Short communication: Evaluation of 5 different ELISA for the detection of bovine leukemia virus antibodies

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ABSTRACT

Although Canadian dairy herds have been infected with bovine leukemia virus (BLV) for years, recent research has put new emphasis on the potential negative effects of this infection. Consequently, BLV control is becoming more favorable; however, BLV control cannot be successful without identifying infected animals. Bovicheck BLV (Biovet, Saint-Hyacinthe, QC, Canada) is currently the only assay licensed by the Canadian Centre for Veterinary Biologics. The first goal of this study was, therefore, to determine the reproducibility of the Bovicheck BLV assay for serum samples derived from Canadian cattle. The second goal was to evaluate and compare 5 different ELISA and determine their test characteristics using serum samples from Canadian herds. The considered ELISA were Bovicheck BLV, ID Screen BLV Competition (IDvet, Grabels, France), Idexx Leukosis Serum X2 Ab Test (Idexx Europe B.V., Hoofddorp, the Netherlands), Svanovir BLV gp51-Ab (Svanova, Uppsala, Sweden), and the Serelisa BLV Ab Mono Indirect (Synbiotics, Lyon, France). Eighty serum samples from Canadian cattle provided by Prairie Diagnostic Services (PDS; Saskatoon, SK, Canada) and an additional 80 serum samples from Canadian dairy and beef herds were used for the study. The Bovicheck BLV assay yielded the same results for all PDS-derived samples, implying a high level of reproducibility and robustness of this assay. Additionally, the comparison of the assays' results showed high agreement between assays, with Cohen's kappa values between $\kappa = 0.91$ and $\kappa = 1$. Furthermore, using original test results of the field samples as true status, relative diagnostic sensitivity and specificity were calculated. Relative diagnostic sensitivity of all tests was 100%. False-positive

results were probable; therefore, the following relative diagnostic specificities were determined: 100% for Bovicheck BLV, Idexx Leukosis Serum X2, and Svanovir BLV; 95% for ID Screen BLV; and 97% for Serelisa BLV. When considering other test characteristics, ID Screen BLV is exceptional due to considerable practical advantages.

Key words: ELISA, BLV, comparison, evaluation

Short Communication

Bovine leukemia virus (BLV) infection is highly prevalent in North American dairy cattle (VanLeeuwen et al., 2005; Scott et al., 2006; Bartlett et al., 2014). The infection not only causes tumors in some infected cattle (Schwartz and Levy, 1994), but recent research also suggests that it might result in decreased milk production, decreased cow longevity, and an impaired immune system (Erskine et al., 2012a; Bartlett et al., 2013; Frie and Coussens, 2015). Due to these effects on animal health and production, efforts have been made to fully understand efficient BLV control (Bartlett et al., 2014; EFSA Panel on Animal Health and Welfare, 2015; Nekouei et al., 2015). However, as with all retroviruses, BLV integrates its DNA into the host genome, thereby creating a provirus (Goff, 2013), making it impossible for the host to clear the virus despite the production of neutralizing antibodies. Therefore, successful control of BLV is dependent on the accurate identification and removal or segregation of infected animals. Whereas using PCR allows for the detection of the provirus as early as 7 d after infection, detecting antibodies is only possible at least 3 wk postinfection (Klintevall et al., 1994; Nagy et al., 2007). However, the detection of antibodies against BLV has proven to be a reliable, cost-effective, and quick way to identify infected animals (Burny et al., 1980; Monti et al., 2005; Nekouei et al., 2016). It should be noted, though, that the performance of diagnostic tests also depends on the

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characteristics of the population in which they are used (Dohoo et al., 2014). It is therefore valuable to evaluate these tests before use in the context of a control program. Bovicheck BLV (Biovet, Saint-Hyacinthe, QC, Canada) is currently the only assay licensed by the Canadian Centre for Veterinary Biologics (<http://inspection.gc.ca/active/netapp/veterinarybio-bioveterinaire/vetbioe.aspx#table-heading>). Because BLV control and testing go hand in hand, the robustness of an assay is a very important test characteristic. Therefore, the first goal of our study was to determine the reproducibility of the Bovicheck BLV assay for serum samples derived from Canadian dairy cows. Various diagnostic tests are commercially available; thus, the second goal of our study was to evaluate and compare the performance of 5 different commercially available ELISA and their test characteristics using serum samples from Canadian herds.

Five different commercially available assays were evaluated: Bovicheck BLV, ID Screen BLV Competition (IDvet, Grabels, France), Idexx Leukosis Serum X2 Ab Test (Idexx Europe B.V., Hoofddorp, the Netherlands), Svanovir BLV gp51-Ab (Svanova, Uppsala, Sweden), and the Serelisa BLV Ab Mono Indirect (Synbiotics, Lyon, France).

Serum samples from 220 animals of western Canadian dairy herds were provided by Prairie Diagnostic Services (**PDS**; Saskatoon, SK, Canada). These samples (referred to as PDS samples) were submitted between November 2015 and March 2016 to PDS for BLV diagnostics and included 108 samples with a previous positive, as well as 112 samples with a previous negative test result. Whereas the specific bovine breed for each sample was unknown, it should be noted that Canadian dairy herds consist to 93% of Holstein cattle (Canadian Dairy Information Centre, 2016). The PDS samples allowed an evaluation of the reproducibility for Bovicheck BLV in 2 different laboratories. In addition, we collected serum samples from BLV antibody-positive cows ($n = 54$), as well as serum samples from BLV antibody-negative cows ($n = 62$); collectively they will be referred to as field samples. The 54 positive field samples were obtained from 3 Alberta dairy herds. Inclusion criteria for the BLV antibody-positive samples were previous testing of the milking herd for BLV and the willingness of the farmer to make these results available. These samples were initially analyzed with a milk ELISA (supplied by AntelBio Inc., Lansing, MI) by CanWest DHI (Guelph, ON, Canada) or a serum ELISA (Svanova, no more details available to authors) by Idexx Laboratories (Markham, ON, Canada). The results of these tests were used to classify animals as positive. Whereas different antibody tests have successfully been used to eradicate BLV from infected herds

and for export purposes (Shettigara et al., 1986; Suh et al., 2005; World Assembly of the OIE, 2016), currently no reference test exists for BLV diagnostics. However, the probability of a true positive test result rises with age (Erskine et al., 2012b). Therefore, 40 samples were selected from the pool of 54 positive field samples from animals over 4 yr of age and were included as true positive. The 62 negative field samples were collected from a certified BLV-free Alberta beef herd ($n = 31$) and the dairy herd of the University of Guelph ($n = 31$). This herd is considered to be free of BLV, as all adult animals tested negative in at least 2 consecutive tests (Bovicheck BLV) performed by the Animal Health Laboratory (Guelph, ON, Canada). Forty randomly selected samples, 20 from each BLV-free herd, were included as true negative samples. All field samples were collected between April and July 2016. Each serum sample was divided into 2 to 4 aliquots and stored at -40°C until used. The study was approved by the Veterinary Sciences Animal Care Committee of the University of Calgary (VSACC AC15-0159).

Tests were performed and analyzed per the manufacturers' instructions (available upon request from first author), including both positive and negative controls as supplied with each kit. The European Union requires the detection of the E05 standard BLV serum in a 1/10 dilution before assays can be licensed for diagnostic purposes (World Assembly of the OIE, 2016). This standard serum was included on each plate in a serial dilution in sterile PBS Buffer (10^0 – 10^{-4}) as an additional control (kindly provided by the Enzootic Bovine Leukosis OIE Reference Laboratory, Leipzig, Germany).

To control for day-to-day variation, 1 ELISA plate from each kit was evaluated per day. Included on each plate were duplicates of a positive and negative control as provided by the ELISA company, a blank (empty wells), the EU 05 standard serum dilution series, and 40 samples. The same sample aliquot was used for all 5 plates. In these series of tests, a total of 160 different samples were tested (80 PDS samples and 80 field samples): 40 negative and 40 positive samples were randomly selected from the PDS sample collection. Of these selected PDS samples, 20 positive and 20 negative samples were added in random order to each of 2 test plates. The 40 positive field samples were chosen depending on the sampled cow's age, and the 40 negative field samples were chosen randomly from the 2 negative herds, as described above. The person executing the tests was not blinded to the original results. All assays met the validity criteria, required by the manufacturers' instructions.

The statistical analysis was performed in R 3.3.1 (R Foundation for Statistical Computing, Vienna, Aus-

tria). To evaluate agreement beyond chance between original tests and tests of the current study, as well as between tests in the current study, Cohen's kappa-values were calculated. These calculations were done by using the results of all samples. Additionally, relative diagnostic sensitivity and specificity were calculated based on results of the field samples only, as they were considered true positive and true negative. Samples, whose outcome was classified as suspect according to the assay's instructions, were considered positive for the calculation of results.

The fact that the test executing person was not blinded to the original test results might introduce potential bias into the study. However, randomization of the samples and the automated analysis of the test plates should minimize this bias. All PDS-derived samples yielded the same results when retested with Bovichek BLV in our study, executed in Calgary, indicating a high level of reproducibility and robustness of this assay.

Additionally, the original test results agreed almost perfectly ($\kappa = 0.94$) to perfectly ($\kappa = 1$) with the results of all tests evaluated in our study (Table 1). Based on previous evaluations, a high agreement between assays was expected (Simard et al., 2000; Monti et al., 2005); this was confirmed in our study. Represented by high Cohen's kappa values, the agreement between tests was classified as almost perfect ($\kappa = 0.91$) to perfect ($\kappa = 1$), suggesting very similar performances (Table 2). Nevertheless, whereas Bovichek BLV and Idexx Leukosis Serum X2 agreed with all original test results, ID Screen BLV, Svanovir BLV, and Serelisa BLV disagreed with some of the original test results (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-13626>). All samples previously classified as positive (PDS and field samples), yielded a positive result in all assays in our study. Results only varied for 8 samples previously classified as negative (Supplemental Table S1). One sample (sample 5; Supplemental Table S1) was classified as positive or suspect by 2 tests, whereas 7 samples (samples 1–4, 6–8; Supplemental Table S1) were classified as positive or suspect by only 1 assay. These discrepancies between results can be due to incorrect identification as positive or negative in the original tests or the tests used in our study, seroconversion since the last recorded testing, or a more sensitive cut off that resulted in lower specificity. However, 3 of the samples (samples 6–8, Supplemental Table S1) for which test results did not agree, originated from the Canadian Food Inspection Agency-accredited BLV-negative herd in Alberta (samples evaluated with Bovichek BLV and Svanovir BLV; Bianca Morel, supervisor, Laboratory Veterinary Services, Canadian Food Inspection Agency, St. Hyacinthe Laboratory-Diagnostic and Methods

Validation, Animal Health, Saint-Hyacinthe, Quebec, Canada, personal communication). Although we were unable to obtain additional samples from these animals, this herd is a closed herd and retested yearly by the Canadian Food Inspection Agency, which suggests that these were false-positive results. The additional 5 samples with discordant results (samples 1–5; Supplemental Table S1) were part of the PDS sample group. As no further information on the tested animals was available, no assumption about their true status could be made. However, verifying an individual animal's status could be done by retesting, because antibody titers rise over the first weeks following infection (Cockerell and Rovnak, 1988; Nagy et al., 2007; EFSA Panel on Animal Health and Welfare, 2015). This means that initially low titers might result in a false-negative or suspect result due to insufficient analytic sensitivity, but should be detectable when animals are retested at a later time point. Alternatively, suspect results could be confirmed by PCR, which can detect the presence of proviral DNA in B lymphocytes (Heenemann et al., 2012).

Considering the field samples as truly positive and truly negative, the values for relative diagnostic sensitivity and specificity for the tests evaluated in our study were as follows. All samples originally classified as positive were identified as positive by all tests in our study. Therefore, relative diagnostic sensitivity of all tests was 100% (95% CI = 87–100%). Comparing the original test results and test results from our study for field samples, false-positive results can be observed. Consequently, relative diagnostic specificities were determined for Bovichek BLV, Idexx Leukosis Serum X2, and Svanovir BLV to be 100% (95% CI = 87–100%), for ID Screen BLV to be 95% (95% CI = 83–99%), and for Serelisa BLV to be 97% (95% CI = 87–100%). We were not provided with any additional information on the animals that were tested by PDS. Therefore, we could not make any inferences about the true infection status of these animals and did not include these in the calculations; however, discrepancies between the results for PDS derived samples indicated that the estimated values for specificity were likely overestimates. A 100% relative diagnostic sensitivity is unlikely to be true; for example, acutely infected animals before seroconversion would not present as positive in our tests. These uncertainties could be reduced, for example, by increasing the sample size or through parallel testing of the samples using PCR. Even though the PDS samples were selected randomly and represent most stages of infection, a valuable addition would be a controlled infection trial.

In addition to test agreement, relative diagnostic sensitivity and specificity, other assay characteristics were

Table 1. Cohen's kappa values (95% CI) for original test results and test results found in this study

Item	All samples	PDS samples ¹	Field samples ²
Bovicheck BLV ³	1.00 (1.00–1.00)†	1.00 (1.00–1.00)†	1.00 (1.00–1.00)†
ID Screen BLV ⁴	0.98 (0.94–1.00)†	1.00 (1.00–1.00)†	0.95 (0.88–1.00)†
Idexx Leukosis Serum X2 ⁵	1.00 (1.00–1.00)†	1.00 (1.00–1.00)†	1.00 (1.00–1.00)†
Svanovir BLV ⁶	0.98 (0.94–1.00)†	0.95 (0.88–1.00)†	1.00 (1.00–1.00)†
Serelisa BLV ⁷	0.94 (0.88–0.99)†	0.9 (0.80–1.00)†	0.98 (0.93–1.00)†

¹Samples made available by Prairie Diagnostic Services (PDS, Saskatoon, SK, Canada; n = 80).

²Samples from Alberta dairy herds, Alberta Bovine Leukemia Virus (BLV)-free beef herd, and Ontario BLV-free dairy herd. (n = 80).

³Bovicheck BLV (Biovet, Saint-Hyacinthe, QC, Canada).

⁴ID Screen BLV Competition (IDvet, Grabels, France).

⁵Idexx Leukosis Serum X2 Ab Test (Idexx Europe B.V., Hoofddorp, the Netherlands).

⁶Svanovir BLV gp51-Ab (Svanovir BLV; Svanova, Uppsala, Sweden).

⁷Serelisa BLV Ab Mono Indirect (Serelisa BLV; Synbiotics, Lyon, France).

† $P < 0.01$.

investigated (Supplemental Table S2; <https://doi.org/10.3168/jds.2017-13626>). First, instructions are vital for diagnostic test assays, as different people with different background knowledge and expertise should be able to perform these tests (Crowther, 2009). Whereas Bovicheck BLV, Idexx Leukosis Serum X2, and Svanovir BLV provide instructions in at least 2 languages, ID Screen BLV and Serelisa BLV only provide instructions in English. Second, handling of tests was examined; Bovicheck BLV suggested to perform sample preparation on a different plate before administering it to the wells of the coated assay plate, resulting in an additional laboratory step. Similarly, Bovicheck BLV and ID Screen BLV required the dilution of the washing buffer and the preparation of the test conjugate. Incubation

times were the shortest for ID Screen BLV, at 90 min, and the longest for Serelisa BLV, at 150 min per plate. Overall, ID Screen BLV had the shortest turnaround time. This assay also allowed incubation of samples and test conjugate at room temperature. All other assays required incubation at 37°C, necessitating additional equipment. Finally, except for Svanovir BLV, all assays allow for the classification of results as suspect, which identified them as needing confirmation or retesting.

Overall, all assays performed well; however, 2 tests should be highlighted. First, Bovicheck BLV is readily available and the only BLV antibody assay licensed in Canada. Second, ID Screen BLV has considerable practical advantages due to its short turnaround time, its ability to test entirely at room temperature, and ease of

Table 2. Agreement between the 5 ELISA as determined by Cohen's kappa (95% CI)

Item	Bovicheck BLV ¹	ID Screen BLV ²	Idexx Leukosis Serum X2 ³	Svanovir BLV ⁴	Serelisa BLV ⁵
Bovicheck BLV		0.98 (0.94; 1.00)†	1.00 (1.00; 1.00)†	0.98 (0.94; 1.00)†	0.94 (0.88; 0.99)†
ID Screen BLV	0.98 (0.94; 1.00)†		0.98 (0.94; 1.00)†	0.95 (0.90; 1.00)†	0.91 (0.85; 0.98)†
Idexx Leukosis Serum X2	1.00 (1.00; 1.00)†	0.98 (0.94; 1.00)†		0.98 (0.94; 1.00)†	0.94 (0.88; 0.99)†
Svanovir BLV	0.98 (0.94; 1.00)†	0.95 (0.90; 1.00)†	0.98 (0.94; 1.00)†		0.94 (0.88; 0.99)†
Serelisa BLV	0.94 (0.88; 0.99)†	0.91 (0.85; 0.98)†	0.94 (0.88; 0.99)†	0.94 (0.88; 0.99)†	

¹Bovicheck BLV (Biovet, Saint-Hyacinthe QC, Canada).

²ID Screen BLV Competition (IDvet, Grabels, France).

³Idexx Leukosis Serum X2 Ab Test (Idexx Europe B.V., Hoofddorp, the Netherlands).

⁴Svanovir BLV gp51-Ab (Svanovir BLV; Svanova, Uppsala, Sweden).

⁵Serelisa BLV Ab Mono Indirect (Serelisa BLV; Synbiotics, Lyon, France).

† $P < 0.01$.

handling. Based on the results of the current study, all evaluated tests could be considered for the accreditation by the Canadian Centre for Veterinary Biologics.

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