

Predicting an increased risk of severe clinical mastitis and economic loss using a threshold value of bovine leukemia virus proviral load

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OBJECTIVE

To establish a threshold value of bovine leukemia virus (BLV) proviral load (PVL) to identify increased risk of severe clinical mastitis, and to examine the prognosis and economic loss of clinical mastitis based on the newly established PVL cut-off value.

ANIMALS

97 lactating Holstein cows with clinical mastitis.

METHODS

Blood and milk samples were collected aseptically from each cow. Youden index was used for receiver-operating characteristic curve analysis with the severity rate of clinical mastitis as the dependent variable and PVL as an independent variable. PVL cut-off value was used as a criterion to compare the severity rate of clinical mastitis, percentage of cows with and without systemic treatments, number of treatments, cost of treatment, and prognosis.

RESULTS

PVL cut-off value was 17.8 copies/10 ng DNA for the dependent variable MILD vs SEVERE. The severity rate of clinical mastitis, percentage of cows given systemic treatments, and technical fees for medical treatment were significantly higher in the group above the PVL cut-off value than in the group below the PVL cut-off value and the negative group. Number of treatments was significantly higher in the group above the cut-off value than in the group below the cut-off value. There was no significant difference in prognosis after mastitis among the 3 groups.

CLINICAL RELEVANCE

These results suggested that PVL cut-off value of 17.8 copies/10 ng DNA was a useful threshold for increased economic losses in BLV-infected cows; it may also serve as a new standard value for the detection and culling of BLV-infected cows in Japan.

Keywords: bovine leukemia virus, cut-off value, economic loss, proviral load, severity of clinical mastitis

The lactational disease is very common in cows, of which mastitis is the most representative and accounts for 30% of cow disease and injury incidences in Japan.¹ Severe mastitis with systemic symptoms causes a marked decrease in milk production;² increased medical costs, death, and culling;³ and tremendous economic damage to the livestock industry in Japan. Furthermore, it is also a major

animal welfare issue, such as increased pain.⁴ The predominant cause of mastitis is a bacterial infection from the teat-end; however, it has been reported that not only the pathogen factor but also the host's immune status is involved in its severity.⁵

Enzootic Bovine Leukosis (EBL) is caused by infection with the bovine leukemia virus (BLV). After infection, BLV is integrated into the animal's genome as a provirus, where these sequences remain for the rest of the host's life.^{6,7} Most BLV-infected cows are asymptomatic but continue to shed the virus (ie, asymptomatic antibody-positive cows). As the disease progresses, about 30% of BLV-infected cows

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develop persistent lymphocytosis, and a small percentage of BLV-infected cows may develop EBL.^{8,9} EBL outbreak reports in Japan are increasing,¹⁰ and it has already been reported that about 35% of cows in Japan are infected with BLV by a large survey conducted between 2009 and 2011.¹¹ The most effective approach for BLV eradication is to cull the BLV antibody-positive cows,¹² but this is not currently a realistic approach in Japan where there are many BLV antibody-positive cows.

It has been reported that BLV proviral load (PVL), that is, the number of provirus copies,¹³⁻¹⁵ increases with the progression of BLV infection,¹⁶ and high PVL in BLV-infected cows is a risk factor for the development of EBL.¹⁷ Additionally, it has been reported that the higher the PVL, the easier it is for BLV to be transmitted both horizontally¹⁵ and vertically,¹⁸ and that horizontal transmission does not occur when PVL is low.¹⁹ It has also been reported that controlling new infections of BLV is possible by culling BLV-infected cows with high PVLs and high lymphocyte counts.²⁰ Thus, research is being conducted with a focus on the amount of PVL, not only as a criterion for evaluating the risk for the onset of EBL but also as a decision criterion for measures to control BLV transmission.

As for the relationship between BLV infection and the productivity of dairy cows, it has been reported that milk production is less and somatic cell count in milk are significantly higher in the early and middle stages of BLV-infected cows at more than their fourth lactation compared to BLV noninfected cows,²¹ and that there is a negative association between milk production and BLV infection at the herd level^{22,23} and the individual level,²⁴ indicating an association between BLV infection and lower milk productivity. As for the recent reports describing the relationships between PVL and productivity and economic losses in dairy cows, it has been reported that cows with high PVL were more susceptible to subclinical mastitis within 52 days after calving than BLV noninfected cows and cows with low PVL, causing significant economic losses in Hokkaido, Japan;²⁵ it has been pointed out that there is an association between PVL and susceptibility to mastitis. Additionally, we previously reported that high PVL are associated with a higher severity of clinical mastitis,²⁶ and the relationship between PVL and the severity of mastitis is becoming clearer. However, the PVL value used in this study was based on the value suggested as a diagnostic index for predicting EBL²⁷ and was not developed as an index for severity risk assessment of clinical mastitis. Therefore, a specific PVL value that could serve as a diagnostic index for assessing the increased risk of severe clinical mastitis has not yet been clarified. Furthermore, the economic loss associated with the treatment of clinical mastitis in BLV-infected cows has also not been reported. Although criteria for culling BLV-infected cows using PVL values obtained by evaluating the frequency of vertical¹⁸ and horizontal infection¹⁹ have been presented as one of the measures to control BLV transmission in Japan, clarification of PVL values determined based

on the risk of severe clinical mastitis would allow for further prioritization in the selection of cows for the detection and culling.

The purpose of the present study was to establish a threshold value of PVL to identify an increased risk of severe clinical mastitis. Next, the prognosis and economic loss of clinical mastitis based on the newly developed PVL value were examined.

Methods

Animals

A total of 104 quarters of 97 lactating Holstein-Friesian cows with clinical mastitis from 15 farms with stall barns in Saitama prefecture were sampled from February 2016 to May 2017. All cows were enrolled in the study after the acquisition of owner consent, and this study was conducted following the regulations of the Azabu University Animal Experimentation Committee (Sagamihara, Japan). The day of onset of clinical mastitis was set as day 0, and on the 7th, 14th, and 21st days, cows were identified by clinical evaluation and diagnosis by a veterinarian. After evaluation and diagnosis, milk samples were collected aseptically from each cow, and peripheral blood was collected from the tail vein using EDTA-2Na-containing vacuum blood collection tubes. The collected milk was stored at -80°C until bacterial culture assessment and somatic cell count measurement. Blood was stored at 4°C until used for BLV detection tests, which were performed by quantitative PCR (qPCR). The number of treatments, costs, and survival data were obtained from the medical records.

Evaluation of severity of clinical mastitis

The severity of clinical mastitis was defined by a veterinarian at the time of onset of clinical mastitis. Based on the International Dairy Federation definitions of mastitis severity²⁸ and previous report,^{29,30} the severity at the onset of clinical mastitis was classified as MILD (those with observable abnormalities in milk, such as clots or flakes, discoloration, with little or no signs of swelling of the mammary gland or systemic illness), MODERATE (those with visibly abnormal milk, as described in MILD, accompanied by swelling in the affected mammary quarter with an absence of systemic signs of illness), or SEVERE (those with milk abnormalities and udder abnormalities as described in MODERATE, and with at least 2 of the following clinical signs suggestive of systemic involvement: (1) rectal temperature $\geq 39.0^{\circ}\text{C}$, (2) anorexia, (3) marked depression, (4) decreased milk production, or (5) shock (increased heart rate, tenting of eyelids, shivering, and slow capillary refill time).

Bacterial culture from milk

Milk samples were cultured on 5% sheep blood agar at 37°C for 24 hours, and the bacteria present in milk samples were identified according to the procedure described by the National Mastitis Council.³¹

Measurement of somatic cell count in milk

Measurement of somatic cell count in milk was performed as previously described.³² The cells were quantified using a DeLaval cell counter (DeLaval International AB). One milk sample from 1 cow was excluded from the analysis as it contained an excessive number of clots and flakes, making it unsuitable for measurement using the DeLaval cell counter. All milk samples used in this study, excluding the 1 milk sample that could not be processed, were confirmed to have a somatic cell count of 350,000 (/mL) or higher. The milk sample that could not be measured was confirmed positive by the Californian Mastitis Test using a PL tester (ZENOAG).

Medical record survey

The number of treatments, drug prices, technical fees for medical treatment, and medical fees (total of drug prices and technical fees for medical treatment) were obtained from medical records. Cows without records of the information mentioned above were excluded. Survival status was determined using individual identification numbers. Four veterinarians performed the treatment according to the standard treatment protocol based on the Livestock Mutual Aid System in Japan.³³ Occurrence periods were classified by season (Spring: March to May, Summer: June to August, Fall: September to November, Winter: December to February) according to the classification of the Japan Meteorological Agency.³⁴

Measurement of BLV PVL

As previously described,³⁵ we measured BLV PVL using a method that is widely used in Japan^{36–38} for quantifying BLV PVL and is regarded as reliable.

Isolation of serum and genomic DNA from blood—Serum was prepared from the blood by centrifugation. The genomic DNA was extracted from the whole blood using a Wizard genomic DNA purification kit (Promega Corp), according to the manufacturer's instructions.

Construction of molecular clones and plasmids—The BLV proviral genome was amplified by PCR using PrimeSTAR GXL DNA polymerase (Takara Bio), and cloned into pSMART LC Amp (Lucigen). The cloned BLV genome sequences were amplified in *Escherichia coli* strain Stbl3. For the construction of mutant clones with partial sequence deletions and insertion in the G4 gene, inverse PCR was performed using the clone as template DNA. The PCR products were then self-ligated using In-Fusion HD Cloning Kit (Takara Bio) and were amplified in *E coli* strain Stbl3.

The BLV LTR sequence, G4 gene sequence, and G4 gene sequence fused with an HA tag sequence were amplified by PCR from the genomic DNA of FLK-BLV. Firefly luciferase gene was amplified by PCR using pCMV-Luc plasmid vector (Promega) as template DNA. The amplified PCR products of LTR together with those of the firefly luciferase gene, the firefly luciferase gene alone, the HA tag alone and HA-tagged G4 were cloned into pBApo-EF1 a-Neo

(Takara Bio) to construct pBApo-LTR-Luc, pBApo-EF1a-Luc, pBApo-EF1a-HA, and pBApo-EF1a-G4-HA, respectively. The clones and plasmids were validated by sequencing.

qPCR for BLV—The qPCR to quantify viral and proviral BLV genome levels was performed for all samples using the 7500 Real-Time PCR system (Applied Biosystems). The primers 5'-GGACAAATGGACTGCTCAAAC-3' and 5'-CTCCCATCTGGTCTTTAGAATTG-3' and the probe 5'-FAM-CTTCCCATGACTCAGGCCCTTTCT-TAM-3' were used to measure BLV PVL in BLV-infected cow and proviral copy number in the supernatant of clone-transfected HeLa cells. The primer and probe used in this study were designed to target the highly conserved gene region encoding BLV polymerase (GenBank accession numbers are LC164083, LC164084, LC164085, and LC164086). A standard curve was generated using serial dilutions of pBLV-FLK as the template, and the proviral copy number was calculated.

Identifying the blood PVL cut-off value, evaluation, and analysis

To identify the cut-off value of PVL, the Youden index was used for receiver-operating characteristic curve analysis with clinical mastitis (MILD vs MODERATE, MILD vs SEVERE, and MODERATE vs SEVERE) as the dependent variable and PVL as the independent variable. The PVL with the highest area under the curve obtained for each dependent variable was set as the cut-off value for this study. Cows were classified and compared based on the cut-off value in this study.

Statistical analysis

To compare host, environmental, and pathogen factors in the MILD, MODERATE, and SEVERE groups, the Kruskal-Wallis test with Bonferroni correction was used for age and days in milk, and Fisher's exact test was used for number of BLV infections, season at onset of mastitis, and pathogens. The Kruskal-Wallis test with Bonferroni correction was also used to analyze the somatic cell count in milk at the onset of clinical mastitis (day 0), the number of treatments, drug prices, and technical fees for medical treatment. Fisher's exact test was used to compare the number of incidents of clinical mastitis (MILD vs MODERATE vs SEVERE) according to PVL, and the Kruskal-Wallis test with Bonferroni correction was used to compare the percentages of cows with and without systemic treatments, the number of treatments, drug prices, and technical fees for medical treatment. The Wilcoxon rank-sum test was used to compare the percentages of cows with and without systemic treatments, the number of treatments, drug prices, and technical fees for medical treatment by detection of coliforms. The Kruskal-Wallis test with Bonferroni correction was used to compare the somatic cell count on day 0, and the Wilcoxon signed-rank test was used for trends in the somatic cell count by PVL for short-term prognosis

evaluation. The Kaplan-Meier method with the log-rank test was used to analyze the 3-year survival rate after mastitis for long-term prognosis evaluation.

IBM SPSS Statistics 27.0 software (International Business Machines Corp) was used for all statistical analyses. Values of $P < .05$ were considered significant.

Results

Cows

Of the 104 quarters of 97 cows included in this investigation, the number of BLV-positive cows with BLV PVL in blood detected by quantitative PCR was 82 (89 quarters), and the number of BLV-negative cows without PVL in blood detected was 15 (15 quarters). Mild clinical mastitis was present in 31 quarters of 29 cows (BLV-positive, 25 quarters of 23 cows; BLV-negative, 6 quarters of 6 cows), moderate clinical mastitis was present in 37 quarters of 36 cows (BLV-positive, 30 quarters of 29 cows; BLV-negative, 7 quarters of 7 cows), and severe clinical

mastitis was present in 36 quarters of 32 cows (BLV-positive, 34 quarters of 30 cows; BLV-negative, 2 quarters of 2 cows).

Comparison of clinical mastitis among MILD, MODERATE, and SEVERE groups

There were no significant differences in the host (number of BLV infections, age, and days in milk) and environmental (season at onset of mastitis) factors among the MILD, MODERATE, and SEVERE groups, but the number of coliform detections was higher in the SEVERE, MODERATE, and MILD groups, in that order ($P = .023$) (Table 1). The number of treatments ($P = .005$), drug prices ($P < .001$), and technical fees for medical treatment ($P < .001$) were significantly higher in the SEVERE group than in the MILD group (Table 2). Drug prices ($P = .018$) and technical fees for medical treatment ($P = .039$) were significantly higher in the MODERATE group than in the MILD group (Table 2). There was no significant difference in somatic cell count on day 0 among the 3 groups (Supplementary Table S1).

Table 1—Comparison of host, environmental, and pathogenic factors by severity of clinical mastitis.

Variables		MILD (31 quarters of 29 cows)	MODERATE (37 quarters of 36 cows)	SEVERE (36 quarters of 32 cows)	P-Value
Number of cows	BLV positive	23	29	30	.198
	BLV negative	6	7	2	
Age (year)	(Mean \pm SD)	4.9 \pm 2.1	4.5 \pm 1.4	4.7 \pm 1.9	.854
Days in milk	(Mean \pm SD)	164.7 \pm 142.7	181.7 \pm 149.5	113.1 \pm 108.2	.11
Season	Spring	6	9	14	.073
	Summer	4	11	6	
	Autumn	3	6	5	
	Winter	16	10	7	
Pathogen	Streptococci spp.	6	8	4	.023*
	Enterococci spp.	1	5	4	
	Coliform	0	2	7	
	Cogalase-negative staphylococci	9	2	3	
	Staphylococcus aureus	6	8	4	
	Corynebacterium bovis	2	0	0	
	Trueperella pyogenes	0	0	2	
	Yeast	0	0	2	
	Prototheca zopfii	0	1	1	
	No growth	7	10	8	
	Unknown	0	1	1	

Season: Spring (March, April, May), Summer (June, July, August), Autumn (September, October, November), Winter (December, January, February). The number of coliform detections is higher in the SEVERE, MODERATE, and MILD group, in that order significantly ($P = .023$).

* $P < .05$.

Table 2—Comparison of the number of treatments and the cost of treatments by severity of clinical mastitis.

Variables	MILD (n = 27)	MODERATE (n = 33)	SEVERE (n = 32)	P-Value
The number of treatments	3.0 (0.0–4.0) ^a	3.0 (3.0–5.0)	4.5 (3.0–7.0) ^c	.005**
Drug prices (yen)	627 (0–1,317) ^a	1,822 (522–3,984) ^b	2,770 (1,562–5,146) ^c	< .05*
Technical fees for medical treatment (yen)	500 (0–2,070) ^a	1,790 (1,260–3,100) ^b	2,825 (1,560–3,700) ^c	< .05*

Excluding house call fee and inspection fee, such as culture tests. Cows without records of information mentioned above were excluded. Data are reported as median and interquartile range. Cost of treatments: total of drug prices and technical fees for medical treatments.

The number of treatments ($P = .005$) and drug prices ($P < .001$), and technical fees for medical treatment ($P < .001$) were significantly higher in the SEVERE group than in the MILD group. Drug prices ($P = .018$) and technical fees for medical treatment ($P = .039$) were significantly higher in the MODERATE group than in the MILD group.

a–b, * $P < .05$. a–c, ** $P < .01$.

Cut-off values of blood PVL

The cut-off value was 16.55 copies/10 ng DNA (area under the curve 0.535, sensitivity 0.472, and specificity 0.69) for MILD vs MODERATE (**Figure 1**). The cut-off value was 17.8 copies/10 ng DNA (area under the curve 0.717, sensitivity 0.781, and specificity 0.69) for MILD vs SEVERE (Figure 1). The cut-off value was 29.45 copies/10 ng DNA (area under the curve 0.678, sensitivity 0.75, and specificity 0.611) for MODERATE vs SEVERE (Figure 1).

Classification and comparison by blood PVL cut-off value

The 97 cows with MILD (n = 29), MODERATE (n = 36), and SEVERE (n = 32) clinical mastitis were classified based on the PVL cut-off value (17.8 copies/10 ng DNA) obtained in this study as the group above the cut-off value (n = 51), the group below the

cut-off value (n = 31), and the negative group (PVL nondetected: n = 15) (**Table 3**).

In the group above the cut-off value, 9 cows (17.6%) had MILD clinical mastitis, 17 cows (33.3%) had MODERATE clinical mastitis, and 25 cows (49.0%) had SEVERE clinical mastitis; in the group below the cut-off value, 14 cows (45.2%) had MILD clinical mastitis, 12 cows (38.7%) had MODERATE clinical mastitis, and 5 cows (16.1%) had SEVERE clinical mastitis; and in the negative group, 6 cows (40.0%) had MILD clinical mastitis, 7 cows (46.7%) had MODERATE clinical mastitis, and 2 cows (13.3%) had SEVERE clinical mastitis (Table 3). SEVERE clinical mastitis occurred significantly more often in the group above the cut-off value than in the group below the cut-off value ($P = .004$) and the negative group ($P = .0034$) (**Figure 2**).

Trends in somatic cell count showed that, compared to day 0, somatic cell count decreased

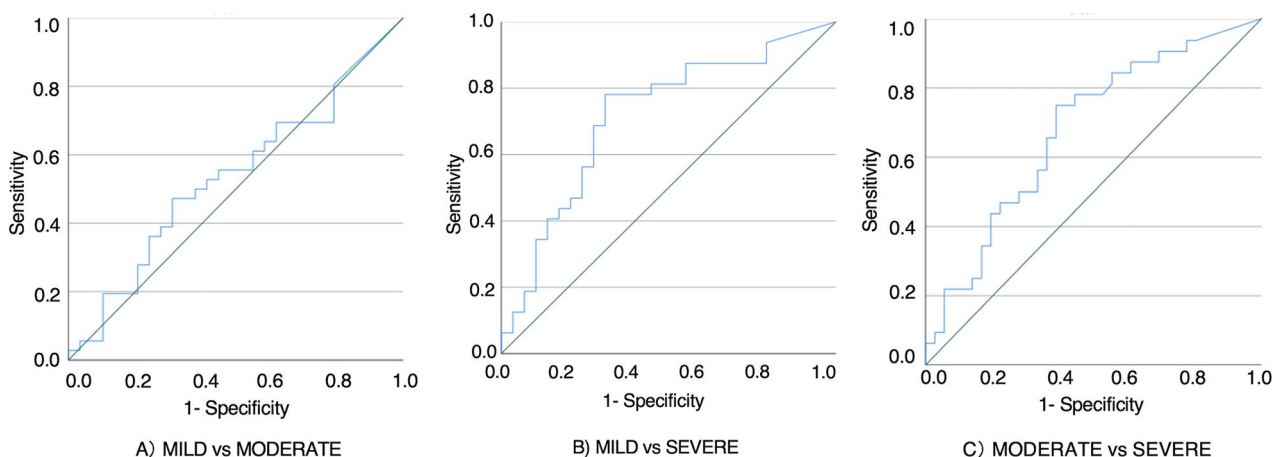


Figure 1—Receiver-operating characteristic curve analysis of BLV PVL for severity of clinical mastitis. Mild clinical mastitis (MILD) (n = 29), Moderate clinical mastitis (MODERATE) (n = 36), Severe clinical mastitis (SEVERE) (n = 32). (A) Cut-off value 16.55 copies/10 ng, sensitivity = 0.472, specificity = 0.69, Area under the curve (AUC) 0.535, 95% CI [0.393–0.677]. (B) Cut-off value 17.8 copies/10 ng, sensitivity = 0.781, specificity = 0.69, AUC 0.717, 95% CI [0.585–0.849]. (C) Cut-off value 29.45 (copies/10 ng), sensitivity = 0.75, specificity = 0.611, AUC 0.678, 95% CI [0.55–0.805]. BLV = bovine leukemia virus. PVL = proviral load.

Table 3—Comparison of the percentage of cows with systemic treatments, the number of treatments and the cost of treatments by bovine leukemia virus (BLV) proviral load (PVL).

Variables	Negative group (0 copies/10 ng DNA) (n = 15)	Below the cut off value group (below the cut off value 17.8 copies/10 ng DNA) (n = 31)	Above the cut off value group (above the cut off value 17.8 copies/10 ng DNA) (n = 51)	P-Value
MILD (n = 29)	6	14	9	
MODERATE (n = 36)	7	12	17	
SEVERE (n = 32)	2	5	25	
The percentage of cows with systemic treatments (%)	46.7 ^c	43.3 ^c	89.4 ^a	< .01**
The number of treatments	3.5 (3.0–5.0)	3.0 (1.0–4.8) ^b	4.0 (3.0–6.5) ^a	.022*
Drug prices (yen)	1,317 (515–3,689)	1,563 (515–3,265)	2,608 (513–4,467)	.291
Technical fees for medical treatment (yen)	880 (500–2,240) ^b	1,410 (500–2,595) ^b	2,060 (1,560–3,520) ^a	< .05*

a–b, * $P < .05$. a–c, ** $P < .01$.

Excluding house call fee and inspection fee, such as culture tests. Cows without records of the information mentioned above were excluded from the analysis. Data are reported as median and interquartile range. The percentage of cows given systemic treatments and technical fees for medical treatment were significantly higher in the above the cut off value group than in the below the cut off value group ($P < .001$) ($P = .023$) and negative group ($P = .007$) ($P = .048$). The number of treatments was significantly higher in the above the cut off value group than in the below the cut off value group ($P = .022$).

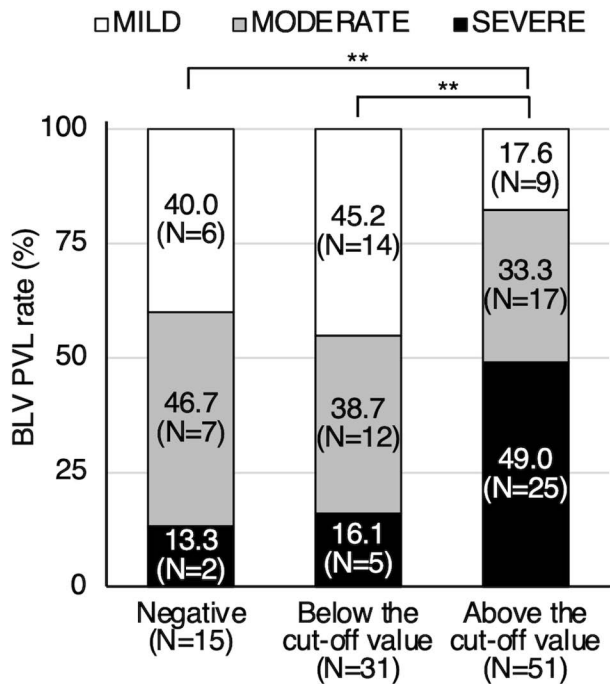
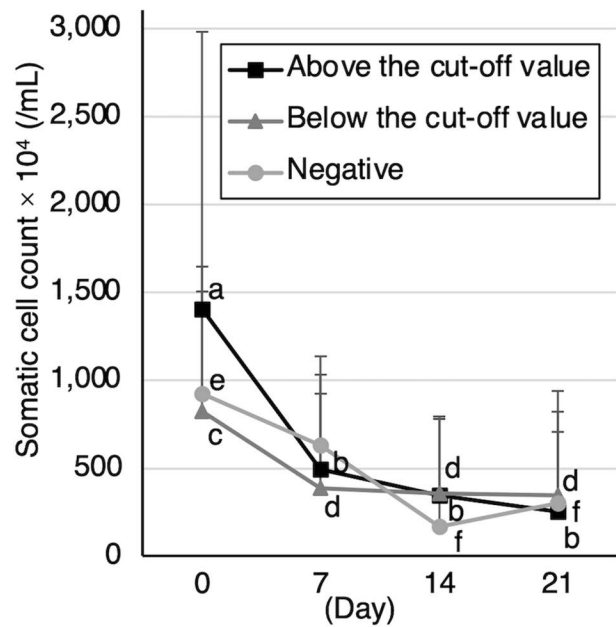


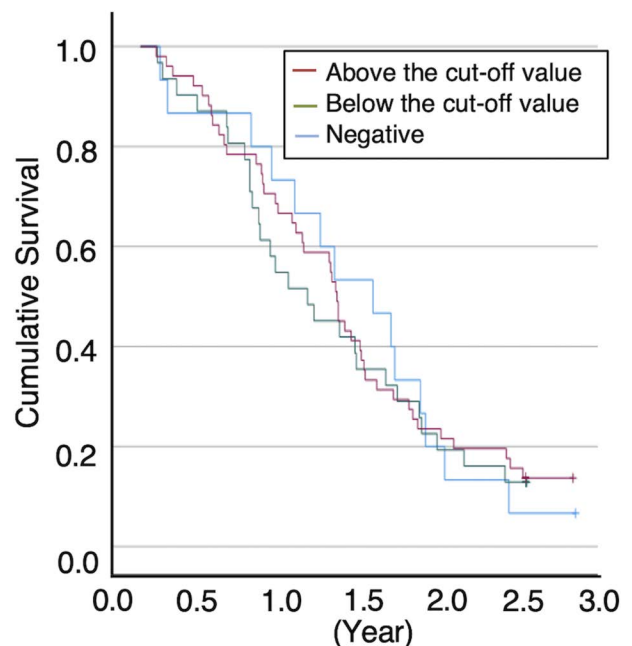
Figure 2—Severity rate of clinical mastitis by BLV PVL using the cut-off value. Cows with PVL not detected-negative (n = 15), cows with PVL-below the cut-off value (n = 31), and cows with PVL-above the cut-off value (n = 51). Mild clinical mastitis (MILD) (white bar), Moderate clinical mastitis (MODERATE) (grey bar), Severe clinical mastitis (SEVERE) (black bar). Negative: MILD (n = 6) (40.0%), MODERATE (n = 7) (46.7%), SEVERE (n = 2) (13.3%). Below the cut-off value: MILD (n = 14) (45.2%), MODERATE (n = 12) (38.7%), SEVERE (n = 5) (16.1%). Above the cut-off value: MILD (n = 9) (17.6%), MODERATE (n = 17) (33.3%), SEVERE (n = 25) (49.0%). BLV = bovine leukemia virus. PVL = Proviral load.

significantly on day 7 ($P < .001$), day 14 ($P < .001$), and day 21 ($P < .001$) in the group above the cut-off value, on day 7 ($P = .001$), day 14 ($P = .002$), and day 21 ($P = .012$) in the group below the cut-off value, and on day 14 ($P = .004$) and day 21 ($P = .013$) in the negative group (**Figure 3**). There was no significant difference in the 3-year survival rate after mastitis (Figure 3).

There were no significant differences in the percentage of cows given systemic treatments, the number of treatments, drug prices, and technical fees for medical treatment between the groups with and without coliform detection for all cows, and for cows in the group below the cut-off value and the group above the cut-off value, respectively (**Supplementary Table S2**). No coliforms were detected in the negative group. The percentage of cows given systemic treatments ($P < .001$) ($P = .007$) and technical fees for medical treatment ($P = .023$) ($P = .048$) were both significantly higher in the group above the cut-off value than in the group below the cut-off value and the negative group (Table 3). The number of treatments was significantly higher in the group above the cut-off value than in the group below the cut-off value ($P = .022$) (Table 3). Although there were no significant differences, drug prices tended to be lower in



A) Short-term prognosis evaluation: Trends in somatic cell count



B) Long-term prognosis evaluation: The 3-year survival rate after mastitis

Figure 3—Prognosis evaluation by BLV PVL. Cows with PVL-above the cut-off value (PVL above 17.8 copies/10 ng DNA at the onset of mastitis) (n = 54) (A: square; B: red), cows with PVL-below the cut-off value (PVL below 17.8 copies/10 ng DNA at the onset of mastitis) (n = 35) (A: triangle; B: green), Cows with PVL not detected-negative (PVL 0 copies/10 ng DNA at the onset of mastitis) (n = 15) (A: circle; B: blue). a-b: $P < .01$. c-d, e-f: $P < .05$. (A) Short-term prognosis evaluation: Trends in somatic cell count in milk. (B) Long-term prognosis evaluation: 3-year survival rate after mastitis. BLV = bovine leukemia virus. PVL = proviral load.

the group above the cut-off value and negative than in the group above the cut-off value (Table 3).

Discussion

The severity of clinical mastitis in the present study was associated with BLV PVL in blood, with a cut-off value of 17.8 copies/10 ng DNA, and an area under the receiver-operating characteristic curve (AUC) of 0.717. The AUC in this study was an indicator of the diagnostic accuracy of PVL for predicting the severity of clinical mastitis (MILD vs SEVERE). Since an AUC close to 1 indicates higher diagnostic accuracy, this cut-off value of 17.8 copies/10 ng DNA was considered to be useful for predicting the severity of clinical mastitis (MILD vs SEVERE). In previous studies examining PVL as an indicator, it was reported that cows with PVL greater than 2,465 copies/50 ng DNA (= 493 copies/10 ng DNA) were more susceptible to subclinical mastitis.²⁵ It has also been reported that cows with PVL less than 100 copies/50 ng DNA (= 20 copies/10 ng DNA) were very low-risk cows that did not transmit BLV both horizontally and vertically under conventional conditions in Japan.¹⁹ The PVL obtained in the present study was consistent with this range of indices for very low-risk cows that do not transmit BLV. In addition, previous studies have shown that polymorphonuclear neutrophils in blood of BLV-infected cows with more than 1,000 PVL copies/10 ng DNA produced a large amount of inflammatory factors, chemokines, and adhesion molecules, triggering inflammatory responses, compared to cows with less than 1,000 PVL copies/10 ng DNA,³⁹ and that the concentration of lingual antimicrobial peptide, an antimicrobial factor, in milk was decreased at the onset of clinical mastitis,²⁶ suggesting that BLV infection affects the immune function of the blood and mammary glands in high-PVL cows. The cut-off value of 17.8 copies/10 ng DNA with the severity of clinical mastitis suggests the possibility of an effect of BLV infection on blood and mammary immune function even in cows with blood PVL lower than 1,000 copies/10 ng DNA. However, since the PVL values obtained in this study differ from existing reports by more than 50-fold, and since this study did not examine the effect of BLV infection on immune function, the specific mechanism behind the increased risk of severe clinical mastitis remains controversial. It is thought that cows with blood PVL above the cut-off value established in the present study have an increased risk of horizontal and vertical transmission of BLV infection, as well as increased economic losses associated with mastitis. As 1 measure to control BLV transmission in Japan, the cut-off value identified in the present study can be used for more efficient control of BLV through the detection, isolation, and culling of BLV-infected cows that exceed this threshold. There was no difference in the number of BLV infections among the MILD, MODERATE, and SEVERE cases of clinical mastitis in the present study, which may have been influenced by the low number of BLV-negative cows in the samples collected.

The occurrence of severe clinical mastitis, the percentage of cows with systemic treatments, the number of treatments, and the technical fees for medical treatment were significantly higher in the above the cut-off value group than in the below the cut-off value group. At the same time, significantly more coliforms were detected in the SEVERE group of clinical mastitis. These results do not contradict reports that more than half of severe clinical mastitis cases were due to coliform infection,^{30,40} and more than 40% of cows with severe clinical mastitis developed bacteremia, usually due to a coliform infection.⁴¹ For the treatment of severe clinical mastitis, it is recommended that the initial treatment be performed within 24 hours of the onset of mastitis, systemic antibiotics selected based on culture be used for 3 to 5 days, and it is strongly recommended that fluids be administered.⁴² Ancillary treatments are required in addition to systemic antibiotic injection and intramammary antibiotic treatment for severe clinical mastitis caused by coliforms,⁴² and the medical fees are known to be high. However, there were no significant differences in the percentage of cows given systemic treatments, the number of treatments, drug prices, and technical fees for medical treatment between the groups with and without coliform detection. These results suggest that, with or without coliforms in the cows in the present study, regardless of PVL, did not affect the costs associated with medical treatment. However, the number of coliforms detected in this study was small, so it is necessary to collect more samples for further investigation. On the other hand, there were no significant differences in short-term or long-term prognosis. It is known that the somatic cell count as an indicator of short-term prognosis recovers to its normal level after the elimination of infection.⁴³ On the other hand, the lifetime until slaughter as an indicator of long-term prognosis was longer in cows that recovered enough to produce marketable milk than in cows with a complete loss of quarter milk production.⁴⁴ In the present study, although recovery of milk productivity after the onset of clinical mastitis was not confirmed, the results suggested that all cows in the above the cut-off value group, below the cut-off value group, and the negative group were treated appropriately with different costs and that the elimination of infection allowed cows to recover productivity enough to remain in the herd regardless of PVL.

These findings suggest that cows above the cut-off value are at high risk for severe clinical mastitis, the prognosis (in this study, somatic cell count and lifetime until slaughter) of mastitis could be ameliorated by appropriate, but costly treatment, which would result in significant economic loss due to the increased severity of clinical mastitis.

However, the relationships between the cut-off values of blood PVL obtained in the present study and immune factors in the host mammary gland and blood remain unclear, and with limited animal samples, it is difficult to say that an accurate PVL threshold to predict the risk of severe clinical mastitis has

been established. Further large-scale cohort studies with more animal samples must be conducted to establish accurate PVL thresholds.

In conclusion, the results of the present study suggested that the PVL cut-off value of 17.8 copies/10 ng DNA is a useful threshold for increased economic losses in terms of increased costs and number of treatments for clinical mastitis in BLV-infected cows, and it may also serve as a new standard value for the detection and culling of asymptomatic BLV-infected cows in Japan.

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Supplementary Materials

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