Determination of proviral load in bovine leukemia virus–infected cattle with and without lymphocytosis

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Objective—To determine proviral load in bovine leukemia virus (BLV)–infected cattle with and without persistent lymphocytosis to assess the potential of transmitting the virus.

Animals—Cattle in 6 dairy herds.

Procedures—Blood samples from infected cows were evaluated 3 times at 6-month intervals for determination of proviral load via PCR assay, serologic results via ELISA, and hematologic status via differential cell counts.

Results—Infected cattle were classified into lymphocytotic and nonlymphocytotic groups. Lymphocytotic cattle consistently had > 100,000 copies of integrated provirus/µg of DNA (ie, high proviral load) in peripheral blood leukocytes. Titers of antibodies against BLVgp51 and BLVp24 indicated a strong immune response. Nonlymphocytotic cattle comprised 2 subgroups: a group with high proviral load and strong immune response, and a group with a weaker immune response, mostly against BLVp24, and a proviral load of < 100 copies/µg of DNA (ie, low proviral load).

Conclusions and Clinical Relevance—Results emphasized the importance of characterizing nonlymphocytotic BLV-infected cattle during eradication programs. The risk of transmitting BLV infection from nonlymphocytotic cattle may differ depending on the proviral load. Nonlymphocytotic cattle with high proviral load could be efficient transmitters (as efficient as lymphocytotic cattle), whereas nonlymphocytotic cattle with low proviral load could be inefficient transmitters under standard husbandry conditions. Because most cattle with low proviral load do not develop anti-BLVp24 antibodies, it appears that lack of an anti-BLVp24 antibody response may be a good marker of this condition. (*Am J Vet Res* 2007;68: xxx–xxx)

Enzootic bovine lymphosarcoma, also known as BLV-associated malignant lymphoma (lymphosarcoma), is the most common neoplasm of dairy cattle. Although beef and dairy cattle are susceptible to BLV infection and BLV-associated lymphosarcoma, the disease is more commonly detected in dairy herds, mostly because of management practices of dairy farms.[1] Bovine leukemia virus is a deltaretrovirus, which is a member of the Retroviridae family. This genus includes human T-lymphotropic viruses I, II, III, and IV[2] and the simian T-cell leukemia viruses I, II, and III. The deltaretroviruses are classified as complex retroviruses because, in addition to the typical structural retroviral genes *gag*, *pol,* and *env*, they have open reading frames that codify regulatory proteins.[3] Deltaretroviruses are associated with nonneoplasic lymphocyte proliferation, lymphoid neoplasia, and progressive myelopathies.[4] Bovine leukemia virus is unique among the Retroviridae family because it infects B lymphocytes and induces a chronic B-cell lymphoproliferative syndrome in cattle.[3] Cattle infected with BLV remain infected for life. Few infected cattle develop the disease; < 10% of infected cattle develop malignant tumors.[5] About 30% of the naturally infected cattle develop a polyclonal nonneoplastic B-cell lymphocytosis (ie, persistent lymphocytosis) within 3 to 6 years after infection.[6] This condition, which primarily involves the $CD5^+$ B-cell subpopulation, is characterized by decreased cell turnover resulting from reduc-

Abbreviations

BLV	Bovine leukemia virus
AGID	Agar gel immunodiffusion
HPL	High proviral load
PBL	Peripheral blood lymphocyte
GSH	Glutathione
PBST	PBS solution containing 0.05% Tween 20
LPL	Low proviral load

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tion of cell death and overall impairment of proliferation.[7] Development of persistent lymphocytosis, which is usually not accompanied by other clinical signs, may depend on genetic factors.[8]

Lymphosarcoma induced by BLV is often found in cattle > 3 years of age, and maximum prevalence is in cattle from 4 to 8 years of age. Approximately two thirds of the lymphosarcomas are preceded by a period of persistent lymphocytosis, but this phase is not necessary for the development of lymphosarcoma.[6] Although persistent lymphocytosis is an indicator of BLV infection, identification of cattle with persistent lymphocytosis by hematologic methods does not detect all infected cattle,[9] despite the fact that for many years, diagnosis of BLV infection was based only on this method.

Because practically all BLV-infected cattle develop specific antibodies, control of BLV infection is based on serologic detection and culling of virus carriers. The AGID test and an ELISA have been widely used for serologic detection of antibodies against gp51, the main antigenic glycoprotein of the viral envelope.[10–12] A PCR assay can also be used to directly detect viral DNA in peripheral blood mononuclear cells[13] and is particularly useful when colostral antibodies interfere with the serologic assays.

In Argentina, control of BLV infection in dairy or beef herds is not compulsory. The most recent (1997 to 1999) serologic surveys conducted in the main dairy regions of Argentina revealed a high prevalence (78% to 86%) of infected herds, with an overall prevalence of infected cattle > 30%.[14,15] In another study,[16] a large number of dairy herds were found with a high prevalence (> 70%) of BLV infection, and some even had a prevalence of 100%. Control of BLV infection by culling of seropositive cattle is not economically feasible in dairy herds with such high prevalences or herds with low prevalence of BLV infection but a high number of lactating cows. To limit dissemination of BLV, infected cattle in such herds are often eliminated on the basis of their risk for transmitting the infection to other cattle. Among BLV-infected cattle, those with persistent lymphocytosis are considered the most efficient transmitters because they harbor a high percentage of infected lymphocytes in peripheral blood[17] and consequently have an HPL (ie, a high number of proviral copies integrated per microgram of DNA from PBLs).[18]

It has been suggested that among BLV-infected cattle, susceptibility to development of persistent lymphocytosis depends on genetic factors, environmental factors, or both.[8,19,20] A study[21] with a limited number of cattle revealed that proviral load in BLV-infected cattle correlates with antibody titer against the envelope glycoprotein (BLVgp51) and the main core protein (BLVp24) of BLV. The purpose of the study reported here was to determine proviral load in BLV-infected cattle with and without lymphocytosis to assess their potential for transmitting the virus.

Materials and Methods

Animals—Cattle infected with BLV from 6 dairy herds from various regions of Argentina were used for the study. Cattle were of the Argentinean Holstein dairy breed. All herds had > 100 lactating cows and a high prevalence (> 75%) of infection with BLV, as determined via serologic examination. Three blood samples from each of 200 BLV-infected cattle were obtained at 6-month intervals during a period of 1 year. Cattle were classified as infected with BLV by testing their plasma by use of an indirect-absorbed ELISA (designated as ELISA 108), a blocking assay that detects anti-BLVgp51 antibodies and is highly specific and sensitive.[16] The proviral load and antibody titer against BLVgp51 and BLVp24 were determined in each of the 3 samples. Blood leukocyte count was determined in 2 blood samples obtained from each animal. The first sample was obtained simultaneously with the sample obtained for serologic examination, and the second sample was obtained 3 months later. All testing was performed after all 3 samples were obtained from each animal.

Sample collection—Ten-milliliter samples of heparinized (5 U/mL) blood were obtained by jugular or coccygeal vein venipuncture. Plasma was harvested after centrifugation of blood samples for 20 minutes at 2,000 × *g*. Sodium azide was added (final concentration, 0.2%), and the plasma aliquots were stored at –20°C until analyzed. Peripheral blood lymphocytes were obtained by mixing the buffy coat with 11 mL of cold ammonium chloride buffer (150mM NH_4Cl, 8mM Na_2CO_3, and 6mM EDTA) for 1 minute to completely lyse the RBCs. The cell pellet obtained after centrifugation at 1,000 × *g* for 7 minutes at 4°C was resuspended in 1 mL of PBS solution, transferred to a 1.5-mL tube, and centrifuged at 10,000 × *g* for 2 minutes. Supernatant was discarded, and the PBLs were stored at –20°C. Typically, > 3×10^6 leukocytes were obtained from each sample. Samples of 1 mL of blood obtained with EDTA (342mM; pH, 7.2) as anticoagulant were used for the total and differential leukocyte counts.

Blood leukocyte count—Total leukocyte count was performed in an automated particle and cell counter.[a] Differential leukocyte count was performed by examining a blood film stained with May-Grünwald Giemsa stain. Four hundred cells were evaluated to determine cell type. Cattle were classified as having or not having persistent lymphocytosis by use of a standard leukosis key for cattle.[22] This key, based on lymphocyte values for BLV-free healthy cattle according to age, was established in Danish cattle. Caution has been recommended regarding diagnosis of persistent lymphocytosis by use of leukosis keys when comparisons are made among geographic locations or breeds of cattle.[23] For this reason, reference values for Argentinean Holstein dairy cows were determined in cattle 3 to 4 years of age and > 4 years of age; no significant differences were detected between those values and values used in the standard leukosis key. In the study reported here, cattle with absolute lymphocyte counts that were 3 or more SDs greater than the mean value for age- and breed-matched BLV-seronegative cattle in 2 successive samples separated by a 3-month interval were considered as having persistent lymphocytosis (ie, lymphocytotic). Bovine leukemia virus–infected cattle that maintained WBC counts within the reference range during the same time interval were considered nonlymphocytotic.

PCR assay—The DNA was organically extracted from PBLs as described elsewhere.[24] Primers from the BLV *pol* gene were used[25]: BLV G07 (position 4441) 5′–CCCTACAACCCCACAAGTTCGG–3′ and BLV G08 (position 4623) 5′–ATGGTGTAGCTCCCATCTG-GTCTT–3′. Amplification was performed in a final volume of 10 µL, which contained 2mM $MgCl_2$, 125µM of each deoxynucleoside triphoshphate, bovine serum albumin (100 µg/mL), 5µM of each primer, appropriate buffer for the enzyme, 1 unit of *Taq* polymerase,[b] and 1 µg of DNA template. Thermal cycling was conducted in a thermocycler.[c] The reaction mixture was denatured for 1 minute at 94°C and amplified for 8 cycles (1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C) followed by another 25 cycles (1 minute at 94°C, 1 minute at 62°C, and 1 minute at 72°C). When the last cycle was finished, samples were incubated at 72°C for 10 minutes.

Determination of proviral load—Proviral load, defined as the number of proviral copies integrated per microgram of DNA extracted from PBLs, was determined via visual comparison of the amplification products. Several dilutions of DNA from fetal lamb kidney cells were used as an internal standard. This cell line carries 4 copies of the BLV provirus integrated into each cell genome.[26] Products of PCR amplification were evaluated after electrophoresis in 12% polyacrylamide gels, staining with ethidium bromide, and UV transilumination. Sensitivity of the PCR assay was determined for each experiment and was consistently 50 copies of provirus/µg of DNA. Specificity of the reaction was determined by cloning the amplified product[d] and subsequent sequencing.

Determination of antibody titer against BLV-gp51—Anti-BLVgp51 antibody titer was determined by testing 2-fold dilutions (from 1:50 to 1:6,400) of plasma samples in an indirect absorbed ELISA (ie, ELISA 108). Characteristics and evaluation of this ELISA have been reported elsewhere.[16]

Determination of antibody titer against BLV-p24—The titer of antibodies against BLVp24 was determined in plasma samples by use of an ELISA designated Rp24. This assay, which uses a recombinant form of BLVp24 as antigen, has been reported elsewhere.[27] Briefly, polystyrene microtiter plates[e] were coated by incubation overnight at 4°C with polyclonal chicken anti-GSH diluted 1:4,000 in 20mM carbonate buffer (pH, 9.6). Plates were washed twice with PBST, and alternate rows were incubated with 100 µL of recombinant fusion protein GSH-p24 ([6 µg/mL]/well; GSH fused to BLVp24) diluted in protein diluent (PBS solution, 10% tryptose,[f] 1% Tween 20, and 0.2% sodium azide) or with diluent alone. After incubation for 60 minutes at 37°C, plates were washed 4 times with PBST. Plasma samples diluted in protein diluent supplemented with 10% normal chicken serum were added to the wells containing the captured GSH-p24 and to control wells that contained only the anti-GSH chicken sera. Plates were incubated at 37°C for 60 minutes, and nonbound antibodies were removed by washing 4 times with PBST. One hundred microliters of biotin-SP–conjugated recombinant protein A/G[g] diluted 1:5,000 in PBST was added to each well; plates were then incubated for 15 minutes at 37°C. After 4 washes with PBST, the reaction was amplified by incubation (15 minutes at 37°C) with 100 µL of peroxidase-conjugated streptavidin[h]/well (diluted 1:5,000 in PBST). After the plates were washed 4 times with PBS, bound peroxidase was detected by adding 90 µL of 3,3′,5,5′-tetramethylbenzidine[i]/well and incubation for 30 minutes at 22°C. The reaction was stopped by the addition of 30 µL of 4N H_2SO_4/well, and optical density was measured at 450 nm with an automated microplate reader.[j] Each plate included duplicate wells in which reference BLV-positive and -negative sera diluted 1:50 were tested as well as 2 wells without plasma that were used as blanks. The optical density value obtained for each sample in the control wells (without antigen) was subtracted from the optical density value obtained in the wells containing the recombinant protein. The cutoff value was defined by use of the 2-graph receiver operating characteristic.[28,29] Antibody titers were determined by testing the samples at 2-fold dilutions from 1:50 to 1:6,400.

Statistical analysis—Mean and median values of the 3 determinations of anti-BLVgp51 and anti-BLVp24 antibody titers for each animal were calculated. For each group of cattle (cattle with or without persistent lymphocytosis and with different amounts of proviral load), the mean, SD, and median values of each animal were almost equal. For this reason, the median of the 3 values was considered representative of the antibody titer for each animal. Statistical comparison of medians was conducted by use of the χ^2 test. The Student *t* test was used to compare proviral load among cattle of various ages. For all comparisons, a value of $P < 0.05$ was considered significant.

Results

Prevalence of BLV-infected cattle in each herd ranged from 20% to 40%, except for 1 herd in which the prevalence was 63%. Overall prevalence was approximately 33%.

Table 1—Proviral and antibody titers against BLVgp51 and BLVp24 in BLV-infected cattle with or without lymphocytosis.

Hematologic status	No. (%) of cattle	Proviral load*	Anti-BLVgp51 antibody titer	Anti-BLVp24 antibody titer
Lymphocytotic	66 (33)	≥100,000	400 to ≥ 6,400	50 to 800
Nonlymphocytotic				
HPL	53 (26.5)	≥100,000	400 to ≥ 6,400	Seronegative to 400
LPL	81 (40.5)	≤ 100	2 to 1,600	Seronegative to 50

*No of proviral copies integrated/µg of DNA from PBLs.

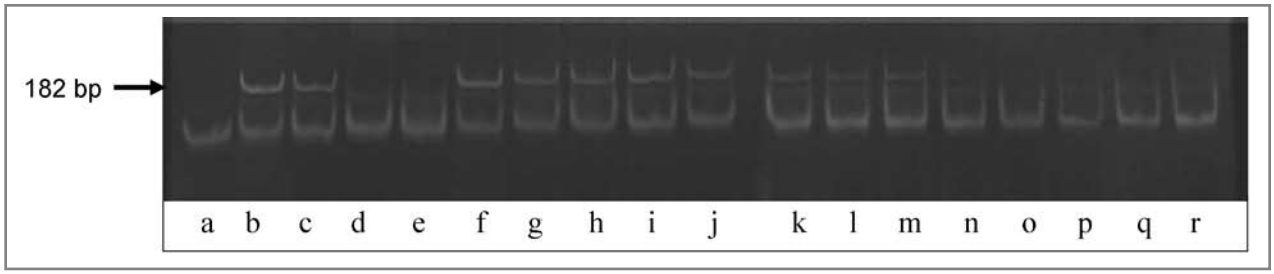

Figure 1—Electrophoretogram of PCR DNA amplification products in a study of BLV in dairy cattle in 6 herds. Lanes were as follows: a, negative control sample (no DNA); b and c, DNA from HPL cattle; d and e, DNA from LPL cattle; f, fetal lamb kidney (FLK) DNA (670,000 copies); g, FLK DNA (300,000 copies); h, FLK DNA (150,000 copies); i, FLK DNA (80,000 copies); j, FLK DNA (40,000 copies); k, FLK DNA (10,000 copies); l, FLK DNA (2,500 copies); m, FLK DNA (1,000 copies); n, FLK DNA (50 copies); o, FLK DNA (25 copies); p, FLK DNA (12 copies); q, FLK DNA (2 copies); and r, DNA from a BLV-free cow. Notice that the 182-bp product is undetectable at 25 or fewer copies of FLK DNA.

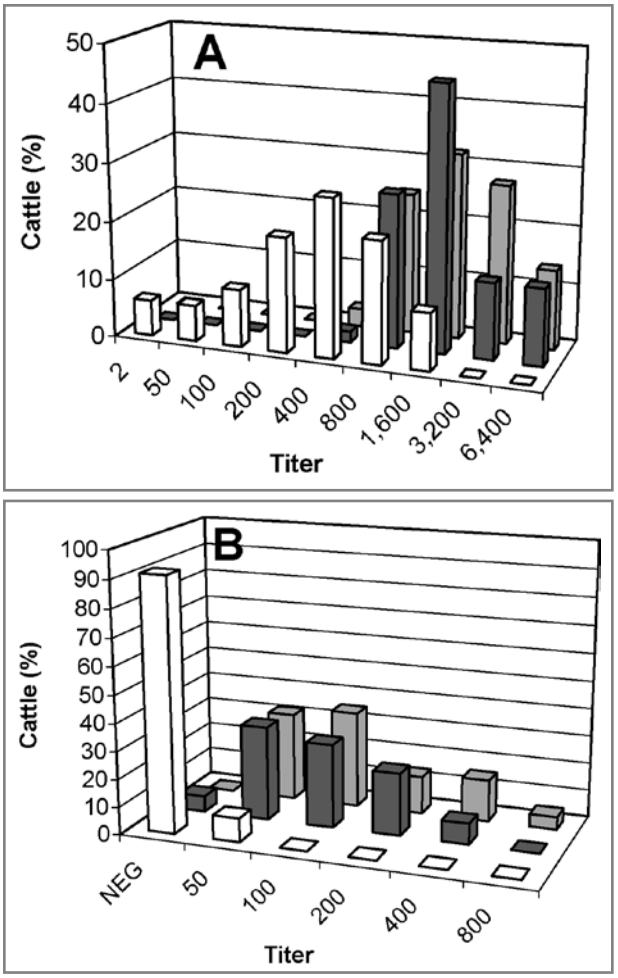

Figure 2—Titers of antibodies against BLVgp51 (A) and BLVp24 (B) in BLV-infected cattle grouped on the basis of proviral load and detection of persistent lymphocytosis. Groups were LPL cattle without lymphocytosis (white bars), HPL cattle without lymphocytosis (dark gray bars), and HPL cattle with lymphocytosis (light gray bars). NEG = Seronegative (no titer detected).

Titers of antibodies against BLVgp51 and BLVp24 did not fluctuate more than 2 successive dilutions in 98% of the cattle during the study period. Ranges for median values of anti-BLVgp51 and anti-BLVp24 antibodies in lymphocytotic cattle were determined (**Table 1**).

All cattle classified as having persistent lymphocytosis had > 100,000 copies of provirus/µg of DNA extracted from PBLs in each of the 3 samples tested (**Figure 1**). Nonlymphocytotic cattle could be segregated into 2 groups on the basis of the number of proviral copies integrated in their genome. Proviral load was not significantly associated with age. Fifty-three of 134 (39.6%) nonlymphocytotic cattle had > 100,000 proviral copies integrated in their genome. Antibody titers against BLVgp51 and BLVp24 in this group were typically slightly lower, compared with titers for cattle with persistent lymphocytosis, but this difference was not significant. Therefore, cattle with > 100,000 copies of provirus were considered HPL cattle, regardless of lymphocyte count. In the remaining 81 (60.4%) nonlymphocytotic cattle, no provirus or < 100 proviral copies/µg of DNA were detected in PBLs. This group was considered LPL cattle. Ranges of median values for anti-BLV antibodies of the 2 groups of nonlymphocytotic cattle were determined (Table 1). All nonlymphocytotic cattle with LPL had anti-BLVgp51 antibodies, although titers were typically lower and significantly different from those of HPL cattle with or without persistent lymphocytosis (**Figure 2**). However, the majority (84%) of LPL cattle had negative results for anti-BLVp24 antibodies in all 3 samples tested. Only 1 of 81 (1.2%) LPL cattle consistently had positive results for anti-BLVp24 antibodies in the 3 samples; the other cattle had negative or positive results, but the antibody titer was never > 50. Conversely, only 3 of 119 (2.5%) HPL cattle had negative results for anti-BLVp24 antibodies in 2 or 3 of the samples tested, whereas 93.2% consistently had positive results (mean titer, 155) for anti-BLVp24 antibodies.

Discussion

In the study reported here, the number of proviral copies in PBLs of most cattle was fairly constant during the 1-year study period. Substantial variation in proviral load was detected in only one of the analyzed cattle. Although this animal had an HPL in the first sample, proviral DNA could not be detected in the 2 subsequent samples. One possible explanation is that the first sample was obtained a few days after the animal was infected and the virus replicated until the specific immune response could control viral dissemination. Regardless of lymphocyte count, almost all cattle with HPL developed a strong humoral immune response against the major envelope glycoprotein of BLV and a humoral response against BLVp24.

The most interesting finding was that approximately 60% (81/134) of the BLV-infected cattle without lymphocytosis did not have a sufficient number of infected cells in their peripheral blood to permit detection by use of a PCR assay with a sensitivity of 50 proviral copies/µg of DNA. In another study[16] conducted by our laboratory group, in which a nested PCR assay was used, it was found that such cattle usually do not have > 2 copies of the provirus/µg of DNA. To avoid problems with carryover, it was decided for the study reported here to use a simple PCR assay, which was to the detriment of sensitivity. Others have also failed to detect BLV proviral DNA by use of DNA hibridization[30] as well as by use of single and double PCR[31,32] assays in samples obtained from seropositive cattle.

Although antibody titers of LPL cattle were significantly lower than those of HPL cattle, anti-BLVgp51 antibodies were consistently detected in LPL cattle by use of a sensitive ELISA. However, anti-BLVp24 antibodies were not detected in 84% of the LPL cattle. It seems that the small number of infected circulating lymphocytes in LPL cattle was not sufficient to induce detectable concentrations of anti-BLVp24 antibodies. The concentration of BLV-infected cells in blood should play a major part in the success or failure of BLV transmission. Development of persistent lymphocytosis has been considered an important risk factor for transmission of BLV because the number of infected lymphocytes in a volume of blood as small as 0.05 µL is sufficient to transmit the infection to sheep.[33]

The risk of transmitting BLV infection from BLV-infected nonlymphocytotic cattle may differ depending on the proviral load. We propose that nonlymphocytotic cattle with HPL are efficient transmitters (as efficient as lymphocytotic cattle), whereas nonlymphocytotic cattle with LPL are inefficient transmitters of the infection under standard husbandry conditions.

Selective culling of seropositive cattle has been recommended to limit the spread of BLV infection when the immediate culling of all seropositive cattle is impractical. Overall seroprevalence in the analyzed herds was 33%, which did not differ substantially from the prevalence of cattle with lymphocytosis (approx 30%) detected in dairy herds with high prevalence of BLV-infected cattle.[6]

The removal of genetically superior cattle may be undesirable, especially from herds that provide breeding stock, semen, and embryos. Criteria for selective culling include development of persistent lymphocytosis and detection of viral antigen in PBL cultures.[34] We propose that the proviral load could also be used for selective culling among BLV-infected nonlymphocytotic cattle. Because most LPL cattle do not develop anti-BLVp24 antibodies, it seems that lack of anti-BLVp24 antibodies would be a good marker of this condition.

Apparently, some cattle have an intrinsic capability for controlling proviral load, and this condition may be associated with a difference in the immune response to the virus, compared with the response for HPL cattle. Additional studies to determine whether the ability to limit BLV dissemination is controlled by genetic factors or is influenced by differences in viral strains are appropriate.

a. Micros 60 OT, Horiba ABX Diagnostics, Montpellier, Cedex, France.
b. *T-plus*, Catalogue No. E1201, Inbio-Highway, Tandil, Argentina.
c. PTC-100, MJ Research Inc, Waltham, Mass.
d. Catalogue No. K2020, Invitrogen, Carlsbad, Calif.
e. Catalogue No. 167695, Nunc, Kamstrup, Roskilde, Denmark.
f. Catalogue No. L47, Oxoid Ltd, Basingtoke, Hampshire, England.
g. Catalogue No. 26-060-085, Jackson ImmunoRes Lab Inc, Philadelphia, Pa.
h. Catalogue No. SA-5100, Vector Lab, Burlingame, Calif.
i. Catalogue No. TMBE-1000, Moss, Pasadena, Md.
j. EL311sx, Bio-Tek Instruments Inc, Winooski, Vt.

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