</table>
Neutrophil-to-lymphocyte ratio—Statistical analyses of the distribution of the neutrophil-to-lymphocyte concentration ratio for all cows (n = 311), BLV-positive cows (208), and BLV-negative cows (103) were summarized (Table 3).

Statistical analysis—The mixed linear model used to predict absolute neutrophil concentration with BLV serology data (negative or positive) revealed that neither days in milk nor lactation number (P > 0.25 for both) was a significant confounder. Bovine leukemia virus serology, possibly due to inadequate power, was nonsignificant (P = 0.062) for predicting neutrophil concentration. A separate mixed linear model was used to explain the effect of BLV serology on absolute lymphocyte concentration with days in milk and lactation as possible confounders. Neither days in milk (P = 0.89) nor days in lactation (P > 0.20) were a significant predictor of lymphocyte concentration. Thus, BLV status remained the sole significant (P < 0.001) predictor of lymphocyte concentration. The Kruskal-Wallis nonparametric test confirmed the association between BLV results and lymphocyte count (P < 0.001).

Discussion

The present study examined the effect of BLV infection on absolute neutrophil and lymphocyte concentrations in 311 healthy lactating Holstein dairy cattle from herds in Michigan, Wisconsin, Iowa, and Pennsylvania; similar leukocyte patterns for BLV-negative dairy cattle of the present (2008 to 2010) study and 2001 University of California-Davis analyses were observed. However, these data are in sharp contrast to commonly used historical (1957) values for dairy cattle of unknown BLV status. We detected a significant (P < 0.001) increase in absolute lymphocyte concentration for BLV-positive versus BLV-negative cattle of the present study. Although mean lymphocyte concentration in the present study was similar to that of BLV-negative cows in 2001, the 1957 data (unknown BLV status) were intermediate between our BLV-positive and -negative data. The mean neutrophil-to-lymphocyte count ratio was > 1 for BLV-negative cows (present and 2001 analyses) but < 1 for BLV-positive cattle (present study) as well as 1957 data for which the BLV status was unknown (Table 4). In addition, absolute neutrophil concentration was lower for BLV-positive than BLV-negative cows of the present study, and 1957 neutrophil values for cows of unknown BLV status more closely approximated our BLV-positive than BLV-negative data. Consequently, ongoing use of the 1957 bovine leukocyte data as reference values for healthy cattle must be discouraged because the BLV status of these animals was unknown. Furthermore, considerable changes in bovine absolute lymphocyte and neutrophil concentrations as well as their interpretation have transpired over the past 50 years.

It is essential to recognize that the present study was not designed to measure herd prevalence of BLV
infection. Although the rate of BLV-infected specimens was 67% in the present study, this was likely impacted by unequal numbers of samples obtained from each herd, with some herds contributing more samples than others. Calculation of a weighted mean prevalence by herd would be a more accurate comparison of our data with previously reported within-herd prevalence.11,12 Moreover, our BLV infection snapshot was not adjusted to reflect proportional cow ages within each herd. Consequently, it is not possible to compare values from the present study with those of other studies of herd prevalence because our data were not balanced for these important variables.

Importantly, striking differences in absolute lymphocyte concentration distribution were observed for BLV-negative versus BLV-positive dairy cattle of the present study. Absolute lymphocyte concentration distribution for the entire population of cattle (without consideration of BLV infection status) was positively skewed with a nonnormal distribution, owing to a combination of the high frequency of BLV infection (with increases in lymphocyte concentration) and a minority of BLV-positive animals with extremely high values. In contrast, the lymphocyte distribution for BLV-negative cattle approached a normal distribution. Our findings highlight the crucial but rarely documented effect of BLV infection on commonly used published reference values.1–6 Accurate, current hematologic reference intervals obtained from BLV-negative cattle, paired with knowledge of the presence or absence of patient BLV infection, are imperative for meaningful interpretation of the biological importance of absolute lymphocyte concentration in dairy cattle. The neutrophil-to-lymphocyte ratio is substantially affected by changes in the denominator caused by a BLV-induced lymphocytosis. This potentially masks the presence of a neutrophilia and underscores the importance of considering absolute neutrophil and lymphocyte concentrations rather than a calculated ratio. It is important to consider that the neutrophil-to-lymphocyte ratio depends on both the neutrophil and lymphocyte concentrations. For example, a cow with a normal neutrophil concentration (4.0 × 10³ neutrophils/µL) and lymphocytosis (8.0 × 10³ lymphocytes/µL) would have a neutrophil-to-lymphocyte ratio of 0.5, interpreted as normal according to most published reports. In contrast, a cow with normal concentrations of neutrophils (4.0 × 10³ neutrophils/µL) and lymphocytes (3.0 × 10³ lymphocytes/µL) would have a neutrophil-to-lymphocyte ratio of 1.3, potentially interpreted as evidence of inflammation. Contrary to expectation, the first cow (with a normal neutrophil-to-lymphocyte ratio) should be tested for BLV infection because of an increased absolute lymphocyte concentration, whereas the latter cow (with increased neutrophil-to-lymphocyte ratio) was more likely to be a healthy animal because absolute concentrations of both neutrophils and lymphocytes were within reference intervals. Mean and median neutrophil-to-lymphocyte ratios were > 1 in BLV-negative cows but < 1 in BLV-positive cows as well as the entire population of cattle evaluated in the present study. This strongly suggests that a neutrophil-to-lymphocyte ratio < 1 is more likely pathological than a ratio > 1.

BLV infection on commonly used published reference intervals.24 The lymphocyte distribution for BLV-negative cattle approached a normal distribution. Our findings highlight the crucial but rarely documented effect of BLV infection on commonly used published reference values.1–6 Accurate, current hematologic reference intervals obtained from BLV-negative cattle, paired with knowledge of the presence or absence of patient BLV infection, are imperative for meaningful interpretation of the biological importance of absolute lymphocyte concentration in dairy cattle. The neutrophil-to-lymphocyte ratio is substantially affected by changes in the denominator caused by a BLV-induced lymphocytosis. This potentially masks the presence of a neutrophilia and underscores the importance of considering absolute neutrophil and lymphocyte concentrations rather than a calculated ratio. It is important to consider that the neutrophil-to-lymphocyte ratio depends on both the neutrophil and lymphocyte concentrations. For example, a cow with a normal neutrophil concentration (4.0 × 10³ neutrophils/µL) and lymphocytosis (8.0 × 10³ lymphocytes/µL) would have a neutrophil-to-lymphocyte ratio of 0.5, interpreted as normal according to most published reports. In contrast, a cow with normal concentrations of neutrophils (4.0 × 10³ neutrophils/µL) and lymphocytes (3.0 × 10³ lymphocytes/µL) would have a neutrophil-to-lymphocyte ratio of 1.3, potentially interpreted as evidence of inflammation. Contrary to expectation, the first cow (with a normal neutrophil-to-lymphocyte ratio) should be tested for BLV infection because of an increased absolute lymphocyte concentration, whereas the latter cow (with increased neutrophil-to-lymphocyte ratio) was more likely to be a healthy animal because absolute concentrations of both neutrophils and lymphocytes were within reference intervals. Mean and median neutrophil-to-lymphocyte ratios were > 1 in BLV-negative cows but < 1 in BLV-positive cows as well as the entire population of cattle evaluated in the present study. This strongly suggests that a neutrophil-to-lymphocyte ratio < 1 is more likely pathological than a ratio > 1.

Infection with BLV causes persistent lymphocytosis resulting from an increase in B lymphocyte concentration.11,12 The pathogenesis of this increased lymphocyte concentration is due in part to BLV-infected lymphocytes delayed in the G₀-to-G₁ cell cycle phase. This hiatus affords protection from apoptosis during cell proliferation, thereby increasing survival time of infected cells. In addition, antigen- or cytokine-induced proliferation of uninfected lymphocytes expands the cell pool susceptible to BLV infection, which, at subsequent infection (in conjunction with prolonged survival time), amplifies the lymphocytosis over time.16–18 Notably, activated lymphocytes express BLV-binding receptor promoting increased infection.12 Lymphocytotic BLV-infected cows have increased T lymphocyte interleukin-2 production. The elevated interleukin-2 concentration results in increased T lymphocyte interleukin-2 receptors and B lymphocyte BLV expression,10,20 thereby promoting increased viral burden. Reduced mitogen-induced proliferation of T cells with resultant decreases in CD3³, CD4⁺, and CD8⁺ cells also promotes a relative increase in B lymphocytes.18 An elevation in total (predominantly B) lymphocyte concentration in turn decreases the neutrophil-to-lymphocyte count ratio.

In addition to a window period when BLV infection may not be detected serologically,23 false-negative reactions are possible.22,23 Cattle with acute to subacute BLV infection may have lymphocyte concentrations that are increased for the individual cow but below the lymphocytosis threshold. Moreover, detection of anti-BLV antibodies from animals in the early stages of BLV infection also may be more challenging. Therefore, an unknown number of BLV antibody test–negative cattle of the present study could actually be BLV-infected, false-negative animals. The pathogenesis of the increased absolute lymphocyte concentration in 1 BLV-negative cow (8.89 × 10³ lymphocytes/µL) was unclear, but could represent a false-negative serologic test result or immune stimulation. It would be important to perform an outlier test on this data point before incorporation into a reference interval.24

Results of this study provide new fuel for the debate regarding whether reference intervals define medically normal versus usual values within a population of interest. Cattle infected with an immunologically suppressive virus would certainly not be considered biologically normal in numerous European countries that have attempted to eradicate BLV for several decades.25–27 However, because of the high prevalence of BLV infection in the United States, it will be necessary for bovine practitioners to clearly distinguish medically normal from usual when selecting and using appropriate reference intervals for interpretation of lymphocyte data. Results of this study demonstrate that clinical implications of absolute lymphocyte concentration cannot be interpreted without knowledge of patient and refer-
ence animal BLV infection status and use of a calculated neutrophil-to-lymphocyte ratio should be abandoned in cattle.

References

15. Stone DM, Norton LK, Davis WC. Spontaneously proliferating lymphocytes from bovine leukemia virus-infected, lymphocytic cattle are not the virus-expressing lymphocytes, as these cells are delayed in G0/G1 of the cell cycle and are spared from apoptosis. J Gen Virol 2000;81:971–981.