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NOTE

Internal Medicine

A clinical case of B-cell lymphoma in a Japanese Black cow with monoclonal proliferation of B-cells as revealed by polymerase chain reaction based on the immunoglobulin light chain gene

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ABSTRACT. A 35-month-old Japanese Black cow was presented to a local veterinarian with a main complaint of premature labor. There was no swelling of lymph nodes on the body surface and no palpable mass on rectal examination. Although atypical lymphocytes were observed in the peripheral blood, no lymphocytosis was recorded. On Day 12, the cow developed hindlimb paresis and was euthanized. Necropsy revealed swelling of some lymph nodes, and white nodules in uterus and right atrial appendage. Histopathological examination revealed B-cell lymphoma. In clonality analysis of peripheral blood and tumor tissue, monoclonal proliferation was detected by PCR for immunoglobulin light chain (IgL) λ, suggesting neoplastic proliferation of B-cell. IgL-PCR is thought to be a possible tool for diagnosis of B-cell lymphoma.

KEYWORDS: B-cell lymphoma, diagnosis, immunoglobulin light chain lambda, monoclonal proliferation

Most lymphomas in cattle are enzootic bovine leukosis (EBL) caused by bovine leukemia virus (BLV) infection [3]. Although clinical symptoms of EBL vary depending on the location of enlarged lymph nodes and the presence or absence of lymphocytosis, EBL is fundamentally B-cell lymphoma [3, 4, 6]. Clinical diagnosis of EBL involves the quantification of the BLV proviral load (BLV-PVL), peripheral blood lymphocyte count, cytology of enlarged lymph nodes, and evaluation of serum lactate dehydrogenase (LDH) and thymidine kinase (TK) activities [5, 9, 13, 15, 16]. In recent years, methods for evaluating the clonal state of lymphocytes have been used to diagnose lymphoma in cattle, taking advantage of the fact that EBL is a neoplastic proliferation of B-cells [12]. Clonality PCR using immunoglobulin heavy chain gene rearrangement (IgH-PCR) has been applied to the diagnosis of B-cell lymphoma in cattle [5] with high specificity, but the sensitivity of IgH-PCR has been reported to be insufficient [12]. In this study, we developed a new clonality PCR method using bovine immunoglobulin light chain λ gene rearrangement (IgL-PCR) to complement IgH-PCR and applied it to a clinical case of B-cell lymphoma in a Japanese Black cow.

The case involved a 2-year 11-month-old Japanese Black female cow that was presented to a local veterinarian with a chief complaint of premature birth and anorexia (Day 1). On Day 1, the patient was inactive but was able to stand on her own. No enlargement of superficial lymph nodes was observed, and no mass was palpable during rectal examination. A premature fetus 70 cm in length was delivered approximately 3 months earlier than expected. As EBL had occurred on the farm 3 months ago, the referral veterinarian requested an additional analysis for lymphoma at the Veterinary Medical Center of The University of Tokyo (VMCUT) on Day 2.

Complete blood counts revealed normal numbers of lymphocytes (4,260 /μL; RI: <4,600 /μL) [1]. In blood smear, most of the lymphocytes were medium in size, however, some of them were large and had a wide cytoplasm like monocytes and had constricted

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or cleaved nuclei with obvious nucleoli (Fig. 1). High levels of lactate dehydrogenase (LDH: 3,818 U/L; RI: 697–1,450 U/L) [10], and thymidine kinase (TK: 82 U/L; RI: <5.4 U/L) [16] activities were recorded. The BLV-PVL in peripheral blood measured using a BLV detection kit (TaKaRa, Kusatsu, Japan) was high at 357 copies/10 *n*g DNA, and the elevated TK activity suggested the onset of EBL [5, 9, 16].

On Day 12, astasia due to hindlimb paralysis was noted. The prognosis was judged to be poor given the inability to stand, with a high possibility of developing EBL; the cow was transferred to VMCUT for necropsy on Day 14. Upon arrival, the animal was euthanized by arterial blood exsanguination under deep anesthesia with xylazine (Xylazine Injection 2% "Fujita," Fujita Pharmaceutical Co., Ltd., Tokyo, Japan) and thiamylal sodium (Isozole, Nichi-Iko, Co., Ltd., Toyama, Japan). Necropsy revealed enlarged mesenteric, sublumbar, and mediastinal lymph nodes. The right uterine horn did not contract sufficiently, the uterine caruncle remained enlarged (Fig. 2A). The uterus was filled with grayish white pus, and white pus was also observed (Fig. 2A). White nodules in the mucosa of the uterine was observed and the cross section of the nodule was milky white in color (Fig. 2B). Similar white nodules were also found in right atrial appendage (Fig. 3), and abomasum wall. There was a large amount of adipose tissue around lumbar and sacral vertebrae in the spinal canal.

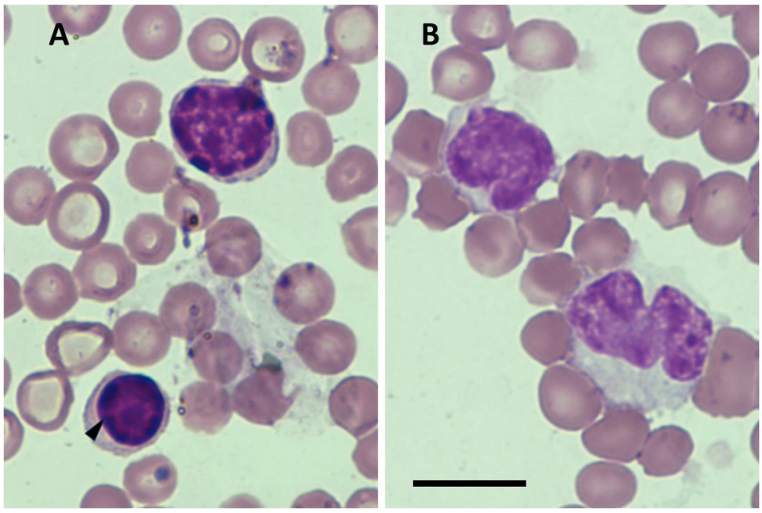

Fig. 1. Lymphocytes found in peripheral blood. (A) Most of the lymphocytes were medium in size, with no significant atypia (arrowhead). (B) Some of the lymphocytes were large and had a wide cytoplasm like monocytes. They had constricted or cleaved nuclei with obvious nucleoli. Wright-Giemsa stain. ×1,000, Bar=10 µm.

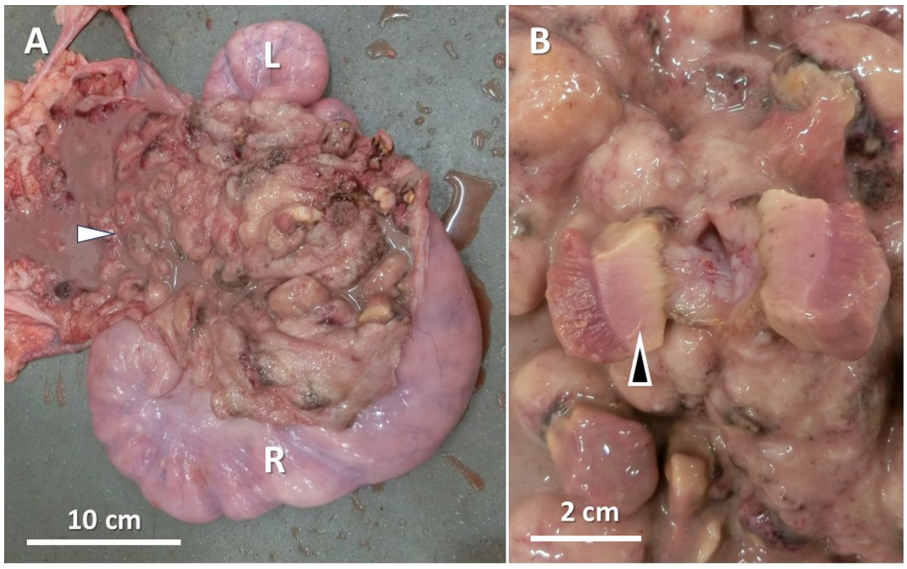

Fig. 2. (**A**) The right uterus horn (R) did not contract sufficiently compared with the left (L), the uterus caruncle remained enlarged. The uterus was filled with grayish pus (white arrowhead). (**B**) White nodules in the mucosa of the uterus were observed. The cut surface of the nodule was milky white in color (arrowhead).

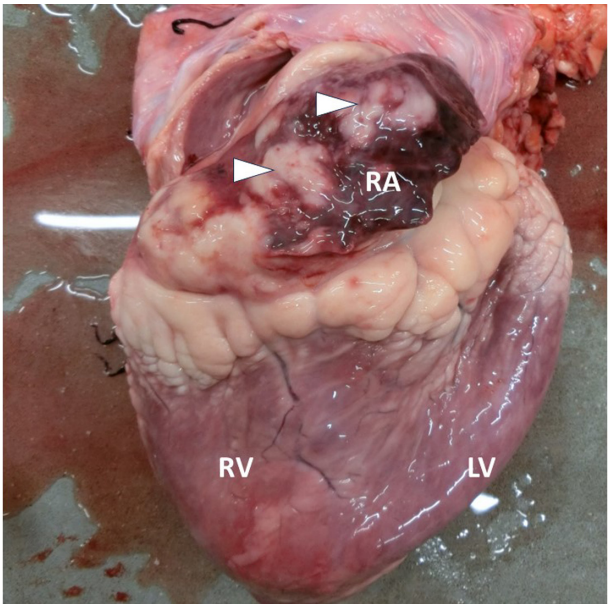

Fig. 3. Multiple white nodules were found in the right atrial appendage of the heart. RA: right atrial appendage, RV: right ventricle, LV: left ventricle.

Histopathological examination revealed the proliferation of medium to large lymphocytes with prominent nucleoli and scattered mitotic figures in the white nodules of the right uterus, heart, abomasum and mesenteric and sublumbar lymph nodes, and in the adipose tissue of the spinal canal (Fig. 4). No microscopic abnormalities were found in the lumbar spinal cord or the spinal nerves examined. The tumor cells were positive for B-cell markers CD20 (PA5-16701, Thermo Fisher Scientific, Foster City, MA, USA) and CD79α (GTX74022, GeneTex, Irvine, CA, USA) and negative for the T cell marker CD3 (IR503, Agilent Technology, Santa Clara, CA, USA) (Fig. 4).

DNA was extracted from both peripheral blood and tumor tissue using the QIAamp DNA Mini Kit (QIAGEN, GmbH, Hilden, Germany) according to standard procedures and stored at −30°C. IgH-PCR was performed using each DNA material based on a previous report [12]. Agarose gel electrophoresis revealed smear patterns in both the peripheral blood DNA and tumor tissue DNA. IgH-PCR demonstrated no monoclonal proliferation of B-cells.

IgL-PCR was also performed using DNA materials from the present case and peripheral blood of a clinically healthy cow without lymphocytosis infected with BLV as a negative control. Bovine IgL λ gene sequences registered in GenBank (Accession Nos. AF015791 to AF015799, Accession Nos. U32249 to U32264) were collected, and the forward primer IGL34F (5'-GCCAGAGGGTCTCCATCA CCTG-3') for the Vλ region and the reverse primer IGL298R (5'-TGTGGTCCCGCTGCCGAAAAC-3') for the Jλ region were designed so that the amplified region would include the complementarity determining region 3 (CDR3). The PCR reaction mixture contained 2.5 µL × 10 PCR buffer, 2.5 µL 2 mM dNTP, 0.75 µL 50 mM $MgCl_2$, 1.0 µL 20 µM forward primer, 1.0 µL 20 µM reverse primer, 0.2 µL Taq DNA polymerase (Invitrogen Taq DNA Polymerase; Thermo Fisher), and 12.05 µL distilled water, to which 5 µL of DNA solution was added for a total of 25 µL. After preactivation at 95°C for 15 min, the DNA amplification reaction was performed for a total of thirty-seven cycles, with denaturation at 95°C for 8 sec, annealing at 58°C for 10 sec, and extension at 72°C for 15 sec. After the PCR reaction, 2 µL of Loading Buffer (Thermo Fisher) was added to 7 µL of the PCR product, and electrophoresis was performed at 100V using a 2% agarose gel containing Midori Green Xtra (Nihon Genetics Co., Ltd., Tokyo, Japan). After electrophoresis, the amplified product was confirmed using FAS-Digi PRO (Nihon Genetics Co., Ltd.). Capillary electrophoresis was also used to evaluate the amplified products (Q sep 1-Lite Bio-Fragment Analyzer, Bioptic Inc., New Taipei City, Taiwan). A monoclonal band was detected in the present case, while a polyclonal band was observed in the control cow by gel electrophoresis (Fig. 5). The capillary electrophoresis also revealed a sharp monoclonal band in the present case and a polyclonal pattern in the control cow (Fig. 5). The IgL-PCR product of the present case was purified using a kit (QIAquick PCR purification kit, QIAGEN), and the nucleotide sequence was determined by direct sequencing at Eurofin Genomics K.K. (Tokyo, Japan). A 291 bp nucleotide sequence of the PCR product without primer regions was obtained, and BLAST analysis (https://www.ddbj.nig.ac.jp/ services/blast.html) revealed that this sequence was most similar to the *Bos taurus* immunoglobulin light chain variable region (accession number AF023843.1) with 99% identity.

In clinical practice of bovine medicine, lymphoma is suspected based on physical examination findings such as superficial lymph node enlargement, exophthalmos, and palpable masses on rectal examination, and is diagnosed based on lymphocytosis with atypical lymphocytes detected in blood smears and cytology of enlarged lymph nodes as well as a high BLV-PVL [3, 4]. However, since there are many cases of bovine lymphoma with no physical examination findings suggestive of lymphoma, the onset of lymphoma is not always suspected before death [13, 15]. In the present case, the absence of enlarged superficial lymph nodes and palpable masses on rectal examination made it difficult to suspect lymphoma based on physical examination. However, given that EBL had previously

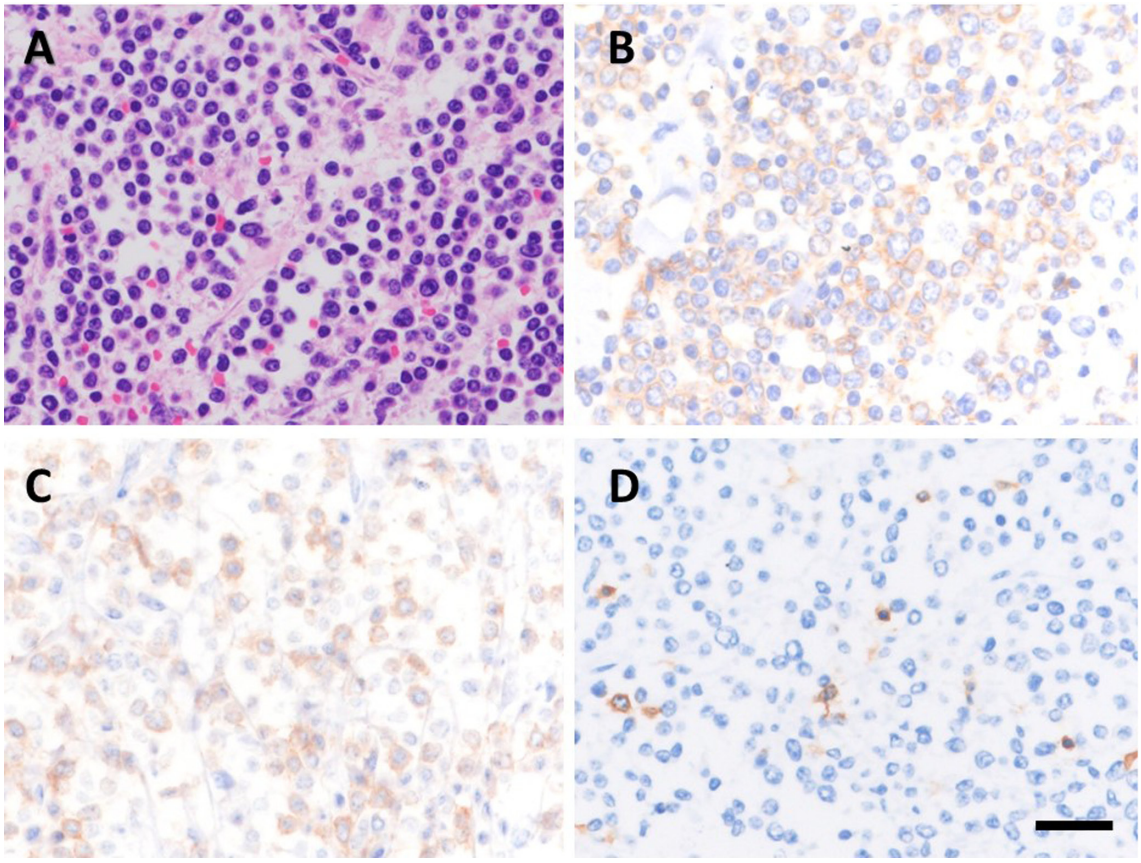

Fig. 4. Histopathological findings of a nodule in the uterine. (**A**) HE stains sections revealed the proliferation of medium to large lymphocytes. Immunohistochemically, the tumor cells were positive for CD20 (**B**) and CD79α (**C**), and were negative for CD3 (**D**), leading to a diagnosis of B-cell lymphoma. Bar=25 µm.

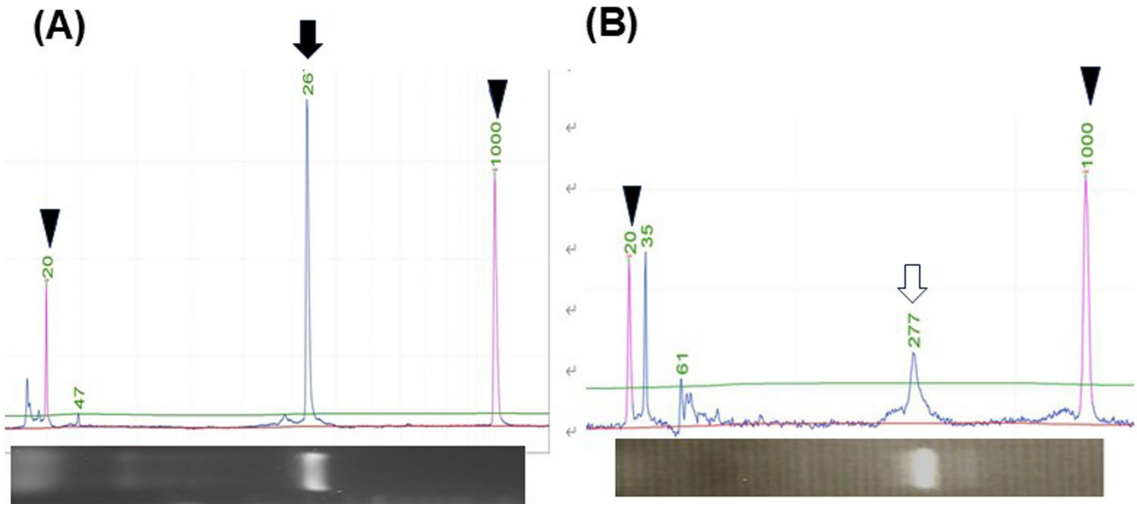

Fig. 5. IgL-PCR was performed to examine the clonal status of peripheral blood B-cells. (**A**) A single monoclonal band (black arrow) was detected in the present case by both capillary and gel electrophoresis, suggesting the onset of lymphoma. (B) A polyclonal band (white arrow) was observed in the non-lymphoma control cow. Arrowheads: size markers.

occurred on the same farm, we considered the possibility of lymphoma and examined serum TK activity, which was found to be high; this strongly suggested the onset of lymphoma. Necropsy revealed enlarged internal lymph nodes and tumor infiltration into organs, and histopathological examination also led to a diagnosis of B-cell lymphoma. In addition, the higher BLV-PVL in the peripheral blood

suggested EBL. In the present case, it was considered that the tumor cells that had infiltrated into the abomasum caused anorexia, and that those around the lumbar and sacral vertebrae that had infiltrated into the spinal canal compressed the spinal cord and resulted in hindlimb paresis. Furthermore, tumor cells had also infiltrated into the mucosa of the pregnant uterus, which may have caused premature birth.

The present case was diagnosed as B-cell lymphoma by immunohistochemistry. However, IgH-PCR, which is a widely used method, failed to detect monoclonal proliferation of B-cells. In human and small animal medicine, clonality testing targeting the IgH gene is mainly used to diagnose B-cell lymphoma, although its sensitivity is not necessarily high [2, 7, 8, 11, 14]. To address the low sensitivity of IgH-PCR, methods focusing on multiple target genes have been introduced, and in both human and small animal practice, IgL κ gene, IGL λ gene, kappa deleting element (KDE) gene, and incomplete IgH D-J rearrangements have been reported [2, 7, 8, 11, 14, 17]. As the gene sequences of IgL λ gene of cattle registered in GenBank were seemed to be most variable among other related gene sequences, a new clonality PCR for cattle was designed based on the IgL λ gene in the present study. And the results suggested monoclonal proliferation of B-cells in the present case. IgL-PCR revealed polyclonal proliferation in a non-lymphoma control cow, suggesting that the developed IgL λ gene-based clonality test may be useful as a new diagnostic tool for bovine lymphoma. Further investigation is warranted to determine the specificity and sensitivity of the developed test in the future.

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