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Detection of the BLV provirus from nasal secretion and saliva samples using BLV-CoCoMo-qPCR-2: Comparison with blood samples from the same cattle

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ABSTRACT

Bovine leukemia virus (BLV) induces enzootic bovine leukosis, which is the most common neoplastic disease in cattle. Sero-epidemiological studies show that BLV infection occurs worldwide. Direct contact between infected and uninfected cattle is thought to be one of the risk factors for BLV transmission. Contact transmission occurs via a mixture of natural sources, blood, and exudates. To confirm that BLV provirus is detectable in these samples, matched blood, nasal secretion, and saliva samples were collected from 50 cattle, and genomic DNA was extracted. BLV-CoCoMo-qPCR-2, an assay developed for the highly sensitive detection of BLV, was then used to measure the proviral load in blood ($n = 50$), nasal secretions ($n = 48$), and saliva ($n = 47$) samples. The results showed that 35 blood samples, 14 nasal secretion samples, and 6 saliva samples were positive for the BLV provirus. Matched blood samples from cattle that were positive for the BLV provirus (either in nasal secretion or saliva samples) were also positive in their blood. The proviral load in the positive blood samples was >14,000 (copies/1×10^5 cells). Thus, even though the proviral load in the nasal secretion and saliva samples was much lower (<380 copies/1×10^5 cells) than that in the peripheral blood, prolonged direct contact between infected and healthy cattle may be considered as a risk factor for BLV transmission.

1. Introduction

Bovine leukemia virus (BLV), which is closely related to human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2), is the etiologic agent of enzootic bovine leucosis (EBL), a disease characterized by a very extended course that often involves persistent lymphocytosis (PL) and culminates in B-cell lymphosarcoma (Aida et al., 2013). Sero-epidemiological studies show that BLV infection is a global phenomenon (Kobayashi et al., 2010; Kurdi et al., 1999; Monti and Frankena, 2005; Schoepf et al., 1997; VanLeeuwen et al., 2006; Wang, 1991; Zaghawa et al., 2002). The world organization for animal health lists EBL as a disease that can have a significant impact on international trade (Rodriguez et al., 2011).

Although BLV uses both horizontal and vertical transmission routes, horizontal transmission is one of the major routes of transmission in most settings (Hopkins and DiGiacomo, 1997). Horizontal transmission occurs through hematophagous insects (Bech-Nielsen et al., 1978; Buxton et al., 1985), prolonged direct contact between infected and uninfected cattle (Kono et al., 1983), and iatrogenic procedures such as dehorning (DiGiacomo et al., 1985), ear tattooing, and the use of infected needles and plastic sleeves (Hopkins and DiGiacomo, 1997; Hopkins et al., 1988). The virus is transmitted to cattle primarily through direct exposure to infected blood, saliva, semen, and milk (Hopkins and DiGiacomo, 1997).

After infecting cattle, BLV integrates into the genomic DNA of host lymphocytes as a provirus (Kettmann et al., 1982), leading to lifelong infection despite the induction of virus-neutralizing anti-

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bodies (Tajima et al., 2003). In addition to conventional serological tests such as agar gel immunodiffusion (Aida et al., 1989; Kurdi et al., 1999) and enzyme-linked immunosorbent assay (ELISA) (Beier et al., 2004; Kurdi et al., 1999; Trono et al., 2001), diagnostic BLV polymerase chain reaction (PCR) techniques are often used to detect the integrated BLV proviral genome within the host DNA (Lew et al., 2004; Nagy et al., 2003; Rola and Kuzmak, 2002; Tajima et al., 1998). In 2010, a new quantitative real-time PCR method (BLV-CoCoMo-qPCR) was developed, which is based on the use of Coordination of Common Motifs (CoCoMo) primers to measure the proviral load of both known and novel BLV variants in BLV-infected animals (Jimba et al., 2010, 2012; Miyasaka et al., 2013; Panei et al., 2013). The BLV-CoCoMo-qPCR technique amplifies a single-copy host gene, the bovine leukocyte antigen *BoLA-DRA* gene, in parallel with viral genomic DNA; this technique effectively normalizes the level of viral genomic DNA. Recently, a modified version of BLV-CoCoMo-qPCR, BLV-CoCoMo-qPCR-2 (which uses optimized degenerate primers), was developed (Polat et al., 2015; Takeshima et al., 2015).

Studies based on nucleotide and amino acid sequence comparisons demonstrate that different BLV variants occur in different geographical regions. Recent phylogenetic studies of the BLV *env* gene from strains isolated across the globe demonstrate that the virus can be classified into eight genotypes (Ababneh et al., 2012; Balic et al., 2012; Matsumura et al., 2011; Moratorio et al., 2010; Polat et al., 2015; Rodriguez et al., 2009; Rola-Luszczak et al., 2013).

Currently, genomic DNA can be isolated from a variety of sources, including whole blood, milk, semen, saliva, and nasal secretions (Foley et al., 2011; Mitsouras and Faulhaber, 2009). Therefore, to confirm whether BLV provirus is detected from these specimens, we collected matched blood, nasal secretion, and saliva samples from 50 animals and extracted the genomic DNA. We then used the BLV-CoCoMo-qPCR-2 to determine the absolute copy number of BLV provirus in nasal secretion and saliva samples, and compared the results with those from matched blood samples. Moreover, we sequenced the partial BLV gp51 *env* gene region to confirm the BLV genotype.

2. Materials and methods

2.1. Samples

Matched blood, nasal secretion, and saliva samples were obtained from 50 cattle (34 Japanese black, six Holstein-Friesian, and ten crossbred) in Japan. Blood samples used for PCR amplification and lymphocyte counts were collected from the neck vein using an ethylenediaminetetraacetic acid (EDTA) vacuum blood collection tube. Serum (used to detect anti-BLV antibodies) was obtained from blood collected from the same cattle using a vacuum blood collection tube. Nasal and saliva samples were collected using the Performagene Livestock PG-100 collection device (DNA Genotek Inc., Ontario, Canada) with minimum restraint. Samples were collected carefully to avoid contamination with blood. All experiments were conducted in accordance with the Guidelines for Laboratory Animal Welfare and Animal Experiment Control set out by the NARO Institute of Livestock and Grassland Science (permit numbers: NILGS-13053101 and NILGS-14112050).

2.2. Genomic DNA extraction

DNA was obtained from blood samples (300 μL) using the Wizard Genomic DNA Purification Kit (Promega K. K., Tokyo, Japan) and dissolved in 100 μL of DNA Rehydration Solution, according to the manufacturer's instructions (Promega K. K.). DNA was extracted from nasal and saliva samples (500 μL) using DNA Genotek purification protocol PD-PR-083 (DNA Genotek Inc.) and dissolved in 20 μL of TE buffer (10 mM Tris–HCl [pH 8.0] and 1 mM EDTA [pH 8.0]).

2.3. DNA quantity and quality

The quantity and quality of the DNA (A260/A280 ratio) were assessed using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific K. K., Kanagawa, Japan), according to the manufacturer's instructions. Furthermore, to test whether the quality of the extracted DNA was suitable for PCR amplification, and to examine the efficiency of amplification (threshold cycle value), DNA from a subset of the paired samples was subjected to real-time PCR to amplify a single-copy bovine gene: *BoLA-DRA* (DDBJ: D37956). A 151 bp fragment of the *BoLA*-DRA gene was amplified in a total volume of 20 μL comprising 1×TaqMan Gene Expression Master Mix containing 50 nM forward primer (5′-CCCAGAGTATGAAGCTCCAGCCC-3′), 50 nM reverse primer (5′-CCCTCGGCGTTCAACGGTGT-3′), 150 nM FAM-DRA probe (5′-FAM-TGTGTGCCCTGGGC-NFQ-MGB-3′), and 150 ng of template DNA. PCR was performed using the ABI 7500 Fast Dx Real-time PCR system according to the following program: Uracil-DNA Glycosylase (UDG) enzyme activation at 50 °C for 2 min, followed by AmpliTaq Gold Ultra Pure (UP) enzyme activation at 95 °Cfor 10 min, followed by 60 cycles of 15 s at 95 °C and 1 min at 60 °C.

2.4. Detection of anti-BLV antibodies in serum samples

Anti-BLV antibodies were detected using ELISA kits according to the manufacturer's instructions (JNC Inc., Tokyo, Japan).

2.5. Measurement of the BLV proviral load using BLV-CoCoMo-qPCR-2

The BLV proviral load was measured by BLV-CoCoMo-qPCR-2, as previously described (Polat et al., 2015; Takeshima et al., 2015), using 150 ng of template DNA. Briefly, the BLV long terminal repeat (LTR) region was amplified using the degenerate primer pair, CoCoMo-FRW and CoCoMo-REV. The *BoLA-DRA* gene (internal control) was amplified using the protocol described in "DNA quantity and quality" above.

2.6. PCR amplification and sequencing of BLV env *gp51*

Nested PCR was performed as described below (the nucleotide number (nt) indicates the position of the FLK-BLV subclone, pBLV913 [DDBJ: EF600696], within the nucleotide sequence). The first round of amplification (the full-length BLV gp51 *env* gene [913 bp]) was performed using the following primers (Moratorio et al., 2010): forward, 5′-ATGCCYAAAGAACGACGG-3′ (nt 4826–4843), and reverse, 5′-CGACGGGACTAGGTCTGACCC-3′ (nt 5738–5718). The second round of amplification (the partial BLV gp51 *env* gene [598 bp]) was carried out using the following primers (Asfaw et al., 2005): forward, 5′-TCTGTGCCAAGTCTCCCAGATA-3′ (nt 5037–5058), and reverse, 5′-AACAACAACCTCTGGGAAGGGT-3′ (nt 5634–5613). All reagents (except the primers) were contained within the PrimerSTAR GXL DNA Polymerase mix (Takara Bio Inc. Shiga, Japan). The final reaction mixture (25 μL/sample) used for the first round of amplification contained 100 ng of sample DNA, each primer (0.2 μM), 200 μM dNTPs, and 0.625 U PrimerSTAR GXL DNA Polymerase. The PCR conditions were as follows: 30 cycles of denaturation at 98 °C for 15 s, followed by annealing at 60 °C for 20 s, and extension at 68 °C for 1 min. The PCR product (1 μL for blood samples and 5 μL for nasal and saliva samples) from the first round of amplification was then used in a second round of amplification