The Journal of Veterinary Medical Science

NOTE

Internal Medicine

Abnormal clonalities of B-lymphocytes in bovine leukemia virus-infected cattle with persistent lymphocytosis

Shiho TAKEZAWA[1)], Masaki MAEZAWA[2)], Satoko TSUZUKU[3)], Junko KAWAKAMI[3)], Yoshinao OOUCI[3)] and Hisashi INOKUMA[2)]*

[1)]Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan
[2)]Veterinary Medical Center, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
[3)]Ibaraki Prefectural Kenpoku Livestock Hygiene Service Center, 966-1 Nakagachicho, Mito, Ibaraki 310-0002, Japan

J. Vet. Med. Sci.
83(12): 1928–1932, 2021
doi: 10.1292/jvms.21-0357

Received: 22 June 2021
Accepted: 15 October 2021
Advanced Epub:
1 November 2021

ABSTRACT. Peripheral B-lymphocyte clonality of 274 bovine leukemia virus-infected cattle with lymphocytosis was analyzed using clonality PCR based on sequences of the variable region of the bovine immunoglobulin H chain. None of the cattle showed monoclonal proliferation, while 10, 31, and 233 showed minor-clonal, oligoclonal, and polyclonal proliferation, respectively. A total of 163 cattle were analyzable the following year, and lymphocytosis was maintained in 157, indicating persistent lymphocytosis (PL). B-lymphocyte clonality of the 157 PL cattle was minor-clonal in 6 (3.8%), oligoclonal in 8 (5.1%), and polyclonal in 143 (91.1%). A higher rate of enzootic bovine leukosis (EBL) onset within a year was observed in PL cattle with minor-clonal (50.0% (3/6)) and oligoclonal (25.0% (2/8)) proliferation compared to those with polyclonal (5.6% (8/143)) proliferation. Minor-clonal and oligoclonal proliferation in PL cattle may be a prognosis factor for developing EBL.

KEY WORDS: bovine leukemia virus, minor-clonal proliferation, oligoclonal proliferation, persistent lymphocytosis, prognosis factor

Enzootic bovine leukosis (EBL) is a disease caused by infection with bovine leukemia virus (BLV), and its incidence has recently been increasing in Japan [9, 10]. Most BLV-infected cattle are asymptomatic, but 30% of them progress to persistent lymphocytosis (PL), and 10% of those with PL develop EBL [2, 3]. Lymphocytes in EBL cattle are tumorigenic and show monoclonal proliferation, whereas lymphocytes in PL cattle are non-tumorigenic and show polyclonal proliferation [4, 6]. In humans, the oligoclonal proliferation phase is observed in patients with adult T-cell leukemia, which results from infection by human T-cell leukemia virus, a virus closely related to BLV [5]. We recently reported an oligoclonal lymphocyte proliferation phase in PL cattle [11]. However, the clinical significance of abnormal clonal proliferation in PL cattle remains unclear, and the percentage of clonal abnormalities in PL cattle caused by BLV infection has never been examined. The present study aimed to examine the clonality status of B-lymphocytes in PL cattle and observe its temporal changes.

A total of 274 blood samples taken from BLV antibody-positive cattle with lymphocytosis from 18 farms in Ibaraki Prefecture in 2018 were used. Of the 274 cattle, 168 were Holstein-Friesian (HF) and 106 were Japanese Black (JB). Criteria for lymphocytosis used in this study were the European Community's leukosis key [8] for HF and the criteria of Akagami *et al*. [1] for JB (Table 1). DNA was extracted from peripheral blood of these 274 cattle using an automated nucleic acid extractor (Automate Express Nucleic Acid Extraction System, Thermo Fisher Scientific, South San Francisco, CA, USA) and stored at −30°C until clonality analysis. DNA samples from lymph nodes or blood of cattle diagnosed with lymphoma were used as positive controls. EDTA peripheral blood samples from 4 BLV-negative and PL-negative cattle were used as negative controls.

For B-lymphocyte clonality analysis, PCR was performed using the primers BoVHF1: 5′-AGC CCT GAA ATC CCG GCT CA-3′ / BoVHR1: 5′-TCC AGG AGT CCT TGG CCC CA-3′, which target the region containing the variable region of the bovine immunoglobulin H (IgH) chain [7]. PCR products were analyzed for clonality using a capillary electrophoresis device (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) and a DNA 1000 Lab Chip kit (Agilent Technologies). Samples showing a single peak exceeding the height of the marker were designated as monoclonal (Fig. 1A). We defined "minor-clonal" as

*Correspondence to: Inokuma H.: ainokuma@g.ecc.u-tokyo.ac.jp
(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

a sample that does not show a high peak like monoclonal, but has a peak that is larger than the maximum height of the polyclonal waveform and does not exceed the height of the marker (Fig. 1B). Those with one or more small peaks were designated as oligoclonal (Fig. 1C). Samples showing a waveform without a peak were designated as polyclonal (Fig. 1D).

Proliferation in 274 samples from cattle with lymphocytosis in 2018 was minor-clonal in 10 (3.7%), oligoclonal in 31 (11.3%), and polyclonal in 233 (85.0%), with none showing monoclonal proliferation (Table 2). Of the 168 HF cattle, proliferation was minor-clonal in 7, oligoclonal in 18, and polyclonal in 143. Of the 106 JB cattle, proliferation was minor-clonal in 3, oligoclonal in 13, and polyclonal in 90.

Blood samples from the same cattle were continuously examined in 2019 to the extent possible. DNA were extracted from the peripheral blood of the PL cattle and analyzed for B-lymphocyte clonality with the same manner. When peripheral blood samples were not available due to death, slaughter, or trading in 2019, the presence or absence of EBL onset was confirmed for dead cattle that could be tracked by interviews with local veterinarians or meat inspection records. The prognosis (dead/alive and presence/absence of EBL) of cattle at the end of 2020 was also tracked by using the cow traceability system (https://www.id.nlbc.gp.jp) and/or interviews. Differences between HF and JB and the rate of EBL onset were analyzed using Pearson's chi-square test. Comparisons of cattle age and numbers of lymphocytes according to clonality were analyzed using the Kruskal-Wallis test. The significance level was set to 5%.

In 2019, blood count and clonality analyses were possible for 163 cattle. Among these, 157 (109 HF and 48 JB) still had lymphocytosis and were considered PL cattle. Proliferation in the 157 PL cattle was minor-clonal in 6 (3.8%), oligoclonal in 8 (5.1%), and

Table 1. Criteria of lymphocytosis used in this study

Breed	Age (years old)	Numbers of lymphocyte (/μl)		
		Normal	Suspected	Lymphocytosis
Holstein-Friesian [8]	<1	<10,000	10,000–12,000	>12,000
	<2	<9,000	9,000–11,000	>11,000
	<3	<7,500	7,500–9,500	>9,500
	<4	<6,500	6,500–8.500	>8,500
	≥4	<5,000	5,000–7,000	>7,000
Japanese Black [1]	<1	<6,300		≥ 6,300
	≥ 1, <2	<5,900		≥ 5,900
	≥ 2, <3	<5,500		≥ 5,500
	≥ 3, <6	<4,500		≥ 4,500
	≥ 6, <10	<4,300		≥ 4,300
	≥10	<3,700		≥ 3,700

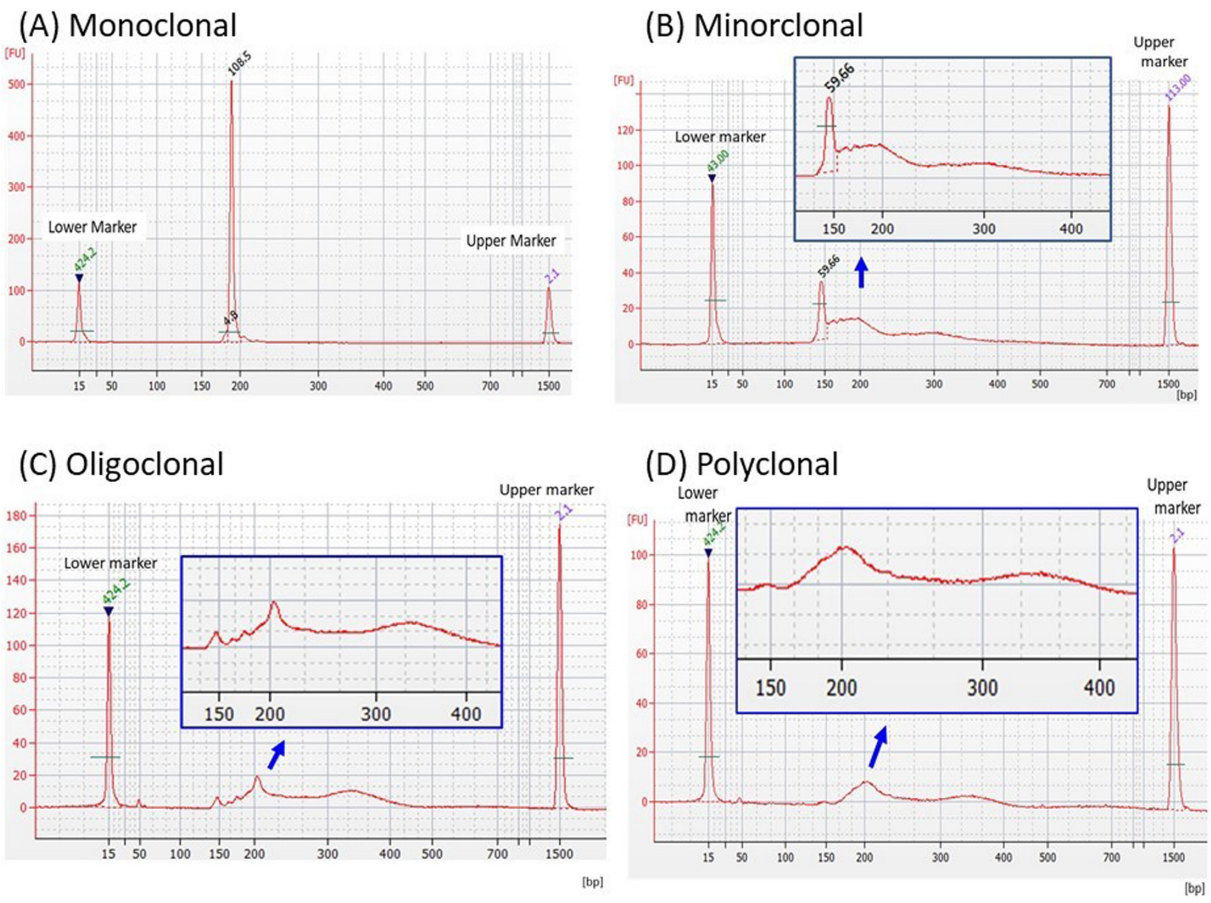

Fig. 1. Representative patterns of **(A)** monoclonal, **(B)** minor-clonal, **(C)** oligoclonal, and **(D)** polyclonal B-lymphocyte proliferation by clonality PCR using a capillary electrophoresis device (Agilent 1000).

Table 2. Time lapse analysis of clonality status in peripheral B-lymphocyte or prognosis of cattle with lymphocytosis in 2018 and with or without persistent lymphocytopsis (PL) in 2019

2018		2019	
Clonality status	Number of cattle	Clonality status or prognosis	Number of cattle
Monoclonal	0	-	-
Minor-clonal	10	PL cattle	
		Monoclonal	0
		Minor-clonal	4
		Oligoclonal	1
		Polyclonal	0
		No PL or unknown cattle	
		Normal lymphocyte counts	0
		EBL (Slaughtered)	0
		Non-EBL (Slaughtered)	2
		Untraceable	3
		Total	10
Oligoclonal	31	PL cattle	
		Monoclonal	0
		Minor-clonal	1
		Oligoclonal	3
		Polyclonal	12
		No PL or unknown cattle	
		Normal lymphocyte counts	2
		EBL (Slaughtered)	2
		Non-EBL (Slaughtered)	5
		Untraceale	6
		Total	31
Polyclonal	233	PL cattle	
		Monoclonal	0
		Minor-clonal	1
		Oligoclonal	4
		Polyclonal	131
		No PL or unknown cattle	
		Normal lymphocyte counts	4
		EBL (Slaughtered)	0
		Non-EBL (Slaughtered)	50
		Untraceable	43
		Total	233
Total	274	Total	274

EBL: enzootic bovine leucosis.

polyclonal in 143 (91.1%), with none showing monoclonal proliferation (Tables 2, 3 and Supplementary Table 1). In the 109 HF cattle, proliferation was minor-clonal in 4, oligoclonal in 7, and polyclonal in 98. In the 48 JB cattle, proliferation was minor-clonal in 2, oligoclonal in 4, and polyclonal in 40. There was no significant difference in the ratio of clonal abnormalities between HF and JB, suggesting that breed-specific differences in clonality might not exist in PL cattle. Median ages of PL cattle with minor-clonal, oligoclonal, and polyclonal proliferation in 2019 were 8, 7, and 5 years, respectively (Table 3). Median ages of cattle with minor-clonal and oligoclonal proliferation were significantly older than that of cattle with polyclonal proliferation (P=0.0004). Median lymphocyte counts of PL cattle with minor-clonal, oligoclonal, and polyclonal proliferation in 2019 were 7,100, 7,800, and 8,000, respectively (Table 3 and Supplementary Table 1), with no significant difference between the 3 groups. The remaining 6 cattle among the 163 showed normal lymphocyte counts in 2019. A total of 59 cattle with lymphocytosis in 2018 were slaughtered in 2019; 2 cattle (6-year-old HF and 12-year-old JB) had EBL onset as determined by inspection at the slaughterhouse, and the remaining 57 were confirmed to be EBL-negative. Other 52 cattle were untraceable due to various reasons, such as death or trading with other farms.

We found that 8.9% of PL cattle showed clonal abnormalities−minor-clonal or oligoclonal proliferation. As the sensitivity of the PCR assay for IgH rearrangement used in the present study was reported to be 68.6% [7], approximately 30% of clonal abnormalities may have been overlooked. Thus, the actual number of PL cattle with minor-clonal or oligoclonal proliferation might be higher than the present results suggest.

Table 3. Time lapse analysis of lymphocyte numbers, B-lymphocytes clonality and prognosis of persistent lymphocytosis cattle with minor- and oligo-clonal proliferation in 2019

Clonality status in 2019	Cattle No.	Breed	Age in 2019	Numbers of lymphocytes in 2018 (/μl)	Clonality Status in 2018	Numbers of lymphocytes in 2019 (/μl)	Prognosis in 2020
Minor-Clonal	1	HF	7	9,700	Minor	10,300	EBL (Slaughtered)
	2	HF	8	5,500	Minor	5,000	EBL (Slaughtered)
	3	JB	12	9,300	Oligo	9,300	EBL (Slaughtered)
	4	HF	4	6,500	Minor	7,100	Alive
	5	JB	14	5,000	Minor	5,000	Alive
	6	HF	9	9,700	Poly	5,600	Alive
		Median	8	6,500		7,100	
Oligoclonal	7	JB	6	5,600	Oligo	5,600	EBL (Slaughtered)
	8	JB	15	5,000	Poly	5,000	EBL (Slaughtered)
	9	HF	9	11,500	Minor	10,000	Alive
	10	HF	2	13,400	Oligo	15,000	Alive
	11	HF	6	10,700	Poly	14,900	Alive
	12	JB	7	6,000	Poly	5,100	Alive
	13	HF	7	5,700	Oligo	5,200	No EBL (Slaugtered)
	14	JB	12	6,100	Poly	4,500	No EBL (Slaughered)
		Median	7	8,350		7,800	

NE: not examined, HF: Holstein-Friesian, JB: Japanese Black, EBL: enzootic bovine leucosis. *: No lymphocytosis.

Oligoclonal proliferation of lymphocytes is recognized as one type of onset in human ATL [5], and is also observed in human patients with chronic lymphocytic leukemia [12]. Thus, it is possible that cattle having oligoclonal proliferation may already have tumors, although direct evidence is lacking. In this study, minor-clonal proliferation was included as a category in addition to monoclonal and oligoclonal proliferation. Both minor-clonal and oligoclonal proliferation suggest the emergence of weak neoplastic clones. The only difference between minor-clonal and oligoclonal proliferation is the number of bands detected. The actual significance of minor-clonal proliferation of lymphocyte is not well understood [3]. Further studies will be needed with more cases of minor-clonal proliferation to better understand the clinical significance of this clonality category.

Among the 6 PL cattle with minor-clonal proliferation in 2019, 4, 1, and 1 cattle had minor-clonal, oligoclonal, and polyclonal proliferation in 2018, respectively (Table 3). A total of 3 cattle (2 HF and 1 JB) among 6 (50.0%) with minor-clonal proliferation in 2019 were confirmed to have EBL in 2020 by inspection at the slaughterhouse, and the remaining 3 were alive at the end of 2020 (Table 3). Among the 8 PL cattle with oligoclonal proliferation in 2019, 1, 3, and 4 had minor-clonal, oligoclonal, and polyclonal proliferation in 2018, respectively (Table 3). Two cattle among 8 (25.0%) with ologoclonal proliferation in 2019 were confirmed to have EBL in 2020 by inspection at the slaughterhouse (Table 3). Other 4 cattle were alive at the end of 2020 (Table 3). A total of 143 PL cattle showed polyclonal proliferation in 2019. Of these, 6 had shifted from oligoclonal proliferation, and the remaining 137 have remained polyclonal since 2018 (Supplementary Table 1). A total of 8 cattle (3 HF and 5 JB) among 137 (5.8%) with polyclonal proliferation in 2019 were confirmed to have EBL in 2020 by inspection at the slaughterhouse (Supplementary Table 1).

Given the lack of data on lymphocyte counts of BLV-infected cattle before 2018, we could not determine whether lymphocytosis detected in 2018 was PL or not. However, our findings suggest that peripheral B-lymphocyte clonality in PL cattle does not always progress in one direction. Rather, it can change reversibly. Clonality changed over time, and not all cattle showing oligoclonal or minor-clonal proliferation developed EBL. This change in clonality may be related to the immune function of cattle and tumorigenesis of B lymphocytes. It will be important to investigate in further detail the relationship between changes in clonality status of B-lymphocytes in PL cattle and EBL cattle in future studies.

With regard to EBL onset, 50.0% (3/6) of PL cattle with minor-clonal proliferation and 25.0% (2/8) with oligoclonal proliferation developed EBL in the following year. These percentages were significantly higher than that of PL cattle with polyclonal proliferation (5.6% (8/143)), suggesting that minor-clonal and oligoclonal status in PL cattle may be a prognosis factor for developing EBL. We also found that cattle with minor-clonal or oligoclonal proliferation were significantly older than those with polyclonal proliferation. This may be related to the high incidence of EBL in older cattle. A larger-scale study will be needed to clarify the significance of clonality abnormalities in PL cattle.

CONFLICTS OF INTEREST. The authors declare no conflict of interest.

ACKNOWLEDGMENT. This work was supported by JSPS KAKENHI Grant Numbers 20H03142 and 20J10567.

REFERENCES

1. Akagami, M., Oya, S., Kashima, Y., Seki, S., Ouchi, Y. and Hayama, Y. 2019. A hematologic key for bovine leukemia virus screening in Japanese black cattle. *J. Vet. Diagn. Invest.* **31**: 568–571. [Medline] [CrossRef]
2. Angelos, J. A. and Thurmond, M. C. 2015. Bovine lymphoma. pp. 1070–1073. In: 140 rge Animal Internal Medicine, 5th ed. (Smith, B. P. ed.), Elsevier, St. Louis.
3. Armand, M., Derrieux, C., Beldjord, K., Wabeke, T., Lenze, D., Boone, E., Bruggemann, M., Evans, P. A. S., Gameiro, P., Hummel, M., Villarese, P., Groenen, P. J. T. A., Langerak, A. W., Macintyre, E. A. and Davi, F. 2019. A new and simple TRG multiplex PCR assay for assessment of T-cell clonality: a comparative study from the EuroClonality Consortium. *HemaSphere* **3**: e255. [Medline] [CrossRef]
4. Coulston, J., Daniel, R. C. W. and Lavin, M. F. 1991. Integration of bovine leukaemia virus at all stages of enzootic bovine leukosis. *Arch. Virol.* **119**: 13–23. [Medline] [CrossRef]
5. Firouzi, S., Farmanbar, A., Nakai, K., Iwanaga, M., Uchimaru, K., Utsunomiya, A., Suzuki, Y. and Watanabe, T. 2017. Clonality of HTLV-1-infected T cells as a risk indicator for development and progression of adult T-cell leukemia. *Blood Adv.* **1**: 1195–1205. [Medline] [CrossRef]
6. Kettmann, R., Cleuter, Y., Mammerickx, M., Meunier-Rotival, M., Bernardi, G., Burny, A. and Chantrenne, H. 1980. Genomic integration of bovine leukemia provirus: comparison of persistent lymphocytosis with lymph node tumor form of enzootic. *Proc. Natl. Acad. Sci. USA* **77**: 2577–2581. [Medline] [CrossRef]
7. Maezawa, M., Watanabe, K., Horiuchi, N., Matsumoto, K., Kobayashi, Y. and Inokuma, H. 2020. Molecular diagnosis of bovine B-cell lymphoma using PCR for 6 immuno- globulin heavy chain gene. *J. Vet. Med. Sci.* **82**: 61–63. [Medline] [CrossRef]
8. Mammerickx, M., Lorenz, R. J., Straub, O. C., Donnelly, W. J., Flensburg, J. C., Gentile, G., Markson, L. M., Ressang, A. A. and Taylor, S. M. 1978. Bovine hematology. III. Comparative breed studies on the leukocyte parameters of several European cattle breeds as determined in the common reference laboratory. *Zentralbl. Veterinärmed. B.* **25**: 257–267. [Medline]
9. Murakami, K., Kobayashi, S., Konishi, M., Kameyama, K. and Tsutsui, T. 2013. Nationwide survey of bovine leukemia virus infection among dairy and beef breeding cattle in Japan from 2009–2011. *J. Vet. Med. Sci.* **75**: 1123–1126. [Medline] [CrossRef]
10. Murakami, K., Kobayashi, S., Konishi, M., Kameyama, K., Yamamoto, T. and Tsutsui, T. 2011. The recent prevalence of bovine leukemia virus (BLV) infection among Japanese cattle. *Vet. Microbiol.* **148**: 84–88. [Medline] [CrossRef]
11. Sakaguchi, K., Maezawa, M. and Inokuma, H. 2020. B-cell clonality in cattle with persistent lymphocytosis caused by bovine leukemia virus infection. *Jap. J. Large Anim. Clin.* **11**: 1–4 (written in Japanese with English summary). [CrossRef]
12. Sanchez, M. L., Almeida, J., Gonzalez, D., Gonzalez, M., Garcia-Marcos, M. A., Balanzategui, A., Lopez-Berges, M. C., Nomdedeu, J., Vallespi, T., Barbon, M., Martin, A., de la Fuente, P., Martin-Nuñez, G., Fernandez-Calvo, J., Hernandez, J. M., San Miguel, J. F. and Orfao, A. 2003. Incidence and clinicobiologic characteristics of leukemic B-cell chronic lymphoproliferative disorders with more than one B-cell clone. *Blood* **102**: 2994–3002. [Medline] [CrossRef]