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Enzootic bovine leukosis in a two-month-old calf

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ABSTRACT

A two-month-old calf was diagnosed with leukosis on the basis of the clinical sign of enlarged, superficial lymph nodes. Serological and genetic tests for bovine leukemia virus (BLV) were performed because the calf was born from a cow infected with BLV. The serum had a weakly positive BLV antibody, and the BLV provirus was detected within neoplastic cells on performing polymerase chain reaction (PCR). Analysis of the BLV provirus integration site using inverse PCR revealed that the BLV integration site location was identical on all chromosomes in all tumor tissues examined. Thus, the tumor cells monoclonally proliferated following BLV infection. The present study shows that enzootic bovine leukosis can occur in a young animal, as in the two-month-old calf in our study.

1. Introduction

Bovine leukemia virus (BLV) is the etiological agent of enzootic bovine leukosis (EBL). BLV is horizontally transmitted by arthropods such as the horsefly or to calves via *in utero* infection and the ingestion of colostrum from a BLV-infected cow. Iatrogenic transmission can occur via surgical instruments or sleeves contaminated with infected blood during rectal palpation. Lymphoma occurs in approximately 5%–10% of BLV-infected cows, predominantly in animals older than 3–5 years (Gutiérrez et al., 2014a). The diagnosis of BLV infection is serologically determined via an agar gel immunodiffusion (AGID) test, an enzyme-linked immunosorbent assay (Hoff-Jørgensen, 1989; Kettmann et al., 1994). Another diagnostic method is the detection of the integrated BLV genome in blood and tissue samples using genomic polymerase chain reaction (PCR) (Fechner et al., 1996). However, the BLV genome and antibody are detectable in some sporadic bovine leukosis (SBL) cases, including the juvenile form, which was unrelated to BLV infection (Jacobs et al., 1992). The confirmation of only one or a few sites of BLV integration within a sampled tumor tissue is diagnostic of EBL because EBL tumors are mono- or oligoclonal in origin (Kettmann et al., 1980; Kettmann et al., 1983; Coulston et al., 1991; Murakami et al., 2011). In the present study, we investigated a case of EBL in a two-month-old calf diagnosed by an integration site analysis of tumor tissues.

2. Materials and methods

2.1. Case description

A two-month-old, emaciated female calf with pneumonia, bronchitis, and enlarged superficial lymph nodes was examined at a veterinary clinical service center of the National Agricultural Insurance Association. A blood examination revealed leukocytosis (23,560 cells/μL) with marked neutrophilia (15,078 cells/μL) including an increased number of stab cells (3063 cells/μL). The monocyte count was also slightly elevated (942 cells/μL), and the lymphocyte count was normal (4476 cells/μL). Eosinophils and basophils were not detected on a blood smear. The serum lactate dehydrogenase activity was 3432 units/L. The calf was humanely euthanized and necropsied. Generalized lymphadenomegaly was noted, and abnormal nodules were observed on the surface and in the parenchyma of the thoracic and abdominal organs (Fig. 1). However, anemia, fever, and symmetrical enlargement of the lymph nodes, which are the general clinical signs of the juvenile form of SBL (Hendrick, 2002), were not observed. A microbiological examination of the swabs of abscesses found in the lung and trachea detected *bovine coronavirus*, *Actinomyces pyogenes*, *Streptococcus bovis*, and *Mycoplasma bovis*.

2.2. Polymerase chain reaction (PCR) analysis of BLV

Genomic PCR was performed to amplify a partial *env* gene fragment of the BLV provirus. The template DNA was extracted from enlarged mammary, superficial cervical, inguinal, and hilar lymph nodes using

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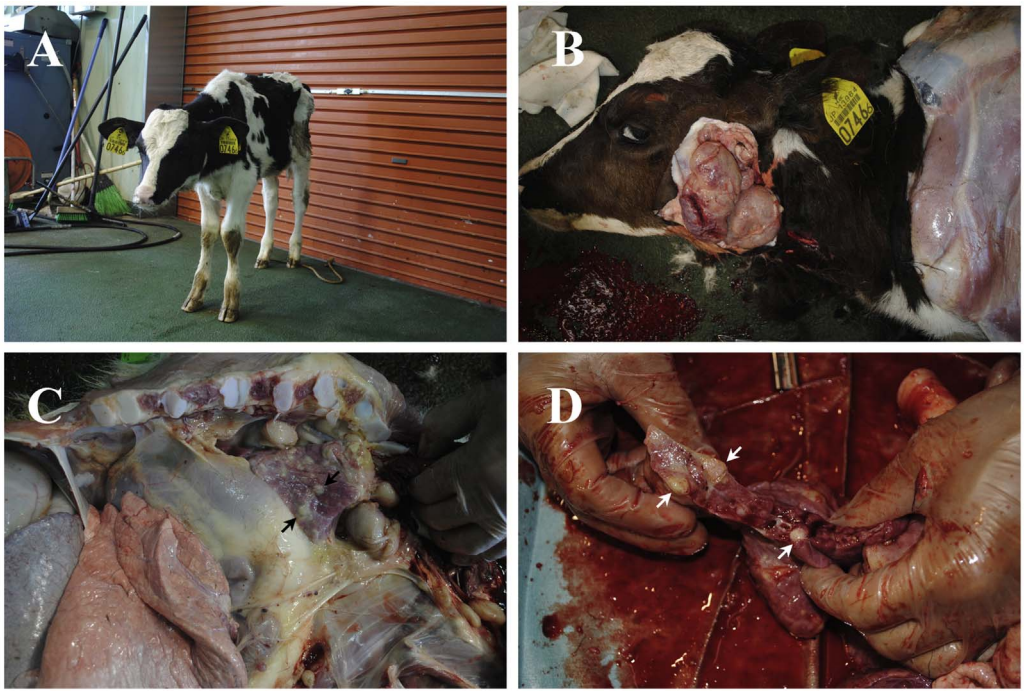

Fig. 1. The appearance of the calf and the gross necropsy findings. (A) Emaciation of the calf. (B) The enlargement of a parotid gland lymph node. (C & D) White nodules in the lung lobe (arrows).

the Gentra Puregene Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Peripheral blood DNA was also extracted using the DNeasy Blood Fechner et al., 1997). The following external primers were used: *env*5032 (5′-TCTGTGCCAAGTCTCCCAGATA-3′) and *env*5608r (5′-AACAACAACCTCTGGGAAGGGT-3′). This results in the amplification of a 598-bp fragment. A 444-bp fragment was produced using the following internal primers: *env*5099 (5′-CCCACAAGGGCGG-CGCCGGTTT-3′) and *env*5521r (5′-GCGAGGCCGGGTCCAGAGCTGG-3′). PCR was performed using the GoTaq Green Master Mix (Promega, Madison, WI) and primers with a final concentration at 0.5 μM. Amplification during the first round of PCR was performed as follows: initial denaturation was at 94 °C for 2 min, which was then followed by 40 cycles of the following 3 steps: at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. The second round of PCR was performed with the same protocol except that the annealing temperature was set at 70 °C and 35 amplification cycles were performed.

2.3. Inverse PCR (iPCR)

The procedure for iPCR was performed as previously reported (Murakami et al., 2011). Tumor tissue DNA samples that were used for genomic PCR were digested with *Bcl*I, *Pst*I, or *Bss*HII and were then self-ligated using Mighty Mix (Takara Bio, Shiga, Japan). The resulting products were used as templates for PCR using inverse primers. PCR products were electrophoresed in an agarose gel, and positive samples were cloned into a pCR2.1-TOPO vector (Thermo Fisher Scientific, Waltham, MA). The product was then sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit with an Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific). The bovine genome sequence adjacent to the 5′ long terminal repeat was determined using the University of California, Santa Cruz Cow BLAT Search (https://genome.ucsc.edu/cgi-bin/hgBlat?command=start) against the October 2011 freeze of the cow genome sequence as previously described (Miyasaka et al., 2015).

2.4. Quantitation of BLV provirus copy number in tumor tissues

The BLV provirus in tumor tissues was quantified by duplex real-time PCR that enabled the simultaneous detection of BLV *pol* and the bovine beta-actin gene *ACTB* in a single PCR tube. To make standard samples for quantitation, a partial BLV *pol* gene fragment was amplified from the genomic DNA of fetal lamb kidney cells persistently infected with BLV (FLK-BLV cells) (Van Der Maaten and Miller, 1975) using a primer set (Forward: 5′-AAGCTCACCCACTGCAACTCT-3′, Reverse: 5′-TCTGATTGTGAGTCCAGAGGG-3′) and cloned into a pCR2.1-TOPO vector (Thermo Fisher Scientific). A partial bovine *ACTB* gene fragment was also amplified from the DNA of Madin-Darby bovine kidney cells using a primer set (Forward: 5′-TACGCCCTTCCCCATGCCATCCTGCG-TCTG-3′, Reverse: 5′-TCTTCATTGTGCTGGGTGCCAGGTCATTGA-3′) and cloned into a pCR2.1-TOPO vector. The *Rsa*I-digested vector containing a *pol* fragment was subcloned into an *Eco*RV-digested *ACTB*-cloned vector. The resultant plasmid containing both *pol* and *ACTB* at a precisely equivalent molecular ratio was adjusted from 10^6 to 10^1 copies by 10-fold serial dilution using EASY Dilution (Takara); these were used as standard samples. Quantitative PCR (qPCR) was performed by a Taqman probe assay using an ABI 7500 Fast thermal cycler (Thermo Fisher Scientific) and Thunderbird Probe qPCR Mix (TOYOBO, Osaka, Japan). The primers and probe for *pol* were as follows: Forward (*pol*-4219F): 5′-TTCACCTACGCTCTGCATGTG-3′, Reverse (*pol*-4339R): 5′-CCAGATGCACTATGGCCTCAA-3′, and probe (*pol*-4256T): 5′-FAM-CTGGAGCTACTCATGC-minor groove binder (MGB)-3′. *ACTB* was quantified using the following primers and probe: Forward: 5′-TCCCTGGAGAAGAGCTACGA-3′, Reverse: 5′-GGCAGACT-TAGCCTCCAGTG-3′, and probe: 5′-VIC-CTTCCTTCCTGGGTGAGTGAG-AAG-MGB-3′. All primers and probes were purchased from Thermo Fisher Scientific. The final concentration of each primer and probe was 0.6 μM and 0.2 μM, respectively. The PCR condition was as follows: 95 °C, 20 s for the first denaturation, then 40 cycles of 95 °C, 3 s and 60 °C, 30 s. Copy numbers of the BLV provirus and *ACTB* gene were analyzed in the DNA from typical EBL tumors developed in 12 adult cows that were provided by a meat inspection center in Japan, in addition to the DNA from four tumor tissues and the peripheral blood of the present calf. qPCR was performed in duplicate. The mean *pol* number in 100 cells of each sample shown in the Results section was calculated as follows: [mean *pol* copy number/(mean *ACTB* copy number/2)] × 100.

2.5. Whole BLV genome sequence determination

The entire proviral sequence of the BLV strain from the infected calf was determined using conventional PCR. Sequencing was facilitated by dividing the provirus into 22 parts and using the primers designed from the BLV sequences obtained from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/pubmed).

2.6. Histological examination of tumor tissues

Tumor tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin. Monoclonal antibodies for immunohistochemistry were purchased from the Monoclonal Antibody Center at Washington State University (Pullman, WA) included the following: MM1A (CD3), CACT138A (CD4), CACT105A (CD5), CACT80C (CD8α), MM10A (CD11b), LCT27A (CD45R/B220), BIG73A (IgM), TH14B (MHC class II), and BIG501E (λ light chain). Anti-IgG2 (BG2-7) was purchased from Sigma Aldrich (Saint Louis, MO). Visualization was performed using LSAB2 Kits, Universal (Dako, Glostrup, Denmark).

2.7. AGID test

The BLV antigen for the AGID test was prepared using FLK-BLV cells. Culture fluids were concentrated using ammonium sulfate, were then dialyzed in distilled water, and were further concentrated with polyethylene glycol using a published procedure (Kono et al., 1982). AGID tests were performed according to the OIE manual. The gel consisted of 1.0% Noble agar, 8.5% NaCl, and 50 mM Tris buffer. The wells were 5 mm in diameter, and six circumferential wells were placed at a distance of 3 mm from the central well. The central well was filled with the antigen, and two opposite exterior wells were filled with the positive control serum. Serum samples were taken from the calf and its dam. Phosphate-bufferd saline (PBS) was used as the negative control. The gel diffusion plate was allowed to stand at room temperature for 48 h, and precipitation lines were observed.

3. Results

3.1. Detection of BLV and iPCR analysis of tumor tissues

Genomic PCR of the four enlarged lymph nodes amplified the BLV *env* gene fragment in the first and second rounds of PCR (Fig. 2). The same templates digested with restriction enzymes were subjected to iPCR, and in total, 29 clones were sequenced. All four tumor DNA samples digested with *Eco*RI and *Pst*I contained the BLV provirus integrated into position 25,702,287 of chromosome 15. *Bss*HII digestion did not give any positive signals. The integrated site was a non-transcriptional unit (Fig. 3).

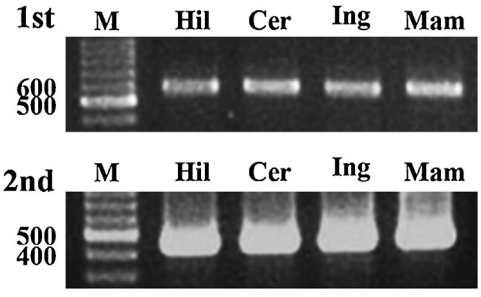

Fig. 2. Detection of BLV in tumor tissues by nested PCR. Templates were genomic DNA extracted from hilar (Hil), superficial cervical (Cer), inguinal (Ing), and mammary (Mam) enlarged lymph nodes. Expected fragment sizes were 577 bp and 423 bp in the first and second PCRs, respectively. PCR products were loaded in an agarose gel with a DNA marker (M).

3.2. Quantitation of BLV in tumor tissues

qPCR of BLV in the EBL calf showed that the BLV provirus was integrated in an average of 57.7% of the total cells examined from the four tumor tissues (median: 57.3%, range: 44.7%–71.6%) and in only 1.8% of the leukocytes in blood (Fig. 4). BLV integration was detected in other 12 adult EBL tumors in an average of 123.6% of the total cells (median: 102.0%, range: 7.2%–322.9%).

3.3. BLV genome sequence

The sequence of the integrated BLV provirus was 8720 bp in length and was deposited in the DNA Data Bank of Japan (AB987702). The entire BLV sequence exhibited a sequence homology of 99.2% with the BLV provirus in FLK-BLV cells (EF600696) and 98.8% with a Japanese BLV reference strain (K02120). The residue at codon 230 of the Env protein precursor was found to be asparagine.

3.4. Histological examination

The normal histological architecture of the enlarged lymph node tissue was almost completely disrupted by the infiltration of large tumor cells. Several mitoses were evident in majority of high-power field views (Fig. 5A). Some cells had atypical nuclei and prominent nucleoli (Fig. 5B). Tumor cells were positive for the following markers: B-cell marker CD45R, λ light chain (Fig. 5C), IgM (Fig. 5D), and IgG2. The cells weakly reacted with MHC class II and had a negative result for other antigens.

3.5. Antibody to BLV in sera from the calf and dam in the AGID test

A weak, but detectable, precipitation line was formed by the serum from the dam, but a scarcely positive reaction was observed from the serum of the leukemic calf (Fig. 6).

4. Discussion

Generally, BLV requires a long latent period to cause EBL. Therefore, bovine leukosis in calves is more likely to present as a BLV-independent sporadic form. Conventional diagnostic methods for BLV infection, which include antibody tests and PCR for the amplification of the gene fragment of the BLV provirus, are unable to distinguish between EBL and SBL cases with BLV infection. However, the location of the BLV integration site within the tumor tissue in an EBL case is identical as EBL tumor cells are monoclonal in nature and proliferate from a single transformed cell. In the present case, the diagnosis of EBL was established by iPCR because BLV had identical integration sites in anatomically different tumors. Although the BLV provirus was detected in approximately half of the total cells examined from the four tumorous lymph nodes, we attribute this to the contamination of BLV-negative stromal cells. A comparable level of the BLV *pol* gene was amplified in adult EBL tumors. Tumor cells were classified as B-2 (conventional B) cells as they were $CD5^{-}/CD11b^{-}$ and T-cell marker negative (Ikeda et al., 2005).

The replacement of asparagine with glutamic acid at codon 230 of the Env protein reportedly enhances the pathogenicity of BLV through the alteration of the N-linked glycosylation status (de Brogniez et al., 2015). However, the BLV strain in the calf in this study maintained asparagine at this site. Therefore, the pathogenesis of BLV analyzed in our study might be related to other sequences or integrated sites in host animal chromosomes.

The calf had a low antibody titer to BLV. This may be attributed to the recent infection with BLV and the rapid progression of the tumor before a clinically detectable amount of BLV antibodies could be produced because newborn calves have a weaker immune response than adults and their antibody-producing ability is relatively inade-

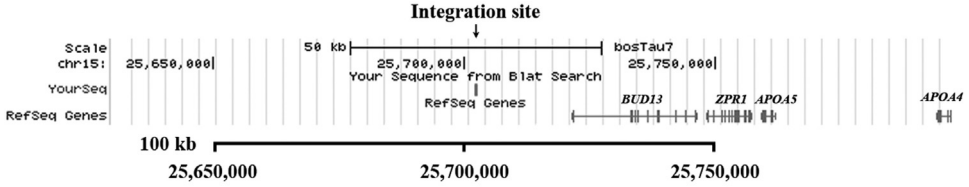

Fig. 3. BLAT analysis of the BLV integration site in chromosome 15 and the surrounding host genomic organization.

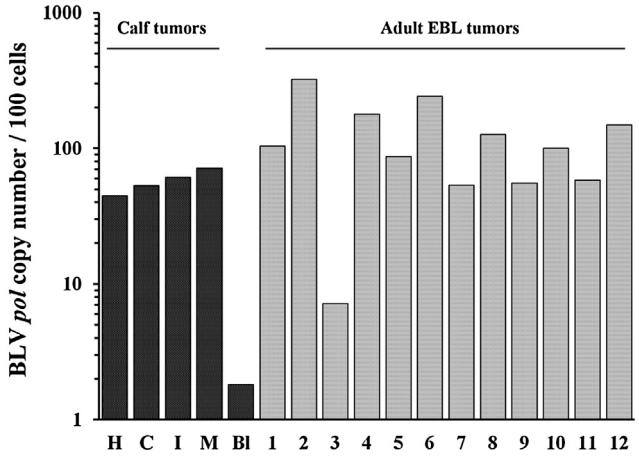

Fig. 4. Quantity of the BLV provirus in tumors and blood from the calf in the present study and 12 typical EBL cases. Percentage of BLV-*pol* gene positive cells in each tumor tissue (ordinate) was standardized by the *ACTB* gene. Four tumorous lymph nodes from the calf: hilar (H), superficial cervical (S), inguinal (I), and mammary (M). Bl indicates peripheral blood.

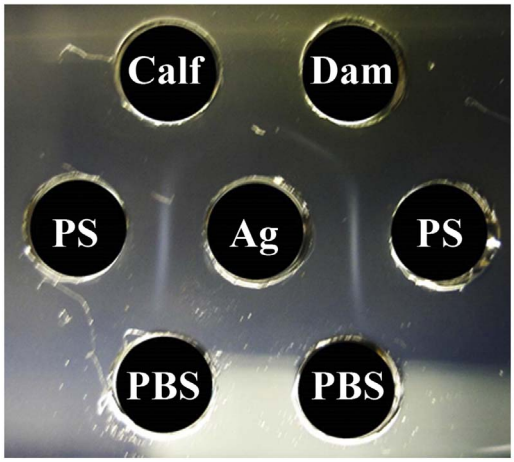

Fig. 6. Antibody titer of the sera from the leukemic calf and her dam. Ag: Antigen. PS: positive reference antiserum. Calf and Dam: Sera from the leukemic calf and her dam. PBS was placed as the negative control.

quate (Ingram and Smith, 1965; Senogles et al., 1979). However, there is the considerable possibility that the host had an immune deficiency against BLV. The AGID test showed a weakly positive BLV antibody, which may have been elicited by remnant maternal antibodies that were transferred from the colostrum and attributable to a weak immunoreaction against BLV. Cases of *in utero* infection may lead to an immune tolerance toward BLV as observed in bovine viral diarrhea virus infection (McClurkin et al., 1984), although this has not been reported. The vertical transmission of human T-lymphotropic virus-I, which is closely related to BLV, has been suggested to be a risk factor for the onset of adult T-cell leukemia (Proietti et al., 2005).

Although the relationship between the transmission route and pathogenesis of EBL remains unclear in the present case, the immunological status of the host, virus infection time, and proviral load may be important factors in the pathogenesis of EBL at an early age. Gutiérrez

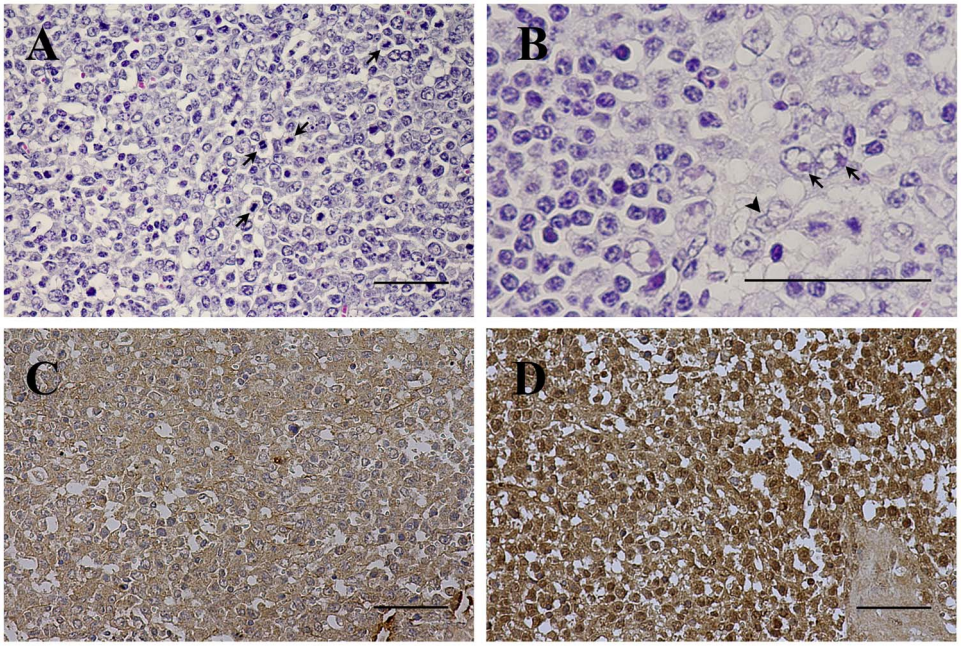

Fig. 5. Histology of the tumor tissues. Photomicrographs of hematoxylin and eosin staining (A and B) and immunohistochemistry of the λ light chain (C) and IgM (D) are shown. Arrows are mitotic figures (A) and nucleoli (B). An arrowhead in (B) is an atypical nucleus. Small round cells shown in (B, left side) are considered to be non-tumor residual lymphocytes. All scale bars are 50 μm.

et al. reported the dynamics of BLV propagation in calves that were infected during the first week of life. Perinatal infection has been shown to lead to a high proviral load in calves without EBL (Gutiérrez et al., 2014b). Some studies have noted a relationship between a high BLV provirus copy number and disease severity (Aida et al., 2013). Accordingly, an analysis of the relationship between the provirus number in blood and EBL development is considered to be important. Because proviral load of our case was as low as 1.8% in peripheral blood nucleated cells, we concluded that the calf had lymphosarcoma without apparent persistent lymphocytosis.

In conclusion, iPCR is an effective method for evaluating the clonal growth of an EBL tumor cell via the identification of the location of the BLV integration site. EBL has been shown to develop in calves at an early age, and a low BLV antibody titer cannot rule out infection. Consequently, EBL may have occurred in calves that were diagnosed with the juvenile form of SBL. BLV infection is becoming more prevalent in Japan (Murakami et al., 2013). Therefore, the precise detection of BLV infection and the diagnosis of EBL are necessary for preventing the transmission of BLV.

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