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Cattle with a low bovine leukemia virus proviral load are rarely an infectious source

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Abstract

Bovine leukemia virus (BLV) is an etiological agent of fatal B-cell leukemia and malignant lymphoma in cattle. Cattle with higher BLV proviral loads represent a higher risk of both horizontal and vertical transmission. Therefore, quantifying the proviral load of BLV is important in identifying major infectious sources and protecting BLV-free cattle from exposure to infected cattle. In this study, we confirmed that cattle with very low BLV proviral loads did not transmit the virus to virus-free cattle under conventional conditions. We observed a total of 7 tests in which a BLV-infected bull was allowed to cohabit with 57 to 92 BLV-free cattle for 12 or 22 months. We then evaluated the frequency of viral transmission. A BLV-infected bull with a "very low proviral load" (i.e., fewer than 100 proviral copies/50 ng of genomic DNA) did not transmit the virus to any virus-free cattle in 2 out of 2 tests. However, a BLV-infected bull with a "low proviral load" (i.e., 100 to 500 copies/50 ng) transmitted the virus to a total of 3 virus-free cattle in 2 out of 5 tests. These results suggest that BLV-infected cattle with "very low proviral loads" do not transmit the virus under conventional conditions, while cattle with "low proviral loads" can transmit the virus, although at low rates. We believe that the results of this study will promote the construction of effective measures to prevent BLV infection and control the spread of BLV.

Key Words: BLV, EBL, infectious source, proviral load, virus transmission

Introduction

Bovine leukemia virus (BLV), a member of the *Retroviridae* family and *Deltaretrovirus* genus, is an etiological agent of fatal B-cell leukemia and malignant lymphoma in cattle which are called enzootic bovine leukosis (EBL). Although more than 90% of BLV-infected cattle remain EBL-free for life, one to five percent of such cattle develop EBL several years after infection[3,23)].

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Australia, New Zealand and many European countries have successfully eliminated BLV. However, other countries, such as Japan, Argentina and the USA, have a high BLV seroprevalence[2,16,22]. BLV can cause lifelong infection, and no vaccines or therapeutic procedures are currently available to prevent BLV infection or the development of EBL. Therefore, preventing cattle from becoming infected is the only feasible measure to reduce the incidence of EBL.

An animal's BLV proviral load fluctuates months after infection and becomes stable over time, similar to the case in human T cell leukemia virus type-1 infection[1,6]. This stable state is called set point. The proviral load in set point varies widely between individual cattle and can range from fewer than 2 copies per 50 ng of genomic DNA to more than 2,000. Cattle with higher proviral loads have a higher risk of virus transmission[10,13,17]. Therefore, quantifying BLV proviral loads in set point and protecting BLV-free cattle from exposure to infectious cattle are important.

The purpose of this study was to evaluate the frequency of viral transmission by cattle with low BLV proviral loads to BLV-free cattle in dairy herds on a private farm under conventional conditions. We observed cohabitation tests of low BLV proviral-load cattle and many virus-free cattle and then evaluated the frequency of viral transmission. The results of this observation show that cattle with very low BLV proviral loads do not transmit the virus to virus-free cattle and that a BLV proviral load higher than 100 copies/50 ng could potentially be used as a criterion to discriminate whether cattle will become an infectious source of BLV under conventional conditions. These results suggests a method to control BLV in farms.

Materials and Methods

Farm and animals used in this observational study: An observational study was conducted on a private dairy farm located in Oita Prefecture, Japan. The animals studied on this farm were mostly Holstein cows, but four were Japanese Black bulls, which were used for natural breeding. This farm contained more than 30 free-stall cowsheds, and this study was conducted in 4 of these cowsheds. Fifty-seven to ninety-two BLV-free healthy cows and one BLV-infected bull were housed in each observational cowshed sufficiently isolated from each other. After artificial insemination was performed, the cows were moved to the cowshed to live with the bull regardless of whether conception occurred. All of the cows could freely contact the bull that was in the same cowshed. As is usual in dairy herds, cows were necessarily replaced according to health management. Only BLV-seronegative cows were brought into the observational cowsheds. The sanitary management of this farm is described below. Artificial insemination was performed using a separate device for each animal. Rectal palpation was performed with a single-use plastic shoulder-length glove. Vaccinations and injections were performed using single-use devices. Stable flies (*Stomoxys calcitrans*) identified as a vector of BLV were present during all seasons and were most prevalent in early and late summer[4].

Farms and animals used in the reference study: Reference studies were conducted on two farms that were located in Miyazaki Prefecture, Japan. Reference farm A was a dairy farm with a high BLV infection rate. Reference farm B was a beef farm with an average BLV infection rate. The sanitary management of these farms was nearly identical to that on the observational farm. Fly screen nets, the mesh size of the net was about 2×2 mm, were installed to separate BLV-free cows from infected cows on reference farm B.

Blood sampling: The number of blood samples collected and the period during which sampling was performed are listed in Table 1. All blood

Table 1. The number of samples and bovine leukemia virus (BLV) infection status found in each test

	Observational farm			Reference farm A		Reference farm B	
	1st test	2nd test	3rd test	1st test	2nd test	1st test	2nd test
Period of sampling	Mar–Apr 2014	Feb–Mar 2015	Feb 2017	Jun 2016	Nov 2016	Nov 2015	Dec 2016
Number of samples	2,106	2,128	2,257	28	28	30	30
Cows	2,093	2,114	2,243	28	28	30	30
Bulls	13	14	14	0	0	0	0
Number of BLV-infected (%)	37 (1.75)	6 (0.28)	7 (0.31)	22 (78.5)	24 (85.7)	9 (30.0)	10 (33.3)
Cows	32 (1.52)	2 (0.09)	4 (0.17)	22 (78.5)	24 (85.7)	9 (30.0)	10 (33.3)
Bulls	5 (38.4)	4 (28.5)	3 (21.4)	–	–	–	–

samples were centrifuged (1,500 × g for 5 min at 4°C), and 500 µl of the resulting serum or plasma was dispensed into the wells of 96 deep-well plates. Blood was collected and serum was separated from all cows in the observational farm by the Oita Livestock Hygiene Service Center as a part of annual activities aimed at eliminating Johne's disease, brucellosis and tuberculosis. Blood was sampled from other cows by local clinical veterinarians. All serum and plasma samples were stored at −20°C until further analysis. All blood samples were refrigerated while stored, and DNA extraction was performed within a week of blood collection.

ELISA test to identify seropositive cattle: All serum and plasma samples were analyzed using a BLV gp51 antibody detection enzyme-linked immunosorbent assay (ELISA) kit (JNC, Tokyo, Japan) according to the manufacturer's instructions. All seropositive cows were immediately moved to a separate cowshed and culled within 2 months. Bulls with a high BLV proviral load or those that were old were culled, and bulls with a "low BLV proviral load" (i.e., bulls R64, R67 and R68) and "very low BLV proviral load" (i.e., bull R69) were used for natural breeding on this farm (Table 2).

DNA extraction and BLV proviral load quantification: Genomic DNA was extracted from BLV-seropositive blood samples using a Wizard Genomic DNA Purification kit (Promega, Fitchburg, USA) according to the manufacturer's instructions. The DNA concentration and purity were determined using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and diluted to a concentration of 20 ng of genomic DNA/µl. The proviral load was quantified using a LightCycler 96 System (Roche Diagnostics, Indianapolis, USA). Amplifications were performed in a reaction mixture containing 5 µl of 2x Cycleave PCR Reaction Mix (TaKaRa Bio, Kusatsu, Japan), 0.2 µl of Probe/Primer Mix for BLV (TaKaRa Bio), 0.6 µl of a template DNA sample and PCR-grade water, which was used to bring the reaction volume to 10 µl. To determine the proviral load, calibration curves were generated from measured concentrations of a dilution series of positive control plasmid containing the BLV *tax* gene (TaKaRa Bio). Each amplification procedure was performed in duplicate and is expressed as the number of proviral copies per 50 ng of genomic DNA.

BLV transmission route: The BLV infectious source was confirmed using both the history of the residents of the same cowshed and a partial sequence of the BLV *env* gene. The detail information about mobile histories among cowsheds of every cattle were managed by software Salesforce cloud system (Salesforce.com, San Francisco, USA). Sequencing and phylogenetic analyses of partial BLV *env* gene sequences were performed as described our previous studies[12,14].

Accession numbers: The partial *env* sequences of BLV that were used in this study were submitted to DDBJ under Accession No. LC310854-63.

Table 2. Details about each test and the results used to determine bovine leukemia virus (BLV) incidence rates

Test ID	Shed R64-1	Shed R67-1	Shed R67-2	Shed R68-1	Shed R68-2	Shed R69-1	Shed R69-2	Reference A	Reference B
Bull cattle ID	R64	R67	R67	R68	R68	R69	R69	NA	NA
Copy number (copies/50 ng)	93.9–143.0	348.5	348.5–350.1	81.9–101.2	101.2–123.1	5.3–18.6	18.6–30.6	NA	NA
Classification of proviral load	Low	Low	Low	Low	Low	Very low	Very low		
Number of BLV-infected cattle	1	1	1	1	1	1	1	28	9
High proviral load[a]	0	0	0	0	0	0	0	22	1 (separated)[d]
Low proviral load[a]	1	1	1	1	1	0	0	3	0
Very low proviral load[a]	0	0	0	0	0	1	1	3	8 (separated)[d]
Number of BLV-free cattle	65–86	57–82	80–85	79–82	64–84	61–86	55–92	8	21
Contact ratio with infected cattle[b]	0.011–0.015	0.012–0.017	0.011–0.012	0.012	0.011–0.015	0.011–0.016	0.010–0.018	3.25	0.42
The period of observation (mo)	12	12	22	12	22	12	22	5	13
New BLV-infected cows	0	1	0	0	2	0	0	2	1
Incidence of BLV (+) conversion[c]	0.00	1.21–1.75	0.00	0.00	1.29–1.70	0.00	0.00	60.00	4.39

[a]High, low and very low proviral are defined as BLV-infected cattle with proviral loads of more than 500, 100–500 and fewer than 100 copies/50 ng, respectively
[b]Contact ratio with infected cattle: The number of BLV-infected cattle/The number of BLV-free cattle
[c]Incidence of BLV (+) conversion: (New BLV-infected cows/number of BLV-free cattle)/period of observation × 12 mo × 100 cattle
[d]separated: Fly screen nets were installed to separate BLV-free cows from infected cows

Results

The seropositive rates for each test are listed in Table 1. The proviruses in BLV-infected bulls were genetically different (Fig. 1). In the observational farm, the fifty-seven to ninety-two BLV-free cows and a BLV-infected bull in each cowshed had opportunities of contact for 12 or 22 months. The contact ratios between BLV-infected bulls and virus free cows for the groups with 12 and 22 months of contract were 0.011 and 0.018 (Table 2). The observational period included 1 or 2 summer seasons during which the cattle were frequently exposed to blood-sucking insects. Despite this extended contact, no new BLV-seropositive cows were detected in the 2 trials (Shed R69-1 and R69-2) in which the bull (R69) had a BLV proviral load lower than 100 copies/50 ng. However, a total of 3 newly BLV-infected cows were found in 2 of the trials (Sheds R67-1 and R68-2) in which the bulls (R67 and R68) had a proviral load of 100 to 500 copies/50 ng. The BLV incidence rate on the observational farm was as low as 1.75 cases per 100 cattle in a year, whereas the rates on the reference farms varied from 4.39 to 60 per 100 cattle in a year.

Discussion

We observed natural infections from bulls instead of cows as an infectious source. We found that bulls with very low BLV proviral loads (i.e., $<$100 copies/50 ng) did not transmit the virus to virus-free cows, while bulls with low BLV proviral loads (i.e., 100–500 copies/50 ng) were probably transmit the virus, but in fewer than 1.75 cases per 100 cattle-year. These incidence rates were lower than those observed at reference farm B, where BLV-infected cattle were segregated from virus-free cattle in the farm. It is assumed that the fly screen nets incompletely separate BLV-free animals from the infected animals. A brief period of open the nets was conducted in this farm due to some management practices

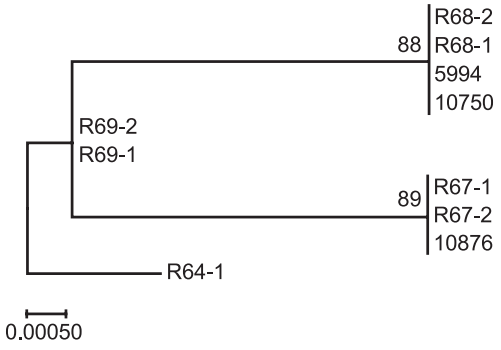

Fig. 1. Phylogenetic analyses of the BLV *env* gene at the nucleotide level. The phylogenetic tree is based on partial sequences (423 nt) of the *env* gene. Detailed information regarding specific bulls (R64, R67, R68 and R69) is listed in Table 2. The numbers 5994, 10750, 10876 indicate the IDs of the newly BLV-infected cows. The evolutionary history was inferred using the Neighbor-Joining method. More than 50% of the bootstrap values (1,000 replicates) are shown next to the branches. The scale bars indicate the number of substitutions per site. The nucleotide sequences for each gene were published on DDBJ (Accession No. LC310854-63).

such as transportation of diet and disposal of feces. It might allow the invasion of the vector fly. Despite same infected bulls were used for the trials, the different results were obtained from shed R67-1 and -2 and R68-1 and -2. It might influence the less chance of viral transmission from low proviral load cattle. We couldn't conclude that the presence of infected bull was only possible exposure. Therefore, the transmission rates from infected-bulls might be lower than 1.75 cases per 100 cattle-year. This result suggests that culling, segregating or moving all the cattle with high BLV proviral loads (i.e., $>$500 copies/50 ng) would be a simple and effective method for controlling BLV.

In this study, the BLV infectious source was confirmed using both the history of which cows and bulls lived in the same cowshed and a partial sequence of the BLV *env* gene. It was unidentified that each viral transmission by the bull was through breeding, casual contact or blood-sucking by the vector fly. If the viral transmission was occurred through breeding, the BLV incidence rate by cows with low BLV proviral loads might fewer than 1.75. The partial sequence of the BLV *env* gene is suitable for BLV genotyping analyses and for determining the infectious route[14,17,20]. A total of two newly BLV-infected cows were identified in cowshed R68-2. We could not clarify the direction of BLV infection or whether both or only one of the cows were infected by the BLV-infected bull. Therefore, the incident rate could be lower than 1.29–1.70 cases per 100 cattle in a year.

Many factors contribute to determining the set point of BLV proviral load in each cattle[11,18,19]. These include the bovine leukocyte antigen (BoLA) *DRB3* gene[9,15]. A previous study showed that BLV-infected cattle that possess the *BoLA-DRB3*0902* allele did not transmit the virus to virus-free cows on a dairy farm[8]. Although the specific mechanism underlying this effect remains controversial, some authors have proposed that a peptide-binding cleft in the BoLA gene might confer strong protective immunity against BLV infection and result in a very low BLV proviral load[5,21]. The results of our large-scale survey demonstrated that BLV-infected cattle that possess the *BoLA-DRB3*0902* allele had a very low proviral load (in Holstein cattle, an average of 13.8 copies/50 ng)[7]. This may well explain why BLV-infected cattle with this BLV-resistance allele did not become an infectious source for virus-free cattle. Our preliminary survey confirmed that none of the BLV-infected bulls used in this study possessed the *BoLA-DRB3*0902* allele.

To prevent BLV infection, quantifying the proviral load of cattle is important because cattle with higher BLV proviral loads have a higher risk of transmitting BLV. However, no criteria were previously available to discriminate between what constitutes high and low proviral loads and what threshold might be useful for preventing the horizontal transmission of the virus under conventional conditions. The data presented in this study provide a method for discriminating cattle with high and low proviral loads of BLV (i.e., a threshold of 500 copies/50 ng) and suggest an effective and realistic measure for controlling

BLV on dairy farms.

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