

Detection of bovine leukemia virus by in situ polymerase chain reaction in tissues from a heifer diagnosed with sporadic thymic lymphosarcoma

Robert B. Duncan, Jr., William K. Scarratt, Gertrude C. Buehring¹

Abstract. An 18-month-old bovine heifer was presented for clinical evaluation after a sudden onset of ventral edema. Clinical and pathological evaluations were consistent with thymic lymphosarcoma, a sporadic form of lymphosarcoma in cattle, which is not generally considered to be associated with bovine leukemia virus (BLV). This heifer was seropositive for BLV at 6 and 18 months of age. Tissues obtained at necropsy were evaluated using in situ polymerase chain reaction. The BLV proviral DNA was detected in lymphocytes of the thymus as well as in epithelial cells of the liver and kidney. This report presents evidence that thymic lymphosarcomas can be associated with BLV infection and that BLV may have a broader cellular tropism than was supposed previously.

Keywords: Bovine leukemia virus; heifers; sporadic thymic lymphosarcoma.

Bovine lymphosarcomas have traditionally been categorized into 2 main types, enzootic bovine lymphosarcoma (EBL) and sporadic bovine lymphosarcoma (SBL). The distinction between the 2 has been based on frequency of occurrence, clinical manifestations and organ distribution, age of onset, and serologic or molecular association with bovine leukemia virus (BLV).¹⁸ These distinctions have been substantiated by many studies.^{7,12,18} Sporadic bovine lymphosarcoma occurs rarely and sporadically, whereas EBL occurs commonly and in enzootic patterns.¹⁸ Sporadic bovine lymphosarcoma is generally observed in animals 6–24 months old and is clinically characterized by either multicentric lymph node involvement (calf or juvenile form, less than 6 months old), thymic involvement presenting as an anterior mediastinal mass (thymic or adollescent form, less than 2 years old), or cutaneous involvement (cutaneous or skin form, 1–3 years old).^{12,18} Enzootic bovine lymphosarcoma occurs in adult cattle usually older than 4 years of age but may occur in cattle as young as 2 years.¹⁸ Almost any organ can be involved; however, heart, abomasum, uterus, and visceral and peripheral lymph nodes are most commonly involved.¹² The neoplastic cell phenotype in EBL is the B lymphocyte. Although 1 study reported SBL to be of T cell origin,⁶ more recently, others have provided evidence that in some cases of SBL (calf form) the tumor cells were of T cell origin, whereas in other cases, they were of B cell origin.^{1,4,17,19} On the basis of

the current literature, SBL is believed not to be associated with BLV infection,⁸ whereas EBL is believed to be caused by infection with BLV. Several studies during the past decade using newer techniques have indicated that the distinctions between SBL and EBL may not be as well defined as was believed previously. In a recent study by Jacobs et al.,⁶ 4 out of 10 calves with SBL were found to be positive for BLV infection either by immunologic assays or polymerase chain reaction (PCR) and 2 out of 11 cattle diagnosed with EBL were negative for BLV by both serology (agar gel immunodiffusion [AGID] assay) and PCR of tumor cell DNA. These results raise the question of whether BLV infection status should be part of the operational distinction between the 2 forms of bovine lymphosarcoma.

The authors report in this study, a case diagnosed as the thymic form of SBL and an investigation to determine its association with BLV infection. An 18-month-old, 360-kg Holstein heifer was presented to the Virginia–Maryland Regional College of Veterinary Medicine Teaching Hospital with nonpainful, pitting subcutaneous edema in the submandibular, ventral cervical, and ventral thoracic regions and proximal forelimbs. The rectal temperature (39.7 C), heart rate (108/minute), and respiratory rate (48/minute) were increased. Left and right jugular veins were distended. With auscultation, heart sounds were normal on right hemithorax and absent on the left hemithorax. Bilaterally, breath sounds were increased dorsally and diminished ventrally.

A complete blood count showed leukocytosis (24,000/ μ l; reference 4,000–12,000/ μ l), band neutrophilia (264/ μ l; reference 0–120/ μ l), and lymphocytosis (19,680/ μ l; reference 2,500–7,500/ μ l). Radiography revealed an increased soft tissue opacity in the ventral thorax to the level of the vena cava, and ultrasonog-

From Department of Biomedical Sciences and Pathobiology, Department of Large Animal Clinical Sciences, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24061 (Duncan, Scarratt), and Infectious Diseases Division, School of Public Health, University of California, Berkeley, CA 94720 (Buehring).

¹Corresponding Author: Gertrude C. Buehring, School of Public Health, University of California, Berkeley, CA 94720.

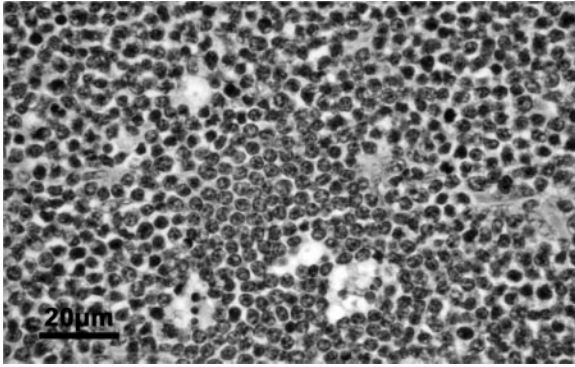


Figure 1. Lymphoid tumor from the thymus of the heifer diagnosed with SBL (hematoxylin and eosin–stained tissue section). Note architectural effacement by a monotonous population of small, round (lymphoid) cells arranged in dense sheets, with round, coarsely granular, basophilic nuclei and scant amphophilic cytoplasm.

raphy showed pleural effusion in the left and right hemithorax. Thoracentesis of the right hemithorax drew hazy, orange fluid containing an abundance of small lymphocytes (90%) and lymphoblasts (10%) with an occasional mitotic figure, suggestive of lymphosarcoma.

Necropsy confirmed marked ventral subcutaneous and interstitial edema. Approximately 10 liters of light yellow, transparent pleural fluid was present. Located in the cranial mediastinum was a large, homogeneous, pale tan mass with similar cut surfaces. There were fibrous adhesions between the visceral and parietal pleura; multifocal, pale tan nodules were on the visceral pleura and attached to margins of the lung lobes. Retropharyngeal, mediastinal, and tracheobronchial lymph nodes were enlarged and effaced by similar homogeneous tissue.

Histologically, dense sheets of small, discrete, (lymphoid) round cells with round hyperchromatic, coarsely granular nuclei and scant amphophilic cytoplasm effaced the previous architecture of the thymus, lymph nodes, and multifocal areas of the visceral pulmonary pleura (Fig. 1). This tumor was classified as a diffuse, large, noncleaved lymphoma, according to consensus classification schemes.⁵ Immunologic typing^a showed that lymphoblasts of the tumor bore CD79a markers but were negative for CD3 markers, thus identifying their B cell origin. On the basis of clinical and pathologic evaluations, a diagnosis of the thymic form of SBL was made.

The heifer was born, raised, and maintained on a farm with a high incidence of BLV infection and was seropositive for BLV by enzyme-linked immunosorbent assay (ELISA)^b at 6 months of age and by AGID^c at the time of clinical presentation. The dam of the heifer was seropositive for BLV by ELISA. These findings led the authors to investigate whether the BLV

seropositivity represented an infection associated with the disease outcome or was merely coincidental. The authors used in situ (IS)–PCR to determine whether BLV was present in the tumor tissue as well as in spleen, liver, kidney, lung, and intestine. As negative controls, the authors evaluated samples of thymus, spleen, liver, kidney, and lung from a BLV-seronegative Holstein calf stillborn to a BLV-seronegative dam. The IS-PCR was used instead of standard solution PCR because the reaction is done directly on a fixed tissue section on a slide, and therefore, the signal can be localized to a specific cell type. In an animal with BLV-positive lymphocytes, any tissue would contain lymphocytes circulating in the blood, and a positive reaction from digested/extracted tissue would, therefore, have little significance in determining viral tropism for different tissue types.

The IS-PCR method was based on a previously published protocol.¹¹ Tissues were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned 4 μ thick, and deparaffinized. In brief, tissue sections and monolayer control cells were pepsin digested, then surrounded with Easiseal gaskets.^d Fifty microliters of PCR reaction mix (4.5 mM MgCl₂, 0.4 mM deoxynucleoside triphosphate, 1 μ M primers, 0.06% bovine serum albumin, 8 μ M digoxigenin-11-deoxyuridine triphosphate,^e and 5 U/ μ l Amplitaq Gold polymerase^f) was applied to the sample, covered with a plastic seal, and the slide placed onto the IS-PCR machine.^d Primer sequences from the tax region of the BLV genome sequence according to Sagata et al.¹⁵ were as follows:

5'-ATGTCACCATCGATGCCTGG
3'-CATCGGCGGTCCAGTTGATA.

Cycling parameters used were: 1 cycle of 94 C for 2 minutes, 56 C for 1.5 minutes; then 30 cycles of 94 C for 30 minutes, 56 C for 1.5 minutes, 72 C for 2 minutes; followed by a soak at 4 C. After removal of gaskets and seals, slides were rinsed once with phosphate-buffered saline, and the PCR product was detected with a standard immunoperoxidase assay, after quenching endogenous peroxidase with 2% hydrogen peroxide. The detection antibody was anti-digoxigenin-11-peroxidase,^e and the chromagen was diaminobenzidine. Controls were run simultaneously and under identical conditions as the experimental samples. The positive control was a smear of C72/BLV cells (a bovine mammary epithelial cell line transfected with the BLV genome)¹⁰ reacted with the complete PCR reaction mix. The negative controls were a smear of C72/BLV cells and an adjacent serial section of each tissue reacted with the PCR reaction mix minus the primers. The final outcome measurement was a semiquantitative judgment of color density; only ratings of $\geq 2+$ (tan color) were reported as positive. The positive control cell line and a section of a control tissue from a

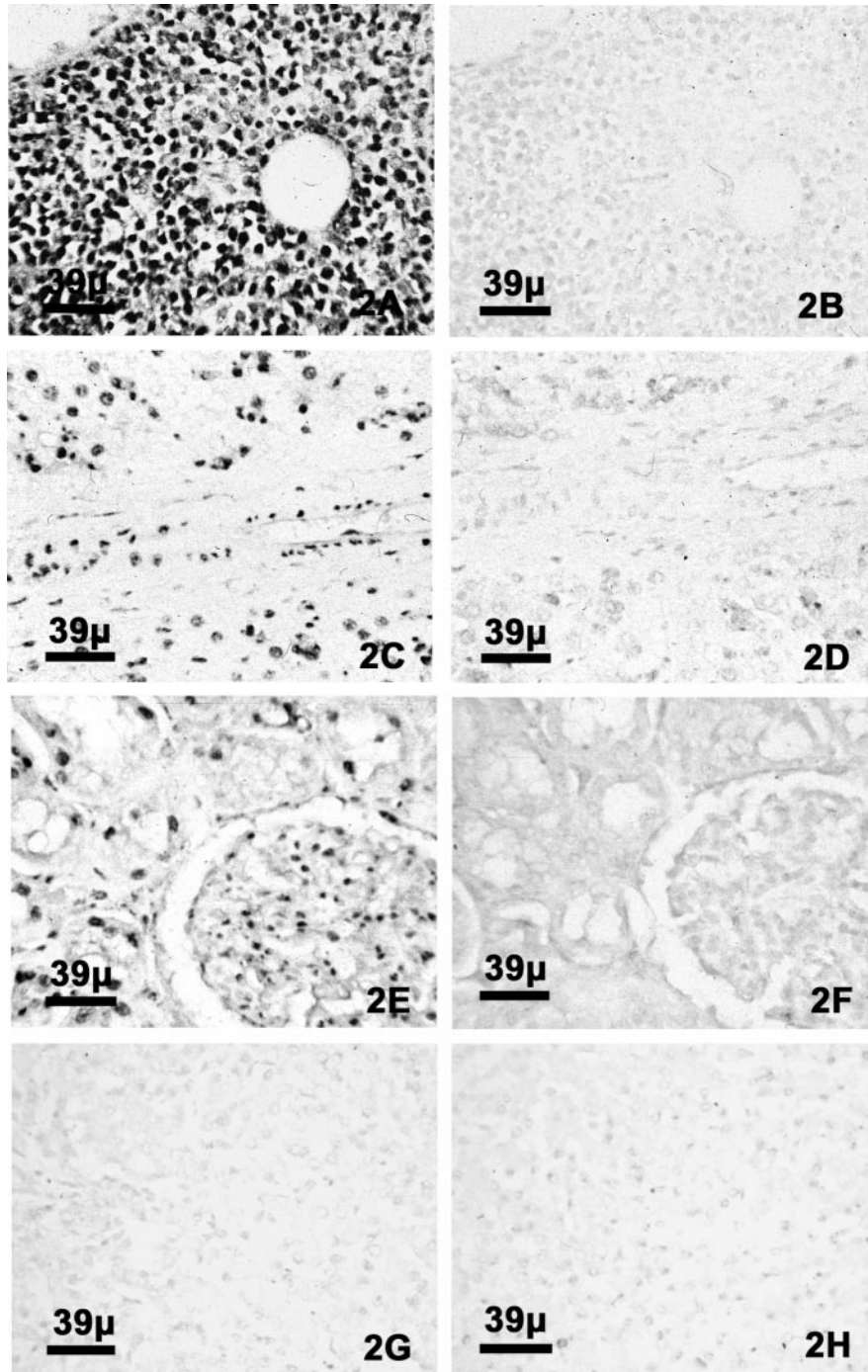


Figure 2. Adjacent serial tissue sections showing on the left of each horizontal pair, reaction with PCR mix containing primers and on the right, reaction with PCR mix without primers, as a negative control. Sections were lightly counterstained with Diff-Quik[®] to delineate nonreactive cells. **A and B**, Thymic lymphoma from the heifer seropositive for BLV. Note BLV-positive cells with dark nuclei in **A**. **C and D**, Liver from the heifer seropositive for BLV. Note BLV-positive cells with dark nuclei in **C**. **E and F**, Kidney from the heifer seropositive for BLV. Note BLV-positive cells with dark nuclei in **E**. **G and H**, Thymus from the control calf seronegative for BLV. Note lack of reactivity in **G**. Note the lack of reactivity in all of the negative controls **B, D, F, and H**.

different animal with mammary epithelium positive for BLV by IS-PCR were also tested by standard solution PCR and found to be positive for BLV.²

Thymic lymphoid cells from the heifer with SBL

were positive for BLV proviral DNA by IS-PCR (Fig. 2A). Quite unexpectedly, approximately 50% of the epithelial cells in the liver (Fig. 2C) and kidney (Fig. 2E) were positive, and an occasional large, round cell

was positive in the spleen and lung (not shown). No BLV-positive cells were seen in any of the tissues from the BLV-seronegative calf (Fig. 2G).

Cattle with sporadic forms of bovine lymphosarcoma are defined as being seronegative for BLV.¹² That fact is perplexing regarding this case because this heifer was born to a seropositive dam and was unexpectedly seropositive itself at 6 and 18 months of age. Several explanations are possible. The seropositivity at 6 months of age could represent passive transfer of maternal antibodies through perinatal consumption of colostrum,⁷ and the seropositivity at 18 months could represent seroconversion because of BLV infection. Alternatively, the seropositivity at 6 months could have resulted from BLV transmission at birth because of traumatic parturition. Four to eight percent of calves born to seropositive dams in naturally infected herds are infected with BLV at birth, as documented by positive sera collected before administration of colostrum.⁷ Such calves would likely seroconvert at least 2- to 3-weeks postpartum, and seropositive status would be sustained. Regardless, bovine lymphosarcoma with features of the sporadic thymic form are not typically associated with BLV-positive titers.

Findings of persistently seronegative, PCR-positive and seropositive, PCR-negative cattle indicate that BLV cannot be excluded as a causative agent in SBL.¹² The authors documented proviral DNA by IS-PCR, confirming BLV infection of lymphocytes in the tumor. These findings corroborate those of others⁶ who showed that sporadic forms of bovine lymphosarcoma may be associated with BLV infection. Although such situations may simply represent a coincidental infection, they raise the question of whether some cases of the thymic form of SBL could actually be EBL in a younger animal and emphasize the difficulty of making a sharp distinction between SBL and EBL.

Bovine leukemia virus cellular tropism was once believed to involve only B lymphocytes because immunohistochemistry (IHC) failed to identify BLV in any other cell types.^{3,13} More recently, BLV has been identified in T lymphocytes (by PCR),¹⁶ mammary gland epithelium (by PCR and IHC),² and endothelial cells (by PCR and IHC).¹⁴ The results of this study suggest that BLV tissue tropism may be even broader. Kettman et al.,⁹ using solution hybridization, found BLV DNA in kidney and liver. However, their technique could not distinguish whether the positive reactions were attributable to infection of organ-specific epithelium or to infected B lymphocytes present in those tissues. The finding of this study by IS-PCR of BLV proviral DNA localized in kidney and liver epithelium suggests that the results of Kettman et al. could have been because of BLV infection of kidney and liver epithelium. This expanded cellular tropism the authors observed broad-

ens the perspective of the pathogenesis and transmission of BLV.

Acknowledgements. The authors are grateful to Jill Songer, Luther Vest, Meg Berger, Brent Sugimoto, Christopher Woo, Rebecca Liu, and Hua Min Shen for technical assistance and to Dr. K. Yeon Choi for helpful advice.

Sources and manufacturers

- a. Diane Naydan, Immunohistochemistry Laboratory, Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California, Davis, CA.
- b. HerdChek® Bovine Leukemia Virus Antibody Test, Idexx Laboratories Inc., Westbrook, ME.
- c. Leukassay B® Bovine Leukemia Virus Antibody Test Kit, distributed by Schering-Plough Corporation, Kenilworth, NJ.
- d. Easiseal gaskets, Hyb-Aid in situ PCR machine, distributed by Continental Lab Products Inc., San Diego, CA.
- e. Digoxigenin-11-dUTP, anti-digoxigenin-11-peroxidase, Roche Diagnostics Corp., Indianapolis, IN.
- f. Amplitaq gold polymerase, Applied Biosystems, Foster City, CA.
- g. Diff-quick® stain, Baxter Healthcare Corporation, McGaw Park, IL.

References

1. Asahina M, Kimura K, Murakami K, et al.: 1995, Phenotypic analysis of neoplastic cells from calf, thymic, and intermediate forms of bovine leukosis. *Vet Pathol* 32:683-691.
2. Buehring GC, Kramme PM, Schultz RD: 1994, Evidence for bovine leukemia virus in the mammary epithelial cells of infected cows. *Lab Invest* 71:359-365.
3. Ferrer JF, Cabradilla C, Gupta P: 1980, Bovine leukemia virus: a model for carcinogenesis. *Cold Spring Harbor Conf Cell Prolif* 7:887-899.
4. Ishiguro N, Matsui T, Shinagawa M: 1994, Differentiation analysis of bovine T-lymphosarcoma. *Vet Immunol Immunopathol* 41:1-17.
5. Jacobs RM, Messick JB, Valli VE: 2002, Tumors of the hemolymphatic system. *In: Tumors in domestic animals*, ed. Meuten DJ, 4th ed. Iowa State Press, Ames, IA.
6. Jacobs RM, Song Z, Poon H, et al.: 1992, Proviral detection and serology in bovine leukemia virus-exposed normal cattle and cattle with lymphoma. *Can J Vet Res* 56:339-348.
7. Johnson R, Kaneene JB: 1992, Bovine leukemia virus and enzootic bovine leukosis. *Vet Bull* 62:287-312.
8. Jones TC, Hunt RD, King NW: 1997, *Veterinary pathology*, 6th ed. Williams and Wilkins, Baltimore, MD.
9. Kettman R, Burny A, Cleuter Y, et al.: 1978, Distribution of bovine leukemia virus proviral DNA sequences in tissues of animals with enzootic bovine leukosis. *Leuk Res* 2:23-32.
10. Motton DD, Buehring GC: 2003, Bovine leukemia virus alters growth properties and casein synthesis in mammary epithelial cells. *J Dairy Sci* 86:2826-2838.
11. Nuovo GJ: 1997, *PCR in situ hybridization: protocols and applications*, 3rd ed. Lippincott-Raven, Philadelphia, PA.
12. Radostits OM, Gay CC, Blood DC, Hincheliff KW: 2000, *Veterinary medicine: a textbook of the diseases of cattle, sheep, pigs, goats, and horses*, 9th ed. WB Saunders, Philadelphia, PA.
13. Reinacher M, Thurmond M, Onuma M, et al.: 1989, Immunohistological demonstration of virus and tumor associated antigen in tissues in experimental and spontaneous bovine leukemia virus (BLV) infection. *Vet Immunol Immunopathol* 22:223-231.
14. Rovnak J, Casey JW, Boyd AL, et al.: 1991, Isolation of bovine

- leukemia virus infected endothelial cells from cattle with persistent lymphocytosis. *Lab Invest* 65:192–202.
15. Sagata N, Yuanaga T, Tsuzuku-Kawamura J, et al.: 1985, Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses. *Proc Natl Acad Sci USA* 82:677–681.
 16. Stott ML, Thurmond MC, Dunn SJ, et al.: 1991, Integrated bovine leukosis proviral DNA in T helper and T cytotoxic/suppressor lymphocytes. *J Gen Virol* 72:307–315.
 17. Tani K, Asahina M, Wu DL, et al.: 1997, Further analysis of the phenotype and distribution of tumor cells in sporadic B-cell and T-cell lymphomas in the lymph node and spleen of cattle. *Vet Immunol Immunopathol* 55:283–290.
 18. Thurmond M: 1996, Bovine lymphosarcoma. *In: Large animal internal medicine*, ed. Smith BP, 2nd ed. Mosby, St. Louis, MO.
 19. Yin SA, Makara M, Pan Y, et al.: 2003, Relationship between phenotype of tumor cells and clinicopathology in bovine leukosis. *J Vet Med Sci* 65:599–606.

J Vet Diagn Invest 17:194–197 (2005)

Neuronal ceroid-lipofuscinosis in a Holstein steer

S. Hafner,¹ T. E. Flynn, B. G. Harmon, J. E. Hill

Abstract. A young, partially blind Holstein steer was affected by mild cerebral atrophy. Formalin-fixed cerebral gray matter was diffusely yellow brown. Microscopically, there were eosinophilic, autofluorescent granules primarily in the cytoplasm of cerebral neurons. There was also extensive retinal atrophy with complete loss of the rod and cone layers. Ultrastructural examination of affected cerebral neurons revealed a mixture of granular osmiophilic and lamellar patterns in the cytoplasmic storage bodies. This suggests the existence of neuronal ceroid-lipofuscinosis in the Holstein breed.

Key words: Bovine; ceroid; Holstein; immunohistochemistry; lipofuscin.

Neuronal ceroid-lipofuscinosis (NCL) has been described as a naturally occurring disease in many animal species. The disease is encountered most frequently in dogs, affecting various breeds including the Australian Cattle Dog, Border Collie, Chihuahua, Cocker Spaniel, Corgi, Dachshund, Dalmatian, English Setter, Golden Retriever, Gordon Setter, Japanese Retriever, Labrador Retriever, Miniature Schnauzer, Polish Owczarek Nizinny, Standard Poodle, Saluki, Tibetan Terrier, and Yugoslavian Shepherd.^{1,2,10,13–15,17} Neuronal ceroid-lipofuscinoses have also been described in Icelandic/Peruvian Paso horses,¹⁶ Siamese and Domestic cats, ferrets, Nubian goats, Merino, Rambouillet, South Hampshire, and White Swedish Landrace sheep,^{3,10} *mnd* and *nclf* mice,^{5,10} a monkey,¹⁴ and Devon and Beefmaster cattle.^{6,8,12} The term NCL is traditional but not accurate because storage often is not limited to neurons, and the storage bodies in many affected animals (including Devon cattle) have been shown to be composed primarily of an extremely hydrophobic protein—subunit C of mitochondrial adenosine triphos-

phate synthase (SCMAS)—rather than ceroid or lipofuscin.⁷ In some affected animals, especially Miniature Schnauzers and White Swedish Landrace sheep, sphingolipid activator proteins (SAPs) are the primary component of storage bodies.³

In humans, NCLs are a common cause of neurodegeneration in children, with some forms additionally or primarily presenting in adults. Human NCLs traditionally have been classified by age of onset combined with ultrastructural phenotype: granular osmiophilic deposits characteristic of SAPs versus the lamellar appearance of SCMAS.^{5,9} However, most human NCLs are now classified on the basis of predicted or defined defects in 7 genes as CLN1 through 8. The CLN1 encodes palmitoyl-protein thioesterase, and the storage material accumulating in affected infants are SAPs, but SCMAS is the main component of the storage material in CLN2-8.^{5,7} The inheritance of many animal and human NCLs has been determined to be recessive, and vision loss has been documented in both human and animal NCLs.^{5,9,16}

A 15–18-month-old Holstein steer with a history of progressive blindness was presented for slaughter. Grossly, the eyes were unremarkable with the exception of dilated pupils. The animal appeared to have a marked visual deficit, but was afebrile, placid, and well conditioned. Because of the possibility of a central nervous system disorder, the animal was excluded from

From the USDA-FSIS, Eastern Laboratory, Russell Research Center, Athens, GA 30604 (Hafner, Hill), the USDA-FSIS Field Operations, 2810 Crossroads Drive, Madison, WI 53718 (Flynn), and the Department of Veterinary Pathology, University of Georgia, Athens, GA 30602 (Harmon).

¹Corresponding Author: S. Hafner, USDA-FSIS Eastern Laboratory (Pathology), PO Box 6085, Russell Research Center, 950 College Station Road, Athens, GA 30604.