Timing of seroconversion and acquisition of positive polymerase chain reaction assay results in calves experimentally infected with bovine leukemia virus

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Objective—To determine the interval to provirus and serum antibody detection (via PCR assay and ELISA, respectively) in calves after experimental inoculation with bovine leukemia virus (BLV).

Animals—8 colostrum-deprived, BLV-negative Holstein bull calves (≥ 6 weeks old).

Procedures—Via IM injection, each calf received a fresh whole-blood inoculum (day 0) calculated to contain 2×10^6 lymphocytes. Blood samples for the ELISA and PCR assay were collected from calves immediately prior to inoculation and weekly thereafter for 7 weeks. Mean and median number of weeks to PCR-detected conversion of BLV status and seroconversion were calculated. Point sensitivity and cumulative sensitivity of the 2 assays were calculated at each sample collection. At each sampling time, the proportion of calves identified as infected by the cumulative weekly ELISA and PCR assay results was compared by use of a Fisher exact test.

Results—In 5 calves, conversion of BLV status was detected via PCR assay before seroconversion was identified. However, seroconversion preceded PCR-detected conversion in 2 calves. In 1 calf, both assays yielded positive results at the same test date. These differences were not significant.

Conclusions and Clinical Relevance—In experimentally inoculated BLV-negative calves, conversion of BLV status was detected via PCR assay more quickly than via ELISA; this difference was not significant and probably not clinically important. The PCR assay may be useful as a confirmatory test in animals of exceptional value; tests based on viral identification may become critically important if vaccines against BLV infection are developed and marketed. (*Am J Vet Res* 2007;68:72–75)

Bovine leukemia virus is an oncogenic retrovirus of the human T-cell lymphotropic virus/BLV group.[1] Approximately 44% of dairy cattle and 10% of beef cattle in the United States are infected with BLV.[2,3] Cattle infected with BLV mount immune responses to both the viral surface and core antigens. Consequently, serologic tests for BLV performed on calves that ingest colostrum from BLV-infected cows will yield positive results, regardless of the infection status of the calves. Results of a previous study[4] indicate that these positive serologic assay results may persist for as long as 6 months.

Transmission of BLV in the perinatal period can occur in utero or through the ingestion of infected colostrum.[5-8] Among bovids, in utero infection of fetuses and infection of neonates via colostrum ingestion have been investigated and transmission is estimated to be 3% to 18% and 12%, respectively.[5-9] Infection of offspring during parturition has been postulated to occur but has not been investigated in depth.[10,11] In a recent study,[10] the transmission rate in colostrum-deprived calves born to BLV-infected cows was 33%; in a control group of colostrum-fed calves born to BLV-infected dams, transmission rate was 0%. It was postulated that exposure to the virus in the colostrum-deprived group occurred at parturition. Thurmond[11] postulated that exposure to maternal blood was a normal and expected event during parturition; however, the source of these parturition-associated infections has not been conclusively determined.

Blood inoculation of naïve calves via ID, IV, IM, and SC routes efficiently transmits BLV.[12] Seroconversion after blood inoculation can occur as early as 3 weeks and as late as 14 weeks.[12] One study[13] revealed that detection of seroconversion via AGID lags behind PCR identification of provirus by 2 to 4 weeks. In another study[14] of BLV-inoculated calves, the AGID assessment, ELISA, and PCR assay were compared; detection of provirus via PCR assay occurred from day 7 to day 56 af-

Abbreviations

BLV	Bovine leukemia virus
AGID	Agar gel immunodiffusion
OD	Optical density

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ter inoculation (depending on challenge dose), and all test results for 3 animals remained negative throughout the study. Seroconversion lagged behind PCR-detected conversion of BLV status in all calves; however, data were not presented to determine whether the timing was similar for all calves. Most studies that compare serologic findings and PCR detection of BLV infection use an AGID assay as the serologic test comparison. The purpose of the study reported here was to compare the interval to provirus and antibody detection (via PCR assay and ELISA, respectively) in calves after experimental inoculation with BLV. We hypothesized that PCR-detected conversion of BLV status would precede seroconversion.

Materials and Methods

Calves—Eight colostrum-deprived Holstein bull calves (≥ 6 weeks old) purchased from the University of Missouri Foremost Dairy were used for the study. Prior to enrollment in the study, BLV-specific ELISA and PCR assay results were negative at each of 5 sequential, weekly assessments for each calf; this ensured that the study calves were not infected with BLV at the start of the study. Calves were housed at a university facility (under veterinarian oversight) in individual stalls and had no contact with other cattle. The calves were fed a commercial milk replacer twice daily and had access to grass hay ad libitum. Calves were weaned at approximately 10 weeks of age. No dehorning or vaccinations were completed prior to or during the study period. All animal care and husbandry procedures were reviewed and approved by the institutional animal care and use committee.

Inoculation procedure—Blood (10 mL) was collected into 10-mL evacuated acid-citrate-dextrose tubes from a known BLV-positive cow that did not have persistent lymphocytosis. The blood was kept at 4°C for approximately 1 hour until each inoculum was prepared and administered to the study calves. Each calf was injected IM (day 0) with an inoculum (0.3 mL) containing approximately 2×10^6 lymphocytes.

Specimen collection and processing—Blood samples for ELISA and PCR assay were collected via jugular venipuncture from calves immediately prior to inoculation to ensure BLV-negative status prior to inoculation and weekly thereafter for 7 weeks. Samples for assessment via ELISA were collected into tubes with no anticoagulant, and serum was collected after centrifugation. Samples for assessment via PCR assay were collected into tubes containing EDTA. Samples were stored at 4°C and processed within 48 hours. Lymphocytes were isolated from the whole-blood samples by use of a commercially available erythrocyte lysis buffer.[a] Lymphocytes were washed 3 times with erythrocyte lysis buffer and stored at –70°C for subsequent analysis. The DNA was extracted by use of a commercially available kit following the manufacturer's protocol.[b]

ELISA—A commercial assay was used to detect serum anti-BLV antibodies.[c] The OD of samples was assessed at 620 nm; a positive test result was defined as an OD reading greater than the mean of the positive control samples, and a negative test result was defined as an OD reading less than the mean of the negative control samples. Samples were analyzed by use of a commercial plate reader.[d] For test validation, the mean OD value of the positive control samples was ≥ 0.250 and the mean OD value of the negative control samples was ≤ 0.200.

PCR assay—Forward (5′-TGG CTA TCC TAA GAT CTA CTG-3′) and reverse (3′-AGA GGG AAC CCA GTC ACT GTT-5′) primers from the env-gp51 region of the BLV viral genome were selected on the basis of a published report.[14] The protocol was altered to a single run from the described nested PCR procedure. Sensitivity and specificity data of this protocol have been published previously.[15] The DNA sequences were amplified in a 25-µL reaction mixture containing 0.5µM of each primer and 1.0 unit of *Taq* DNA polymerase in the manufacturer's buffer, which contained 2.0mM $MgCl_2$ and 0.2mM of each dNTP.[e] The reaction was performed in a programmable thermocycler.[f] An initial incubation of 12 minutes at 95°C was followed by 10 cycles of denaturation at 95°C for 30 seconds, annealing at 70°C for 30 seconds, and extension at 72°C for 90 seconds. Annealing temperature was reduced by 1°C each cycle. An additional 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 90 seconds were performed, followed by a final extension at 72°C for 7 minutes. Amplification with BLV-specific primers yielded a product of 330 bp. Amplification products were isolated in a 2% agarose gel and stained with ethidium bromide.[16] Each laboratory run was performed with known positive and negative samples as controls. A laboratory run may include as many as 94 test samples.

Data analysis—Mean and median intervals from inoculation to detection of conversion of BLV status via PCR assay and from inoculation to seroconversion were calculated. At each sampling time, the sensitivities of the PCR assay and ELISA in the detection of early postinfection events were calculated. At each sampling time, the cumulative sensitivity (defined as the proportion of calves for which at least 1 weekly assay result was positive [either via ELISA or PCR assay] divided by the number of calves that eventually yielded positive results via either assay procedure at each weekly sampling time) was calculated. The proportion of calves identified as infected by cumulative weekly ELISA and PCR assay results was compared at each sampling time by use of a Fisher exact test. An approximation of ELISA sensitivity in known infections was calculated by dividing the number of positive test results by the total number of tests performed in calves that had previous positive ELISA results. A similar calculation was performed for the PCR assay.

Results

Following inoculation, all 8 calves became infected with BLV. Each calf had a minimum of 4 positive ELISA test results and 1 positive PCR assay result prior to the completion of the study. The intervals to conversion of BLV status as detected via PCR assay and via seroconversion were calculated (**Table 1**). In 5 calves, PCR-detected conversion of BLV status preceded se-

Table 1—Number of weeks to seroconversion (via ELISA) and detection of provirus (via PCR assay) in blood samples collected from 8 calves after experimental inoculation with BLV.

	No. of weeks to first positive result	
Calf	PCR assay	ELISA
1	2	3
2	3	5
3	5	4
4	5	3
5	4	4
6	2	4
7	2	3
8	2	3
Mean	3.125	3.625
Median	2.5	3.5

Table 2—Proportion of calves identified as infected by cumulative test results of an ELISA and a PCR assay (detection of serum anti-BLV antibody and provirus, respectively) used to evaluate blood samples collected at weekly intervals from 8 calves after experimental inoculation with BLV.

Week after inoculation with BLV	Cumulative sensitivity	
	PCR assay	ELISA
1	0.00	0.00
2	0.50	0.00
3	0.63	0.50
4	0.75	0.88
5	1.00	1.00
6	1.00	1.00
7	1.00	1.00

roconversion. Seroconversion preceded PCR-detected conversion of BLV status in 2 calves. In 1 calf, both assays yielded positive results at the sampling time. Once serum anti-BLV antibodies were detected, calves were consistently seropositive for the remainder of the study (equivalent to a sensitivity of 1.00). Six calves had at least 1 false-negative PCR assay result after the initial positive result. After the initial positive PCR assay results were obtained, 20 of 30 tests yielded positive results (equivalent to a sensitivity of 0.67 in the detection of infections). The proportion of calves identified as infected by cumulative weekly ELISA or PCR assay results was not significantly different among the sampling times (**Table 2**).

Discussion

In the present study, the ability of a PCR assay to detect early infection with BLV in calves after experimental inoculation with the virus was evaluated. Compared with the ELISA findings, positive test results were detected earlier by use of the PCR assay. Four of 8 calves had positive PCR assay results within 2 weeks after inoculation, yet no positive ELISA results were detected until week 3. The intervals to seroconversion among the calves were similar to those reported previously.[12,14] The intervals to proviral detection via PCR assay were similar to those reported in calves with low-challenge exposure to BLV.[14] Mean and median intervals to detection of BLV status conversion were shorter by use of the PCR assay than the ELISA. However, the difference in detection times was not significantly different and is probably not clinically relevant. In addition, 2 calves were positive for serum anti-BLV antibodies prior to proviral detection via the PCR assay. At least 1 false-negative PCR test result after initial provirus detection was obtained in each of 6 calves. This is consistent with the reported sensitivity of the assay.[15] In contrast, no calf had negative ELISA results after its first positive ELISA result was obtained.

To the authors' knowledge, the assay used in the present study is the only BLV PCR assay that has been validated in a herd setting with known BLV-positive and -negative animals.[15] Although the assay lacks some sensitivity, it has high specificity, which virtually eliminates false-positive test results. Other BLV PCR assays that are capable of detecting proviral DNA had been developed prior to the assay used in this study[13,14]; however, in 1 of those previous studies,[14] it was determined that decreasing infectious dose increased the interval to proviral detection. In the present study, the true infectious dose can only be estimated and the effect of varying the dose was not examined. Although early detection of BLV infection in bovids has merit, it also has a limited application in modern cattle production.

Serologic conversion of bovids to BLV-positive status has been reported to be detectable at 3 to 14 weeks after experimental inoculation with the virus.[12-14] Studies[13,14] that jointly examined serologic and PCR assay methods have revealed that PCR-detected conversion of BLV status in cattle occurred prior to seroconversion by a period of 14 to approximately 30 days. If the PCR procedure routinely identifies BLV-positive animals 30 days before serologic evidence of infection is available, then there is merit in use of the PCR assay to reduce the duration of on-farm quarantine restrictions. However, if the PCR assay identifies BLV-positive animals only 7 days prior to seroconversion, as our data suggest, there is minimal benefit to implementing the former testing strategy. On assessment of possible measures of sensitivity, either cumulative sensitivity with serial testing programs or point sensitivity, it appears that the proportion of infected adult bovids detected by use of the ELISA will be higher than that detected by use of the PCR assay. Cost of the ELISA and the ease with which it can be adapted to mass sample processing also support the routine use of serologic evaluation to identify BLV-infected cattle.

Despite the failure of the PCR assay to identify all BLV-infected calves prior to seroconversion in the present study, the procedure has clinical usefulness. The high specificity of this assay means that it is ideal as a confirmatory test in animals of exceptional value that may otherwise be culled on the basis of a positive serologic test result. Additionally, testing that is based on viral identification may become critically important if vaccines against BLV-associated disease are developed and marketed for use in cattle. As a result of vaccination of cattle, more positive serologic test results may be obtained routinely and more definitive viral detection methods may become necessary.

The PCR assay also may play a role in the identification of infection in neonatal calves that have absorbed anti-BLV antibodies from ingested colostrum. The ability to identify provirus will allow identification and removal of infected calves prior to commingling and processing events that promote amplification of low-level infections. It should be

noted that in the study of this report, serial weekly testing detected BLV infection in 4 of the 8 calves within 2 weeks after experimental inoculation and in all calves by 5 weeks after inoculation. On most modern dairy farms, calves are typically housed singly in calf hutches until 8 to 10 weeks of age. Under those conditions, the potential for horizontal transmission is negligible. We envision the development of programs in which calves infected transplacentally, at parturition, or through ingestion of colostrum may be detected by application of serial PCR assay testing cycles and then culled, thereby generating BLV-negative cohorts with minimal risk for horizontal transmission.

a. Erythrocyte lysis buffer, Qiagen Inc, Valencia, Calif.
b. QIAamp DNA mini kit, Qiagen Inc, Valencia, Calif.
c. Bovine leukemia virus antibody test kit, ELISA, VMRD Inc, Pullman, Wash.
d. Multiskan RC, MTX Lab Systems Inc, Vienna, Va.
e. HotStarTaq, Qiagen Inc, Valencia, Calif.
f. Perkin-Elmer 9700, Perkin-Elmer Inc, Shelton, Conn.

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