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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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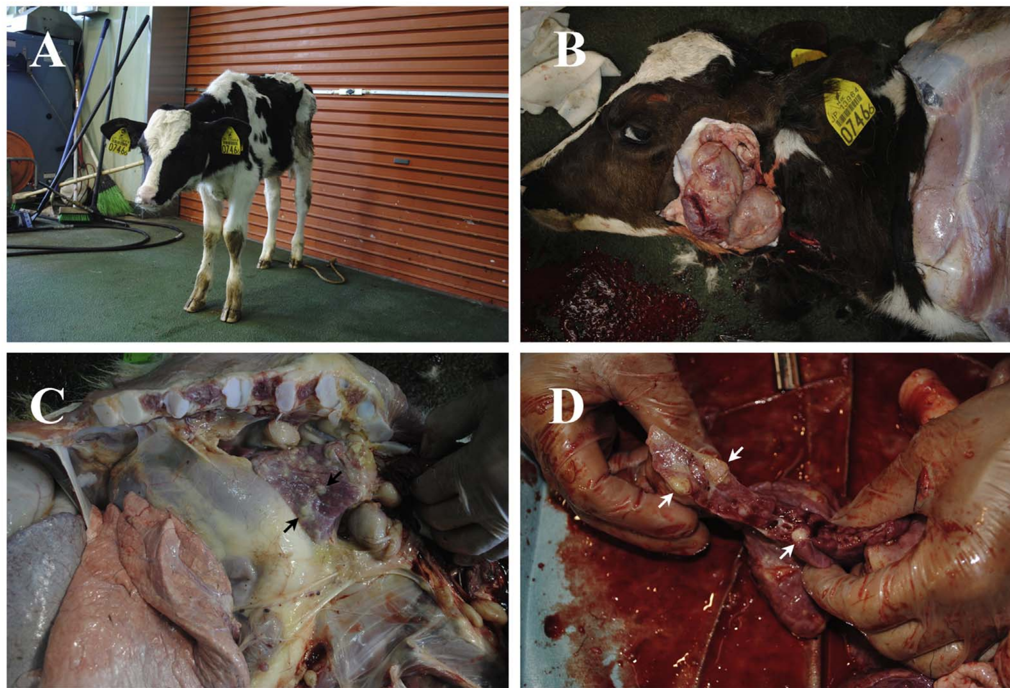

Fig. 1. The appearance of the calf and the gross necropsy findings. (A) Emaciation of the calf. (B) The enlargement of a parotid gland lymph node. (C & D) White nodules in the lung lobe (arrows).

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.

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