The Journal of Veterinary Medical Science

NOTE

Pathology

Bovine leukemia virus-associated B cell lymphoma with severe pleomorphism in a steer

Akiyo HAGIWARA[1)#*], Asami NISHIMORI[2)#], Shinichi HATAMA[2)], Osamu MIKAMI[3)], Yoshiharu ISHIKAWA[3)], Koichi KADOTA[3)#]

[1)]Saitama Prefecture Meat Inspection Center, Saitama, Japan
[2)]National Institute of Animal Health, National Agriculture and Food Research Organization, Ibaraki, Japan
[3)]Sapporo Research Station, National Institute of Animal Health, National Agriculture and Food Research Organization, Hokkaido, Japan

J. Vet. Med. Sci.
84(9): 1272–1276, 2022
doi: 10.1292/jvms.21-0607

Received: 16 November 2021
Accepted: 1 July 2022
Advanced Epub:
1 August 2022

ABSTRACT. We examined a 26-month-old steer with neoplastic lesions in the spleen, lymph nodes, heart and kidneys, characterized by pleomorphic lymphoid cells that were immunohistochemically positive for CD20. The presence of bovine leukemia virus (BLV) at >200,000 copies per 100,000 cells by quantitative RT-PCR was considered to be due to random integration of the provirus into the neoplastic cells´ genomes. Inverse PCR identified the presence of one, two, two and three different malignant clones in the heart, spleen, mesenteric node and blood, respectively. Because BLV can rapidly induce lymphoma and a high proviral load facilitates B-cell carcinogenesis, multiclonal tumor development was suspected in the present case.

KEYWORDS: bovine leukemia virus, cattle, inverse polymerase chain reaction, multiclonality, pleomorphic lymphoma

In humans, adult T cell leukemia/lymphoma (ATLL) is caused by the malignant transformation and clonal outgrowth of T cells infected with human T cell leukemia virus type 1 (HTLV-1). It develops following a clinically latent period of approximately 60 years after mother-to-child transmission via breastfeeding [1]. Bovine leukemia virus (BLV), which is closely related to HTLV-1, causes enzootic bovine leukosis (EBL), a B cell lymphoma. This disease generally develops after an average incubation period of 7 years [15]. However, EBL or pleomorphic B cell lymphoma in calves has also been reported [5, 12, 21] and is presumably caused by transplacental infection of bovine leukemia virus (BLV) in dams with a large viral load [16]. This suggests that BLV-associated lymphoma can develop after a relatively short latency period, and that the tumorigenesis process is different for EBL and ATLL.

In humans, multiple HTLV-I integrations can be detected by Southern blotting as bands with different intensities, which can arise from one tumor cell clone carrying multiple copies of the provirus, or from multiple cell clones, each carrying one copy of the provirus [7, 17, 18]. Similar results were obtained in EBL cases, but some bands of numerically minor cell clones which theoretically should exist were not visible [13]. This phenomenon is partially due to the fact that ordinary Southern blot analysis is less sensitive than polymerase chain reaction (PCR)-based methods such as inverse PCR (iPCR) [1]. In the present paper, we report a case of pleomorphic B cell lymphoma, in which multiple BLV integrations suggestive of true multicentric occurrence of lymphoma were detected by iPCR.

A crossbred (Japanese Black × Holstein) steer aged 26 months appeared healthy on examination before slaughter. However, macroscopically, multiple milky-white homogeneous tumor nodules or masses 3–8 cm in diameter were observed on the auricle and atrium and in the ventricular myocardium. The spleen was twice normal size, and had a fragile consistency. Numerous 0.3–0.6 cm diameter milky-white nodules were present in the cortex of both kidneys. The mesenteric, bronchial, internal iliac and popliteal lymph nodes were enlarged, with milky-white and homogeneous cut surfaces.

Tissue samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (HE). Immunostaining was performed using the streptavidin-biotin complex/horseradish peroxidase (SAB) method, by means of a SAB kit (Nichirei, Tokyo, Japan) with primary rabbit polyclonal antibodies to CD20 (prediluted; Spring Bioscience, Pleasanton, CA, USA), CD3 (1:50; Dako A/S, Glostrup, Denmark), κ light chain and λ light chain (prediluted; Fitzgerald, Acton, MA, USA), and a mouse monoclonal antibody to CD5 (1:400; Pierce Biotechnology, Rockford, IL, USA).

*Correspondence to: Hagiwara A: hagiwara.akiyo@pref.saitama.lg.jp, Saitama Prefecture Meat Inspection Center, 5-18-24 Kamiochiai, Chuo-ku, Saitama 338-0001, Japan
#These authors contributed equally to this work.

DNA obtained from spleen, mesenteric lymph node, heart and blood was analyzed by quantitative real-time PCR (qPCR) using a Cycleave PCR BLV detection kit (Takara Bio, Kusatsu, Japan) [6], and by qPCR using coordination of common motifs (CoCoMo) primers (Riken Genesis, Yokohama, Japan) [14]. For the demonstration of clonally integrated BLV in lymphoid cells, DNA was also analyzed by iPCR as previously described [9, 10] with modifications to reduce operation time using a restriction enzyme *PstI* [11].

By histology, the mesenteric lymph node and spleen were seen to be nearly completely replaced by lymphoma cells of different sizes. Other lymph nodes such as the bronchial, medial iliac and popliteal lymph nodes were moderately affected. Although areas of smaller lymphoma cells were noticed, the most predominant were large cells with round to oval nuclei in the nodes (Fig. 1A, 1B), and very large atypical cells with irregular nuclei in the spleen (Fig. 1C). Lymphoid follicles were atrophic, but a few were large and composed of mantle cell lymphoma (MCL) cells with round to elongated nuclei, slightly condensed chromatin, inconspicuous nucleoli and moderate amounts of cytoplasm (Fig. 1D, 1E). Some larger cells were admixed with them. Variously sized lymphoma cells were present in the renal stroma and hepatic sinusoids. In the heart, neoplastic infiltrates were of relatively small to medium-sized lymphoid cells with round to oval nuclei and scanty cytoplasm (Fig. 1F). Cytological features of lymphoma cells in the heart, spleen and mesenteric lymph node are presented in Table 1. Immunohistochemically, the lymphoma cells were positive for CD20 (Fig. 2A–C). Less than 10% of lymphoma cells were positive for CD5 (Fig. 2D). Lymphoma cells were negative for CD3 and immunoglobulin light chains.

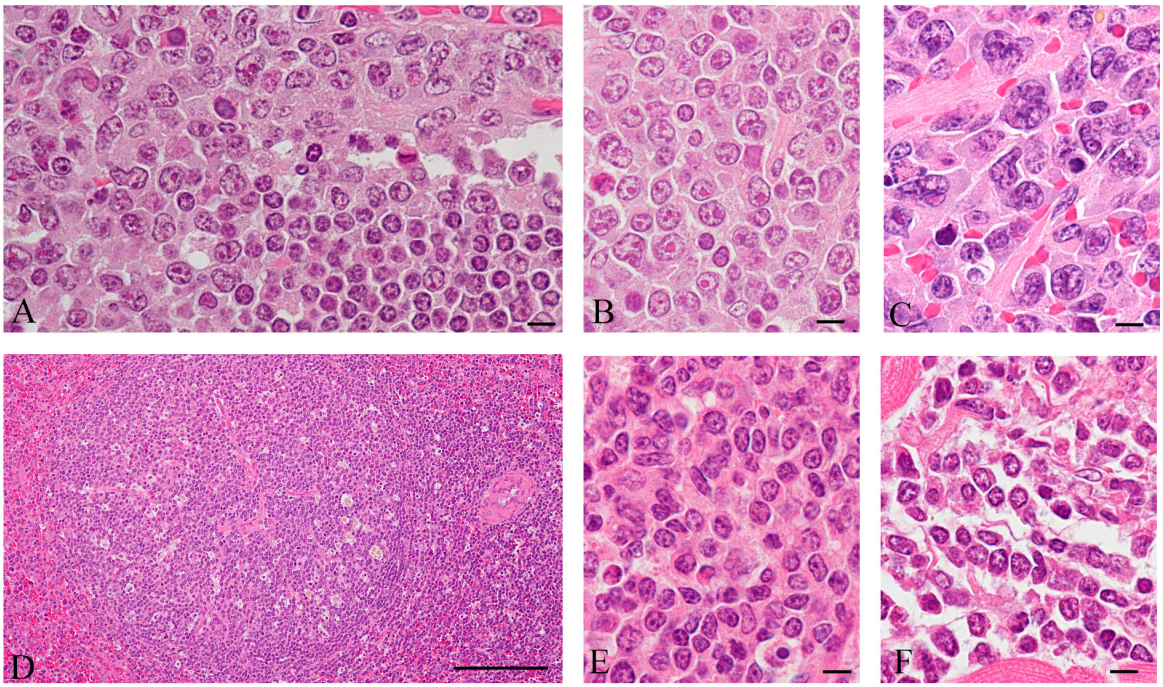

Fig. 1. Histology, hematoxylin and eosin. (**A**) Mesenteric lymph node. Area of large lymphoma cells with irregular nuclei and smaller lymphoma cells (lower right). Bar=5 µm. (**B**) Mesenteric lymph node. Large lymphoma cells with prominent nucleoli. Bar=5 µm. (**C**) Spleen. Atypical giant cells with highly irregular bizarre nuclei. Bar=5 µm. (**D**) Spleen. Lymphoid follicle is replaced by mantle cell lymphoma cells. Bar=100 µm. (**E**) Spleen. Higher magnification of the follicle in Fig. 1D shows mantle cell lymphoma cells of medium size with moderate amount of cytoplasm. Bar=5 µm. (**F**) Heart. Infiltrating lymphoma cells are characterized by scant cytoplasm. Bar=5 µm.

Table 1. Cytological features of lymphoma cells

	Heart	Spleen		Mesenteric lymph node
		Pleomorphic cells	Mantle cell lymphoma cells	
Cell size	Relatively small to medium	Variable. Very large cells predominate	Medium. Larger in some cells	Variable. Large cells predominate
Nuclei	Round to oval, occasionally irregular	Round to markedly irregular	Round to elongated	Round to oval or irregular
Nucleoli	Inconspicuous	Variable in size	Inconspicuous	Small to medium-sized, rarely large
Chromatin	Moderately to fairly condensed	Variable condensation	Slightly condensed	Moderately condensed
Cytoplasm	Scanty	Scanty to abundant	Moderate in amount	Scanty to abundant
Mitosis	Occasional	Frequent	Occasional	Frequent

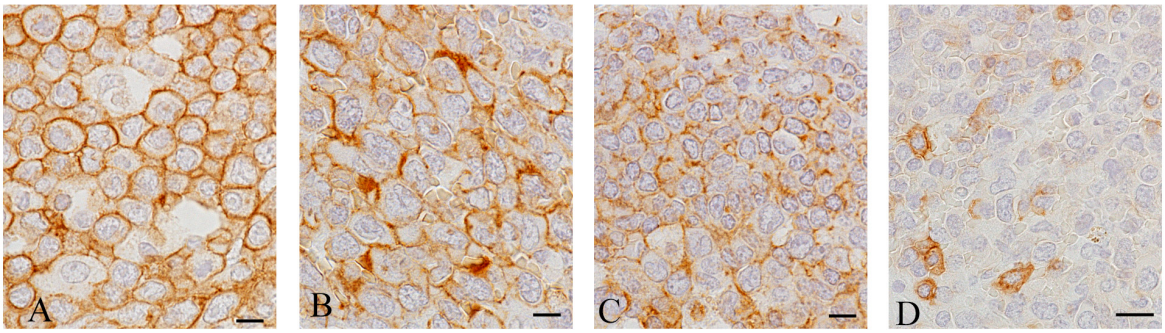

Fig. 2. Immunohistochemistry. (**A**) Mesenteric lymph node. Large lymphoma cells are positive for CD20. Bar=5 µm. (**B**) Spleen. Very Large lymphoma cells are positive for CD20. Bar=5 µm. (**C**) Spleen. Mantle cell lymphoma cells are positive for CD20. Bar=5 µm. (**D**) Spleen. Some lymphoma cells are positive for CD5. Bar=10 µm.

Table 2. Number of bovine leukemia virus proviral copies in Cycleave PCR and CoCoMo qPCR analysis

Samples	Cycleave (copies/10 ng DNA)	CoCoMo (copies/10^5 cells)
Blood	3,735	218,315
Heart	5,950	278,830
Spleen	1,900	197,910
Mesenteric lymph node	6,250	216,923

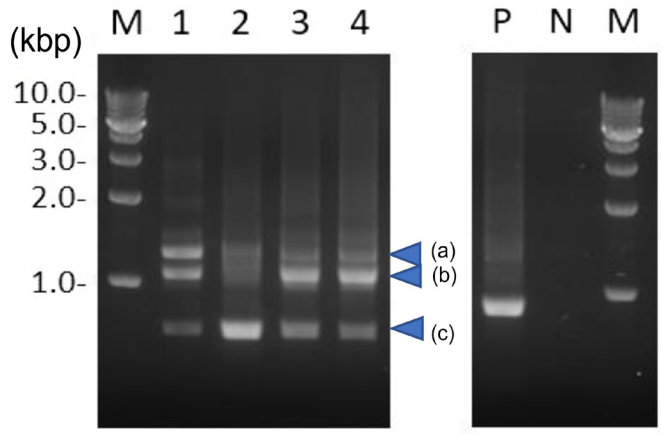

Fig. 3. Clonal integration patterns of bovine leukemia virus proviruses in lymphoid cells. Only one positive band is observed at less than 1 kbp (**c**) in the heart sample (No. 2), and an additional band of ca. 1.1 kbp (**b**) in the spleen (No. 3) and mesenteric lymph node (No. 4). In addition to these bands, another band of ca. 1.4 kbp (**a**) is present in the blood (No. 1). P, positive control, DNA extracted from a BLV-positive bovine cell line (BL2M3); N, negative control, DNA extracted from a BLV-negative bovine cell line (MDBK). M, DNA ladder; (a), 1.4 kbps; (b), 1.1 kbps; (c), <1 kbps.

Results of Cycleave PCR and CoCoMo qPCR analyses are given in Table 2. The highest proviral load was 6,250/10 ng DNA in the lymph node sample by Cycleave PCR, and 278,830 per 100,000 cells in the heart sample by CoCoMo qPCR. Clonal integration of BLV provirus was demonstrated by iPCR, which showed one, two, two and three clonal bands in the heart, spleen, mesenteric lymph node and blood, respectively (Fig. 3).

In the case described here, the neoplastic tissue was characterized by a marked variation of lymphoma cell size and shape both within single tissue sections and between affected organs, and also by the absence of immunoglobulin production [2, 4] and the presence of MCL cells in the spleen. The MCL cells resembled those in MCL with histological progression to pleomorphic lymphoma [3, 19]. Based on these findings, B cell lymphoma with severe pleomorphism was diagnosed [2–4, 19]. Etiological involvement of BLV infection was supported by genetic evidence of monoclonal integration of BLV gene in the heart sample by iPCR [12]. Moreover, the BLV proviral load was 6,250/10 ng DNA by Cycleave PCR analysis of the mesenteric lymph node. Cattle with EBL in which the proviral load of enlarged lymph nodes exceeds 1,000 may be diagnosed with BLV [20].

There was one distinct band in the same position in each of the all four samples examined by the iPCR method, with other additional bands in three samples. The consistently observed band is postulated to represent the original malignant clone that spread (metastasized) extensively to other organs. In contrast, the other bands may indicate clones that developed subsequently. Histologically, smaller cells of the first clone were considered to have metastasized to the heart, some of which may have transformed to highly atypical cells in the spleen [3, 19]. The presence of a single band in the heart sample signifies a malignant clone carrying one copy of the BLV provirus [12,

17, 18], and two bands in the lymphatic organs suggest the presence of two malignant clones. Three bands in the blood sample suggest the presence of a third clone. Similar biclonal or multiclonal proliferation of neoplastic cells has been demonstrated before in EBL by Southern blot or B cell clonality analysis [13, 21]. Considering that HTLV-1 carriers harbor a large number of HTLV-1-infected and immortalized clones [22], it may be possible that several BLV-infected lymphocytes in PL cattle undergo malignant transformation simultaneously or intermittently over short intervals. Such events may lead to the absence of distinct positive bands (faint or smeared bands) in Southern blot samples, as previously suggested [19]. Three tumor cell clones in the current case are interpretable as having populations large enough to be detected by iPCR, and more sensitive methods such as ligation-mediated PCR may demonstrate the presence of smaller populations of additional clones [22].

In the heart of the current case, the BLV proviral load as quantified by CoCoMo qPCR was approximately three times larger than in a previously reported case of pleomorphic B cell lymphoma with 98,462 copies per 100,000 cells in the blood [21]. Considering the presence of a single band in iPCR analysis of the heart sample, it is most probable that multiple random integrations occurred after neoplastic transformation [8]. Lower numbers of BLV proviruses in the other samples may be explained by the admixture of the second or third clone with fewer or absent integration after neoplastic transformation, although more advanced researches such as single-cell genome analysis using next generation sequencing technologies would be required to prove these hypotheses. High BLV proviral loads or multiple tumor cell clones may be detected more frequently in cattle that died or were euthanized due to EBL (advanced cases) than in apparently healthy slaughtered cattle, and may be linked to the degree of cytological pleomorphism and atypia [3, 19]. The present animal appeared healthy, but was considered to be at a relatively advanced stage of lymphoma, because of severe enlargement and neoplastic replacement of the spleen. In humans, patients with multiple tumor cell clones exhibit a more indolent clinical course than patients with a single tumor cell clone harboring multiple proviral copies [17, 18].

In conclusion, the neoplastic tissue was cytologically characterized by the presence of pleomorphic cells and MCL cells. High BLV proviral loads and monoclonal or oligoclonal integration of BLV provirus suggested etiological involvement of BLV infection in lymphoma development. Multicentric origin of BLV-associated lymphoma was suggested in the present study, and Its prevalence and significance should be clarified by further studies on a large number of cases.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

ACKNOWLEDGMENT. The authors would like to thank Prof. Morihiro Saito (College of Nutritional Science, Kagawa Nutrition University) for his help and advice.

REFERENCES

1. Firouzi S, Farmanbar A, Nakai K, Iwanaga M, Uchimaru K, Utsunomiya A, Suzuki Y, Watanabe T. 2017. Clonality of HTLV-1-infected T cells as a risk indicator for development and progression of adult T-cell leukemia. *Blood Adv* **1**: 1195–1205. [Medline] [CrossRef]
2. Hagiwara A, Saito M, Ishikawa Y, Kadota K. 2014. A histological study of lymphoid neoplasms infected with bovine leukosis virus. *Nippon Juishikai Zasshi* **67**: 199–203 (in Japanese with English summary).
3. Hagiwara A, Saito M, Ishikawa Y, Kadota K. 2018. Mantle cell lymphoma with histological progression to pleomorphic lymphoma in two cattle. *Jpn Agric Res Q* **52**: 49–54. [CrossRef]
4. Iwama Y, Inomata T, Ishikawa Y, Kadota K. 2013. Cytology of B cell lymphomas in cattle infected with bovine leukosis virus. *Jpn Agric Res Q* **47**: 103–107. [CrossRef]
5. Kagawa Y, Tomita K, Nakatani H, Sato K, Wada Y, Ishikawa Y, Kadota K. 2009. Immunohistochemical characterization of five types of lymphoid neoplasms in calves. *Jpn Agric Res Q* **43**: 239–245. [CrossRef]
6. Kanno T, Ishihara R, Hatama S, Oue Y, Edamatsu H, Konno Y, Tachibana S, Murakami K. 2014. Effect of freezing treatment on colostrum to prevent the transmission of bovine leukemia virus. *J Vet Med Sci* **76**: 255–257. [Medline] [CrossRef]
7. Kato N, Sugawara H, Aoyagi S, Mayuzumi M. 2001. Lymphoma-type adult T-cell leukaemia-lymphoma with a bulky cutaneous tumour showing multiple human T-lymphotropic virus-1 DNA integration. *Br J Dermatol* **144**: 1244–1248. [Medline] [CrossRef]
8. Levin A, Hayouka Z, Helfer M, Brack-Werner R, Friedler A, Loyter A. 2009. Peptides derived from HIV-1 integrase that bind Rev stimulate viral genome integration. *PLoS One* **4**: e4155. [Medline] [CrossRef]
9. Miyasaka T, Oguma K, Sentsui H. 2015. Distribution and characteristics of bovine leukemia virus integration sites in the host genome at three different clinical stages of infection. *Arch Virol* **160**: 39–46. [Medline] [CrossRef]
10. Murakami H, Yamada T, Suzuki M, Nakahara Y, Suzuki K, Sentsui H. 2011. Bovine leukemia virus integration site selection in cattle that develop leukemia. *Virus Res* **156**: 107–112. [Medline] [CrossRef]
11. Nishimori A, Andoh K, Matsuura Y, Kumagai A, Hatama S. 2021. Establishment of a simplified inverse polymerase chain reaction method for diagnosis of enzootic bovine leukosis. *Arch Virol* **166**: 841–851. [Medline] [CrossRef]
12. Oguma K, Suzuki M, Sentsui H. 2017. Enzootic bovine leukosis in a two-month-old calf. *Virus Res* **233**: 120–124. [Medline] [CrossRef]
13. Onuma M, Sagata N, Okada K, Ogawa Y, Ikawa Y, Oshima K. 1982. Integration of bovine leukemia virus DNA in the genomes of bovine lymphosarcoma cells. *Microbiol Immunol* **26**: 813–820. [Medline] [CrossRef]
14. Panei CJ, Takeshima SN, Omori T, Nunoya T, Davis WC, Ishizaki H, Matoba K, Aida Y. 2013. Estimation of bovine leukemia virus (BLV) proviral load harbored by lymphocyte subpopulations in BLV-infected cattle at the subclinical stage of enzootic bovine leucosis using BLV-CoCoMo-qPCR. *BMC Vet Res* **9**: 95. [Medline] [CrossRef]
15. Rosewick N, Momont M, Durkin K, Takeda H, Caiment F, Cleuter Y, Vernin C, Mortreux F, Wattel E, Burny A, Georges M, Van den Broeke A. 2013. Deep sequencing reveals abundant noncanonical retroviral microRNAs in B-cell leukemia/lymphoma. *Proc Natl Acad Sci USA* **110**: 2306–2311. [Medline] [CrossRef]
16. Sajiki Y, Konnai S, Nishimori A, Okagawa T, Maekawa N, Goto S, Nagano M, Kohara J, Kitano N, Takahashi T, Tajima M, Mekata H, Horii Y, Murata S, Ohashi K. 2017. Intrauterine infection with bovine leukemia virus in pregnant dam with high viral load. *J Vet Med Sci* **79**: 2036–2039. [Medline]

[CrossRef]
17. Shimamoto Y. 1997. Clinical indications of multiple integrations of human T-cell lymphotropic virus type I proviral DNA in adult T-cell leukemia/lymphoma. *Leuk Lymphoma* **27**: 43–51. [Medline] [CrossRef]
18. Shimamoto Y, Kobayashi M, Miyamoto Y. 1996. Clinical implication of the integration patterns of human T-cell lymphotropic virus type I proviral DNA in adult T-cell leukemia/lymphoma. *Leuk Lymphoma* **20**: 207–215. [Medline] [CrossRef]
19. Sochi Y, Takamori H, Sato H, Numabe T, Hagiwara A, Ishikawa Y, Kadota K. 2018. Nodal pleomorphic B cell lymphoma at the earliest stage of tumor development in a cow. *Jpn Agric Res Q* **52**: 353–358. [CrossRef]
20. Somura Y, Sugiyama E, Fujikawa H, Murakami K. 2014. Comparison of the copy numbers of bovine leukemia virus in the lymph nodes of cattle with enzootic bovine leukosis and cattle with latent infection. *Arch Virol* **159**: 2693–2697. [Medline] [CrossRef]
21. Wakatsuki T, Sugiyama S, Takaoka A, Hashida A, Mizukami C, Hirose Y, Kadota K, Takeshima S, Aida Y. 2019. A case of enzootic bovine leukosis in a five-month-old calf. *Nippon Juishikai Zasshi* **72**: 608–613 (in Japanese with English summary).
22. Watanabe T. 2017. Adult T-cell leukemia: molecular basis for clonal expansion and transformation of HTLV-1-infected T cells. *Blood* **129**: 1071–1081. [Medline] [CrossRef]