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A clinical case of enzootic bovine leukosis in a 13-month-old Holstein heifer

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Abstract

A 13-month-old Holstein heifer presented with emaciation and enlarged superficial lymph nodes. Hematological examination showed remarkable lymphocytosis with atypical lymphocytes. Increased activities of serum total lactate dehydrogenase and thymidine kinase were detected. Fine needle aspiration cytology of the subiliac lymph node revealed large lymphoblasts undergoing mitosis. Nested PCR for bovine leukemia virus (BLV) and antibody against BLV were both positive. BLV proviral load was relatively high. Pathological examination confirmed that the case was diffuse large B-cell lymphoma. B-cell monoclonality was also confirmed by PCR for V-D-J junctional variability. Monoclonal integration of BLV provirus in the bovine genome was demonstrated with the inverse PCR method. The heifer was definitively diagnosed with enzootic bovine leukosis based on these findings.

Key Words: heifer, enzootic bovine leukosis, inverse PCR

Bovine leukosis is one of the most common neoplasms observed in cattle and is divided into two types: enzootic bovine leukosis (EBL) caused by bovine leukemia virus (BLV) in cattle older than three years of age, and sporadic bovine leukosis (SBL) in younger cattle^{1,2)}. A two-month-old calf was recently diagnosed with EBL by demonstrating monoclonal integration of BLV provirus, but less information is available on the clinical aspects of EBL in cattle younger than three years of age⁵⁾. In the present study, we describe a clinical case of EBL in a 13-month-old Holstein heifer diagnosed based on the detection

of monoclonal integration of BLV provirus in tumor cells isolated from peripheral blood and superficial lymph nodes.

A 13-month-old Holstein heifer presented with emaciation and diarrhea. The heifer was diagnosed for enteritis and treated with antibiotics. On day 2, swelling of peripheral lymph nodes was noted and pelvic masses were detected by rectal palpation. Hematological examination revealed remarkable lymphocytosis (84,057 / μ l). Bovine leukosis was suspected by a local veterinarian, and the heifer was transferred to the Animal Teaching Hospital at the Obihiro

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Fig. 1. Emaciation was apparent on day 4. Bar = 20 cm.

University of Agriculture and Veterinary Medicine to confirm the diagnosis on day 4.

On initial physical examination at the hospital, normal rectal temperature (39.2°C), tachycardia (140 beats/min), polypnea (54 breaths/min), emaciation, and pallor of the mucosal membranes were noted (Fig. 1). In addition, swelling of peripheral lymph nodes, including mandibular, superficial cervical, subiliac, and mammary lymph nodes, was observed (Fig. S1). Rectal palpation revealed several masses (4–9 cm) in the pelvic cavity. Hematological examination showed anemia (RBC count, $2,500 \times 10^3 /\mu\text{l}$; hemoglobin concentration, 4.3 g/dl; packed cell volume, 11%) and lymphocytosis (100,828 $/\mu\text{l}$). More than 90% of lymphocytes were morphologically atypical with wide cytoplasm, fine nuclear chromatin, and nucleoli (Fig. S2). Serum biochemical analysis revealed decreased total cholesterol level (53 mg/dl) and increased total LDH (2,950 U/l) and TK (649 U/l) activities. LDH isozyme analysis showed elevated activities of LDH-2 (1,297 U/l) and LDH-3 (531 U/l). Findings of fine needle aspiration (FNA) cytology of the right subiliac lymph node revealed large lymphoblasts (64.5%) undergoing mitosis.

DNA samples were obtained from the peripheral blood of the heifer and its dam for genetic diagnosis of BLV infection using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's

instructions. Nested polymerase chain reaction (PCR) for BLV 5' long terminal repeat (LTR) was performed with the following primers: F1/R2 (F1: 5'-GTG-CTC-AGC-TCT-CGG-TCC-TGA-GC-3', R2: 5'-AAA-CAA-ACG-CGG-GTG-CAA-GCC-AG-3') for outer PCR and F2/R1 (F2: 5'-TCT-CTT-GCT-CCC-GAG-ACC-3', R1: 5'-AGG-AGG-CAA-AGG-AGA-GAG-T-3') for inner PCR. These primers were designed based on the sequence of LTR. Taq DNA polymerase (Invitrogen, California, USA) was used for the PCR reaction. The first amplification program was carried out as follows: initial incubation at 95°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 70°C for 45 sec, and extension at 72°C for 45 sec, and a final extension at 72°C for 7 min. The second PCR was performed with the same protocol, except that the annealing temperature was set at 58°C. Genomic DNA from a typical EBL cow (7-year-old, BLV-positive, B-cell lymphoma) and distilled water (DW) were used as positive and negative controls, respectively. PCR products were electrophoresed on a 2% agarose gel. A single band was detected using the nested PCR samples. BLV Antibody Test ELISA kit (JNC, Tokyo, Japan) was used to detect antibodies against BLV gp51 molecules according to the manufacturer's instructions. The serum sample was positive.

B-cell clonality of peripheral blood and swollen subiliac lymph node tissue obtained by FNA was examined using PCR to amplify the variable region of the immunoglobulin heavy chain gene. DNA extracted from peripheral blood and superficial lymph node aspirate were used as templates for PCR, which was performed with HotStarTaq DNA Polymerase (QIAGEN). The following primer pair was designed based on sequence information of the immunoglobulin heavy chain variable region (GenBank accession No. U55195-U55204): BoVHF1: 5'-AGC-CCT-GAA-ATC-CCG-GCT-CA-3' and BoVHR1: 5'-TCC-AGG-AGT-CCT-TGG-CCC-CA-3'. Genomic DNA isolated from the peripheral blood of a typical EBL cow (5-year-old, BLV-positive, B-cell

lymphoma) was used as a positive control. Genomic DNA from enlarged mammary lymph nodes of a cow with mastitis and DW were also used as negative controls. The amplification program was carried out as follows: initial incubation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 8 sec, annealing at 60°C for 10 sec, and extension at 72°C for 15 sec. PCR products were electrophoresed on a 3% agarose gel. A single band was detected in both peripheral blood and lymph node tissue samples (Fig. S3). These findings supported B-cell monoclonality in the peripheral blood and lymph node.

BLV proviral load was quantified using the DNA samples obtained from the heifer and its dam with CoCoMo[®]-BLV Primer/Probe (Riken Genesis, Tokyo, Japan) and TaqMan[™] Gene Expression Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. BLV copy number was relatively high in the heifer (blood: 876 copies/10 ng DNA, lymph node tissue: 1,618 copies/10 ng DNA), and relatively low in the dam (45 copies/10 ng DNA)⁶.

The BLV provirus integration site was analyzed using inverse PCR (iPCR). iPCR was performed as previously reported³. Briefly, DNA samples which were used for the B-cell clonality analysis were digested with *Pst*I (Takara Bio, Shiga, Japan) and were then self-ligated using Mighty Mix (Takara Bio). The resulting products were used as templates for PCR using inverse primers (Pst-F and Pst-R)⁵. PCR products were electrophoresed on a 2% agarose gel. PCR products for DNA sequencing were purified with QIAquick PCR purification kits (QIAGEN). The amplification products were submitted for bidirectional sequencing using the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, California, USA). Sequences were determined using an ABI 3500 Genetic Analyzer (Applied Biosystems). The bovine genome sequence adjacent to the 5'LTR was determined using the University of California, Santa Cruz

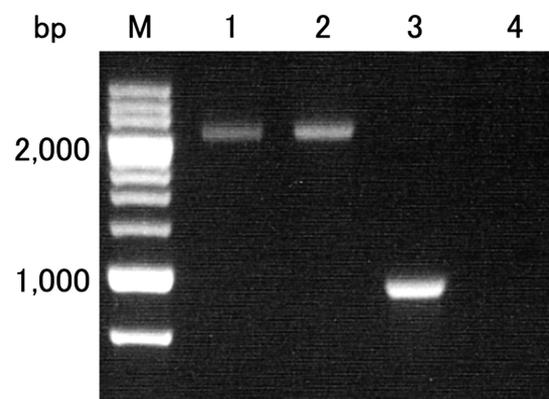


Fig. 2. Results of inverse PCR. Lane 1: DNA from blood; Lane 2: DNA from lymph node tissue obtained by FNA; Lane 3: positive control (typical EBL); Lane 4: negative control (DW); M: molecular weight marker.

Cow BLAT Search (<https://genome.ucsc.edu/cgi-bin/hgBlat>) against the October 2011 freeze of the cow genome sequence, as previously described. Our results showed a single band which supported monoclonal integration of BLV provirus in the bovine genome (Fig. 2). DNA samples from both peripheral blood and lymph nodes contained the BLV provirus integrated into position 98,276,892 of chromosome X. The integration site was a non-transcriptional unit.

The animal was euthanized under anesthesia on day 8 for necropsy. On the gross level, multiple peripheral lymph nodes (e.g., mandibular, superficial, subiliac, mammary, medial, and iliac lymph nodes) were swollen. The cut surface of affected lymph nodes was firm, homogenous, and white to grey. Similar white foci, 1–3 cm in diameter, were disseminated in the kidneys and lungs. The spleen was enlarged (55.5 × 16 × 3.5 cm) and swollen. The small and large intestines were diffusely thickened. The sternal bone marrow was gelatinous and yellow to white. Histopathologically, neoplastic cells diffusely proliferated in the affected lymph nodes; the normal structure of the lymph nodes was lost. The neoplastic cells were large and round with a small amount of cytoplasm, and showed irregular thickening of nuclear membranes, crafted nuclei with nuclear atypia, and granular-patterned chromatin (Fig. 3). Mitoses were common (10–15

per high power field). The neoplastic cells infiltrated the liver, spleen, thymus, tonsil, heart, lungs, diaphragm, adrenal gland, pancreas, bone marrow, uterus, and lamina propria of the intestines. Immunohistochemical examination showed that neoplastic cells were positive for BLA-36 (Biogenex Laboratories, California, USA) and negative for CD3 (Biogenex Laboratories) (Fig. 4). The histological diagnosis was diffuse large B-cell lymphoma.

EBL typically occurs in cattle that are older than three years because BLV requires a long latent period to cause the disease^{1,2}. Therefore, it is difficult to distinguish EBL from the juvenile form of SBL with BLV infection in young cattle.

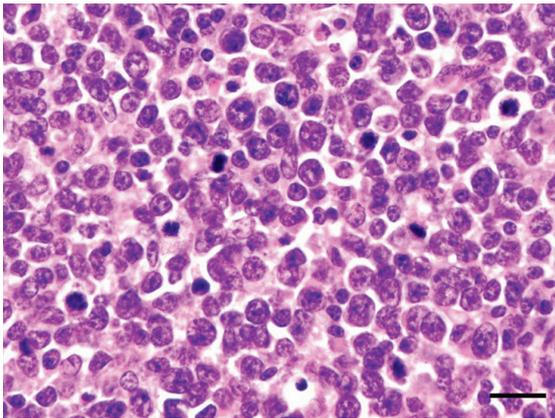


Fig. 3. Histopathology of subiliac lymph nodes. Neoplastic cells were large, round, and had a small amount of cytoplasm, and exhibited irregular thickening of nuclear membranes, crafted nuclei with nuclear atypia, and granular-patterned chromatin. Hematoxylin and eosin stain. Bar = 20 μ m.

In the present case, we demonstrated the occurrence of EBL in a 13-month-old Holstein heifer based on clinical and pathological findings, immunohistochemical analysis, PCR for B-cell clonality, quantitative PCR for BLV proviral load, and iPCR.

Bovine leukemia has recently been classified into five groups (classic EBL (cEBL), polyclonal EBL, B-cell-type SBL, T-cell-type SBL, and nontypeable cases) according to results of cell marker expression, B-cell clonality, and BLV provirus loads⁴. cEBL is defined as a monoclonal or oligoclonal B-cell lymphoma associated with BLV infection. The present case was considered a cEBL case, due to B-cell marker positivity in immunohistochemical analysis, B-cell monoclonality in PCR for B-cell clonality, relatively high proviral load, and monoclonal integration of BLV provirus findings.

BLV infection during the fetal period and BLV provirus integration site are possible factors associated with early onset EBL. In the present case, however, the onset of EBL was unlikely to be related to these factors, since the proviral load of the dam was relatively low, and BLV provirus was integrated in a non-transcriptional unit. Factors that have led to the early onset of EBL in the present case remain unclear.

In a previous study, a two-month-old calf was diagnosed with EBL using iPCR⁵. In the present case, we also demonstrated EBL in a 13-month-old Holstein heifer. This report thus

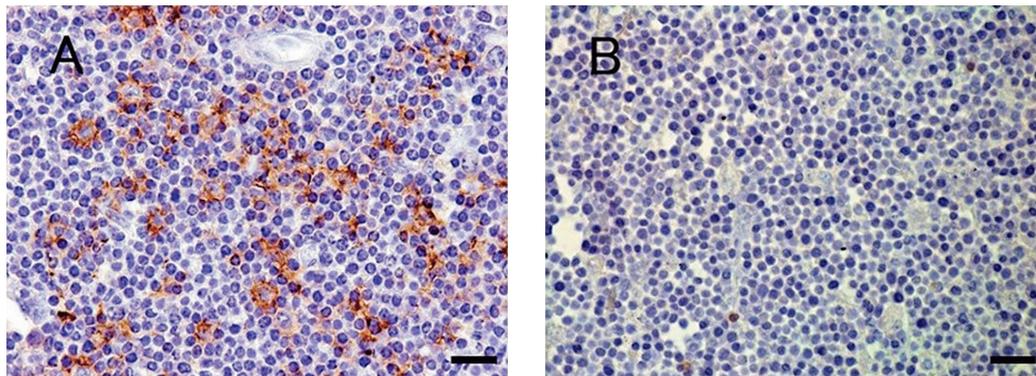


Fig. 4. Immunohistochemistry of subiliac lymph nodes. Neoplastic cells were immunopositive for BLA-36 (A) and negative for CD3 (B). Mayer's hematoxylin counterstain. Bar = 20 μ m.

provides additional evidence for the occurrence of EBL in cattle younger than three years of age and emphasizes the importance of distinguishing between EBL and the juvenile form in young cattle with BLV.

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Supplemental data

Supplemental data associated with this article can be found, in the online version, at <http://dx.doi.org/10.14943/jjvr.66.3.209>

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