

Comparison of the copy numbers of bovine leukemia virus in the lymph nodes of cattle with enzootic bovine leukosis and cattle with latent infection

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Abstract To establish a diagnostic index for predicting enzootic bovine leukosis (EBL), proviral bovine leukemia virus (BLV) copies in whole blood, lymph nodes and spleen were examined by quantitative real-time PCR (qPCR). Cattle were divided into two groups, EBL and BLV-infected, based on meat inspection data. The number of BLV copies in all specimens of EBL cattle was significantly higher than those of BLV-infected cattle ($p < 0.0001$), and the number of BLV copies in the lymph nodes was particularly large. Over 70 % of the superficial cervical, medial iliac and jejunal lymph nodes from EBL cattle had more than 1,000 copies/10 ng DNA, whereas lymph nodes from BLV-infected cattle did not. These findings suggest that the cattle harboring more than 1,000 BLV copies may be diagnosed with EBL.

Introduction

Two types of bovine leukosis can be distinguished on the basis of their epidemiology: enzootic bovine leukosis (EBL), a disease caused by bovine leukemia virus (BLV), a member of the genus *Deltaretrovirus* in the family *Retroviridae*, and sporadic bovine leukosis (SBL), which is not transmissible. The majority of BLV-infected cattle do not display clinical signs of the disease. It is also worth noting that the disease does not appear to have a negative economic impact. However, approximately 30 % of BLV carriers develop a form of the disease known as persistent lymphocytosis (PL), characterized by an increase in the number of B lymphocytes, and only 1–5 % of BLV-infected animals develop malignant B-cell lymphosarcomas [10]. The mechanisms of leukemogenesis induced by BLV, as well as the processes underlying the phenomenon of host resistance/susceptibility to BLV infection and disease progression are complex and still remain poorly understood [9].

BLV infection has a worldwide distribution, and EBL is listed by the World Organization for Animal Health as a disease that can have a significant impact on international trade [18]. Bovine leukosis is a notifiable disease and has been subject to passive surveillance in Japan since 1997. Although EBL has been successfully eradicated in certain countries through national control programs, such as those used in Europe [1, 15], EBL is increasing rapidly in Japan. According to the animal hygiene statistics of Japan, 2,090 cases of bovine leukosis, including EBL and a few cases of SBL, were identified on 1,446 farms in 2012, whereas only 159 cases across 157 farms were reported in 2000 [11]. A nationwide survey was conducted from 2009 to 2011, and it revealed that the intra-herd seroprevalence in dairy and beef breeding cattle was 40.9 % and 28.7 %, respectively

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Table 1 BLV copy number in whole blood, spleen and lymph nodes from cattle with enzootic and sporadic bovine leukosis as well as from BLV-infected and BLV-free healthy cattle

Cattle type	Whole blood	Spleen	Superficial cervical Ln ^a	Popliteal Ln	Medial iliac Ln	Jejunal Ln	Mediastinal Ln	Other ^b
EBL								
n	90	35	38	13	47	25	15	6
Average	2.8×10^{3c}	2.8×10^3	2.2×10^3	1.9×10^3	2.6×10^3	3.8×10^3	2.1×10^3	1.3×10^3
Median	1.7×10^3	2.2×10^3	2.0×10^3	2.0×10^3	2.2×10^3	2.7×10^3	1.5×10^3	8.0×10^2
Minimum	2.5×10^1	1.1×10^0	3.8×10^1	1.1×10^2	3.2×10^1	7.9×10^1	3.4×10^2	3.1×10^2
Maximum	2.3×10^4	1.1×10^4	6.8×10^3	4.1×10^3	1.0×10^4	1.7×10^4	9.3×10^3	3.4×10^3
% of $\geq 1,000$ copies	65.6	65.7	76.3	61.5	70.2	76.0	66.6	33.3
BLV-infected								
n	37	12	0	0	3	36	0	0
Average	3.3×10^2	1.4×10^2	-	-	9.2×10^1	3.2×10^1	-	-
Median	1.1×10^2	6.1×10^1	-	-	2.1×10^1	7.4×10^0	-	-
Minimum	0	0	-	-	2.1×10^1	0	-	-
Maximum	2.6×10^3	5.2×10^2	-	-	2.3×10^2	2.2×10^2	-	-
% of $\geq 1,000$ copies	10.8	0	-	-	0	0	-	-
Healthy without anti-BLV antibody								
n	76	52	0	0	52	76	0	0
Average	0	0	-	-	0	0	-	-
Median	0	0	-	-	0	0	-	-
Minimum	0	0	-	-	0	0	-	-
Maximum	0	0	-	-	0	0	-	-
% of $\geq 1,000$ copies	0	0	-	-	0	0	-	-
SBL^d								
n	2	1	0	0	1	1	0	0
Average	2.6	0	-	-	9.3	0	-	-
Median	2.6	0	-	-	9.3	0	-	-
Minimum	0.0	0	-	-	9.3	0	-	-
Maximum	5.2	0	-	-	9.3	0	-	-
% of $\geq 1,000$ copies	0	0	-	-	0	0	-	-

^a Lymph nodes

^b Intercostal Ln (n=2), subilicac Ln (n=3) and parotid Ln (n=1)

^c BLV copies/10 ng DNA

^d Thymic form of sporadic bovine leukosis

[14]. The number of condemned EBL cattle at the time of meat inspection in slaughterhouses is also increasing.

After infecting cattle, BLV is integrated within the cellular genome as a provirus and enters a period of latency, during which expression is blocked at the transcriptional level [10]. Screening for antibodies has been the primary means of detecting the presence of infection. Agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA) are commonly used for routine detection of antibodies against BLV [18]. However, the use of these methods is prevented frequently by the detection

of BLV-infected cattle in which BLV antibody titers are low, transient, or undetectable [6, 7]. Therefore, diagnostic BLV polymerase chain reaction (PCR) techniques, which detect the integrated BLV proviral genome within the host genome, are commonly used [2, 12, 24].

EBL is usually diagnosed based on pathology tests at the time of meat inspection in slaughterhouses. In general, pathological procedures take a long time to yield a result, and some diseases, such as lymphadenopathy, are difficult to diagnose by pathology. Using only detection of BLV antibodies by AGID and ELISA, detection of the BLV

genome by conventional PCR, and BLV isolation it is not possible to distinguish cattle with EBL from BLV-infected cattle. Here, BLV copy numbers, determined by analysis of whole blood, lymph nodes and spleens of EBL cattle at the time of meat inspection, were compared in BLV-infected and healthy cattle by quantitative real-time PCR (qPCR). We intend to contribute to a new diagnostic index for diagnosing EBL without clinical signs in cattle.

Materials and methods

Animals and antibody test

Ninety whole blood, 144 lymph node and 35 spleen samples from 102 EBL cattle and 37 whole blood, 39 lymph node and 12 spleen samples from 37 BLV-infected cattle were examined. BLV-infected cattle were BLV antibody positive but clinically healthy at the time of meat inspection. EBL cattle were also BLV antibody positive and clinically healthy at the time of arrival at the slaughterhouse but then diagnosed with EBL by pathological examination. Seventy-six whole blood, 128 lymph node and 52 spleen samples from 76 BLV-antibody-negative cattle were used as negative controls. Furthermore, two whole-blood, two lymph node and one spleen sample/s were obtained from three animals that developed a thymic form of bovine leukosis, and one of them had BLV antibody. The regions of lymph nodes used are shown in Table 1.

BLV antibodies of all cattle were examined using a Bovine Leucosis Antibody Assay Kit (Nisseiken, Tokyo, Japan) according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR)

DNA was extracted using a DNeasy Blood and Tissue Kit (QIAGEN, Tokyo, Japan) or an automated DNA extraction system (PNE-2080, Marukomu, Tokyo, Japan) according to the manufacturer's instructions. Extracted DNA was quantified using OD₂₆₀ and OD₂₈₀ values obtained with an ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). One hundred ng of DNA was subjected to a qPCR assay. The BLV proviral load was measured using a Cycleave PCR bovine leukemia virus detection kit (TaKaRa, Shiga, Japan) and an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Cycleave PCR is a specific method that combines a chimeric fluorescence-labeled and quencher-labeled DNA/RNA oligonucleotide probe (cycling probe) and RNase H [3, 13]. PCR primers and probe were designed to amplify the BLV *tax* region. The BLV copy

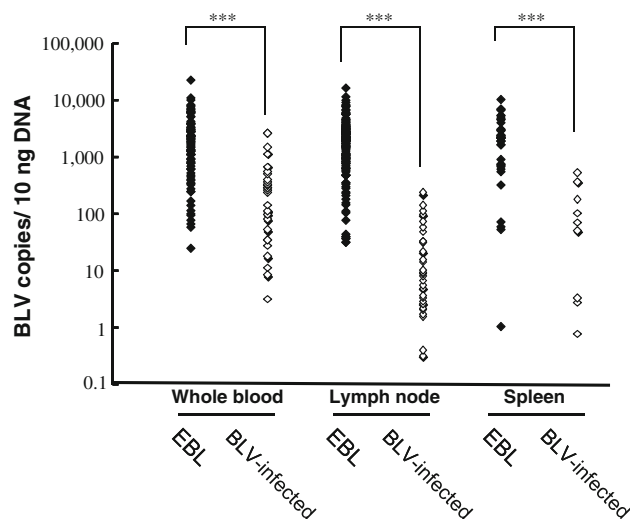


Fig. 1 Comparison of BLV proviral load in whole blood, lymph node and spleen samples between EBL and BLV-infected cattle. EBL, cattle with enzootic bovine leukosis; BLV-infected, cattle infected with bovine leukemia virus. *** $P < 0.0001$

number per 10 ng of DNA was determined for comparison between cattle groups.

Statistical analysis

The Wilcoxon/Kruskal–Wallis test (rank sum test) in JMP (Version 5.0, SAS Institute, Tokyo, Japan) was used to compare the proviral BLV copy number in whole blood and lymph node samples from EBL cattle, BLV-infected, and healthy cattle. $p < 0.05$ was considered a statistically significant difference.

Results

The BLV proviral load in EBL cattle was significantly higher than that in BLV-infected cattle in all three sample categories, i.e., whole blood, lymph node and spleen ($p < 0.0001$, Fig. 1). As shown in Table 1, proviral copy numbers in blood of EBL cattle ranged from 25 to 2.3×10^4 copies, and the median was 1.7×10^3 copies, whereas BLV copy numbers in blood of BLV-infected cattle ranged from 0 to 2.6×10^3 copies, and the median was 1.1×10^2 copies. In blood samples, 65.6 % of EBL cattle had more than 1,000 copies of BLV, and 10.8 % of BLV-infected cattle had more than 1,000 copies.

In lymph nodes of EBL cattle, BLV copy numbers ranged from 32 to 1.7×10^4 copies, and the median was 1.9×10^3 copies. BLV-infected cattle had between 0 and 2.3×10^2 copies, and the median was 6.8 copies. As shown in Table 1, 70.1 % of samples had more than 1,000

copies, and no BLV numbers exceeded 10^3 copies in BLV-infected cattle. Comparing the BLV copy numbers, no significant difference was observed in lymph node samples of EBL cattle. However, the percentage with more than 1,000 copies was in the following order: superficial cervical lymph node (76.3 %) > jejunal lymph node (76.0 %) > medial iliac lymph node (70.2 %) > mediastinal lymph node (66.6 %) > popliteal lymph node (61.5 %) > others (33.3 %).

The BLV copy number in spleen samples from EBL cattle ranged from 1.1 to 1.1×10^4 copies, and the median was 2.2×10^3 copies. Spleen samples from BLV-infected cattle ranged from 0 to 5.2×10^2 copies, and the median was 61 copies. In spleen samples from EBL cattle, 65.7 % of samples also exceeded 1,000 in BLV copy number, and there were fewer than 1,000 copies in all samples from BLV-infected cattle.

The BLV genome and BLV antibody were detected in one of three thymic forms of bovine leukosis. Although the BLV genome was detected in that animal, the copy numbers in whole blood and lymph node samples were only 5.2 and 9.3, respectively.

Discussion

We show here that it would be possible to distinguish EBL cattle from BLV-infected cattle, since there was a significant difference in BLV copy number between EBL cattle and BLV-infected cattle. In particular, comparing the BLV copy number in blood, lymph node and spleen samples in EBL and BLV-infected cattle, the largest difference in the median value was found in the lymph nodes. Interestingly, 70.1 % of lymph node samples and 65.7 % of spleen samples from EBL cattle had more than 1,000 copies, whereas the copy number never exceeded 1,000 in BLV-infected cattle. On the other hand, although 65.6 % of EBL cattle had more than 1,000 copies in blood samples, 10.8 % of BLV-infected cattle had more than 1,000 copies.

Hematologic manifestations do not reflect the pathological feature of bovine leukosis. Burton et al. [5] reported that only 10 % of cattle with bovine leukosis were leukemic, and 25 % had lymphocytosis according to Bendixen's key, which takes into account the normal decrease in lymphocyte count with age [4, 8], whereas 100 % of cattle were positive for lymphosarcoma by biopsy of enlarged peripheral lymph nodes. Given that no significant difference was observed in BLV copy number among lymph nodes of EBL cattle, any lymph node is suitable as a specimen. However, in EBL cases, the degree of enlargement and the degree of the infiltration of the tumor cell vary among lymph nodes from the same individual [17,

21]. In the lymph node specimens from EBL cattle where BLV copy number did not exceed 1,000 copies, enlargement was not observed. On the other hand, lymph node specimens where the BLV copy number exceeded 1,000, enlargement was observed. Therefore, an animal whose lymph node is enlarged and its BLV copy number exceeds 1,000 copies may be diagnosed with EBL. Cattle with fewer than 1,000 copies are difficult to diagnose as EBL, because there are BLV-infected cattle harboring 200–500 copies in their lymph nodes. In that case, reexamination by qPCR using a different lymph node, or in combination with pathological examination, is necessary for verification. In any case, it is recommended that enlarged lymph nodes are collected as specimens.

Taken together, lymph nodes are the most suitable tissues for diagnosing EBL in cattle by qPCR. It is suggested that cattle with a lymph node copy number exceeding 1,000 might be affected by EBL. On the other hand, although there were no essential differences between aleukemic and leukemic cases of EBL in general, a common finding in aleukemic leukosis is that there are practically no proliferative lesions of neoplastic cells in the spleen [16]. Therefore, the diagnostic value of using spleen tissue would be lower at the time of aleukemic attack.

Differentiation between EBL and SBL is also difficult by pathological testing alone. The BLV genome was detected in one out of three cattle that developed the thymic form. However, the BLV copy number in lymph nodes was almost the same as those of BLV-infected and healthy cattle. It was concluded that this was cattle developing the thymic form in the presence of BLV infection.

Recently, EBL cattle numbers have been increasing more rapidly in Japan. EBL cattle show atypical clinical manifestations, e.g., aleukemia without enlargement of superficial lymph nodes [21, 22], atypical mononuclear cells at very low levels in the peripheral blood [16], and unusual clinical manifestations, with cattle showing lymphocytosis [20]. Given that the EBL outbreak continues to increase, the number of cases that are difficult to diagnose will also increase in the field or at the time of meat inspection. During the last few decades, a series of attempts have been made to develop a vaccine against BLV [19]. However, despite advances in research on experimental vaccines, there is as yet no vaccine commercially available for the control of EBL.

Our study suggests that there is a possibility for ante-mortem diagnosis of EBL using a combination of core needle biopsy or fine-needle aspirate of enlarged peripheral lymph nodes and qPCR [23]. Furthermore, as a consequence of using qPCR in combination with pathological examination, the time from sampling to a diagnosis might shorten and improve detection efficiency of EBL at the time of meat inspection.

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