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NOTE

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Bovine leukemia virus genotype surveillance in cattle at a slaughterhouse in Aichi Prefecture, Japan, in 2019 using polymerase chain reaction combined with restriction fragment length polymorphism

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ABSTRACT. Polymerase chain reaction (PCR) combined with restriction fragment length polymorphism (RFLP) is commonly used for genotyping bovine leukemia virus (BLV) in slaughterhouses. However, unclassified BLV genotypes have been sporadically reported. To assess the current status of BLV genetic characterization in cattle, PCR-RFLP was performed on blood samples of 170 cattle (84 Japanese Black, 60 Japanese Black x Holstein, and 26 Holstein) from 17 farms (5 prefectures) at a slaughterhouse in Aichi Prefecture in 2019. A total of 65 samples (38.2%) were BLV positive, and genotype 1 was the most predominant (56/65 samples), followed by genotypes 3 (6 samples) and 5 (1 sample), and two unclassified samples. No relationship between the genotypes and breeds was observed. Sequence and phylogenetic analyses demonstrated that unclassified BLV genotypes clustered with genotype 1 sequences were, therefore, not new genotypes.

KEY WORDS: bovine leukemia virus, epidemiological analysis, genotype, polymerase chain reaction-restriction fragment length polymorphism, sequencing

Bovine leukemia virus (BLV) is a causative agent of enzootic bovine leukosis, a neoplasm of lymphatic tissue in bovine species [1, 4, 5]. BLV belongs to the *Retroviridae* family and the genus *Deltaretrovirus* [14]. The virus is widespread among dairy and beef breeding cattle in Japan, with seroprevalences of 40.9 and 28.7%, respectively [10]. The majority of infected animals did not show signs of infection; however, some develop persistent lymphocytosis, while a small proportion develops lymphoid tumors [5]. If cattle in slaughterhouses in Japan are diagnosed with this disease, they must be disposed of according to the Slaughterhouse Act. Therefore, BLV infections can lead to a significant economic loss for livestock farmers.

Polymerase chain reaction combined with restriction fragment length polymorphism (PCR-RFLP) is widely used for the molecular epidemiological analyses and genetic characterization of BLV in Japan. This genotyping method is based on the digestion of PCR-amplified products of the BLV envelope gene (*env*) using specific restriction enzymes. Genotypes 1 to 6 have been identified based on characteristic restriction enzyme digestion patterns [2, 8, 12]. Previously, PCR-RFLP has been used in some prefectures in Japan [2, 7, 8, 13, 15] to genetically characterize circulating BLV, indicating that genotypes 1 and 3 are predominant in these prefectures. However, unclassified BLV genotypes have been sporadically reported [2, 7]. Moreover, Asfaw *et al*. [2] reported the distribution of BLV genotypes in cattle in 16 prefectures from 2002 to 2003; although about 16 years have passed since the publication of that report, the current nationwide distribution of BLV genotypes among cattle in Japan has not yet been reported. This study aims to identify the current BLV genotypes circulating in cattle in Japan using cattle from a slaughterhouse in Aichi Prefecture. This slaughterhouse is located in central Japan and cattle, more than 7,000 heads annually, are brought here from almost all over the country; this would help clarify the current trend of BLV genotypes circulating in Japan. In

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addition, the data obtained in this study are compared with that from epidemiological reports from Japan.

A total of 170 cattle (84 Japanese Black, 60 Japanese Black × Holstein, and 26 Holstein) brought from 17 different farms in five prefectures to the slaughterhouse between April and June 2019, were used in this study. Blood samples were randomly collected at different days from 10 cattle, from each of the17 farms (Table 1). Serum samples were obtained by centrifugation at 9,100 × *g* for 3 min at 4°C. Seropositivity was assessed using an enzyme-linked immunosorbent assay (ELISA) (data not shown). BLV ELISA kit (JNC, Tokyo, Japan) was used according to the manufacturer's instructions for detection of antibodies against the BLV gp51 protein. Genomic DNA was extracted from the whole blood of 65 BLV-seropositive cattle using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A nested PCR was performed to amplify a 444 bp fragment of the viral *env* gene [3]. The target DNA sequence was amplified in a 25 µl reaction volume. The PCR mix contained 2.5 µl of 10 ×Ex Taq Buffer, 2 µl of dNTP mixture (2.5 mM each dNTP), 0.2 µM of forward primer, 0.2 µM and reverse primer, 0.6 unit of Ex Taq (TaKaRa Bio, Kusatsu, Japan) and 1 µl of the template DNA. Amplification reactions were performed in a TaKaRa PCR Thermal Cycler Dice (TaKaRa Bio). The amplification program consisted of an initial 9 min incubation at 94°C, followed by 40 cycles, of denaturation at 95°C for 30 sec, annealing at 62°C (external primers) or 70°C (internal primers) for 30 sec, an extension step at 72°C for 60 sec, and a final extension at 72°C for 4 min. The PCR products were visualized on 1.5% agarose gels with ethidium bromide using ultraviolet (UV) light.

Next, the PCR products were subjected to RFLP analysis. Each PCR product was digested with 10 U of the *Bcl* I (TaKaRa Bio), *Hae* III (TaKaRa Bio), and *Pvu* II (TaKaRa Bio) restriction endonucleases and incubated at 37°C for 2 hr. The digested PCR products were visualized in a 1.5% agarose gel with ethidium bromide using UV light.

PCR products were also purified for sequencing using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Sequencing was performed with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Forster City, CA, USA) and purified using a DyeEx 2.0 Spin Kit (Qiagen). Sequence analysis was conducted using Genetyx-Win version 13 software (Genetyx, Tokyo, Japan), and phylogenetic analyses were performed using MEGA-X [6]. Phylogenetic trees were constructed using maximum-likelihood estimations, and the reliability of the branches was evaluated by 1,000 replicates. BLV *env* sequences and phylogenetic trees were compared to the available BLV sequences [7, 9, 15].

A total of 65 samples (38.2%, 30 Japanese Black, 29 Japanese Black × Holstein, and 6 Holstein) were BLV positive. The PCR-RFLP results indicated that genotype 1 was predominant (56/65, 86.2%), followed by genotypes 3 (6/65, 9.2%) and 5 (1/65, 1.5%). The remaining two samples (3.1%) could not be ascribed to any known genotypes because PCR-RFLP displayed an atypical restriction enzyme digestion pattern (Fig. 1 and Table 2). In the unclassified two samples, sequence analysis revealed that a single nucleotide was different at the cleavage site of *Bcl* I (accession nos. LC640097 and LC640098) (Fig. 2). On phylogenetic analysis of the *env* gene fragment, the two BLV unclassified genotype sequences were clustered with the genotype 1 sequence (data not shown). No association between the genotypes, breeds (Japanese Black, Japanese Black × Holstein, and Holstein), or regional characteristics was found (Fig. 3 and Table 3).

Table 1. Cattle used in this study and bovine leukemia virus genotypes by polymerase chain reaction combined with restriction fragment length polymorphism

Farm No.	Prefecture	Number of samples tested	Number of BLV-positive	Prevalence (%)	Genotype			
					1	3	5	Unclassified
1	Aichi/West	10	5	50	4	1		
2	Aichi/West	10	4	40	2			2
3	Aichi/West	10	3	30	3			
4	Aichi/West	10	2	20	2			
5	Aichi/West	10	1	10	1			
		50	15	30	12	1		2
6	Aichi/East	10	5	50	3	2		
7	Aichi/East	10	6	60	5	1		
8	Aichi/East	10	6	60	6			
9	Aichi/East	10	5	50	5			
10	Aichi/East	10	3	30	3			
11	Aichi/East	10	1	10	1			
12	Aichi/East	10	1	10		1		
13	Aichi/East	10	1	10			1	
		80	28	35	23	4	1	
14	Aomori	10	7	70	6	1		
15	Ibaraki	10	5	50	5			
16	Shizuoka	10	5	50	5			
17	Kagoshima	10	5	50	5			
Total		170	65	38.2	56	6	1	2

BLV, bovine leukemia virus.

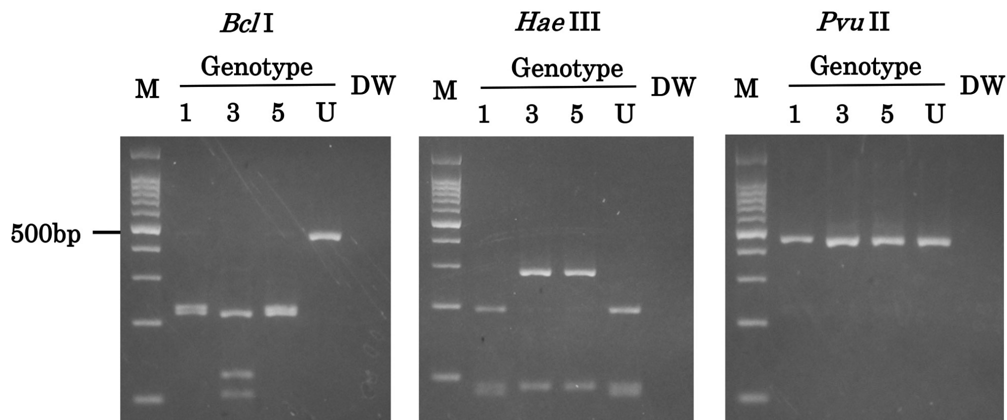

Fig. 1. Polymerase chain reaction combined with restriction fragment length polymorphism (PCR-RFLP) analysis of the 444 bp bovine leukemia virus (BLV) env fragments. Representative patterns of BLV genotyping by PCR-RFLP. Unclassified genotypes were not cleaved by *Bcl* I, but *Hae* III cleavage occurred at three sites. M, marker; U, unclassified; DW, distilled water.

Table 2. Restriction enzyme cleavage products of the 444 bp DNA fragments

Genotype	PCR product (bp)	RFLP fragments (bp)		
		Bcl I	*Hae* III	*Pvu* II
1	444	225, 219	198, 94, 87	444
3	444	219, 121, 104	285, 94	444
5	444	225, 219	285, 94	444
Unclassified	444	444	198, 94, 87	444

PCR, polymerase chain reaction. RFLP, restriction fragment length polymorphism.

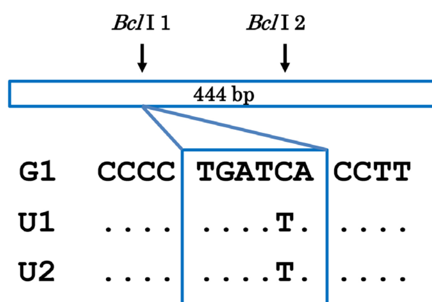

Fig. 2. Nucleotide substitution at the cleavage sites *Bcl* I 1 and *Bcl* I 2 in the unclassified genotypes compared with genotype 1. The *Bcl* I cleavage sites are shown in the 444 bp polymerase chain reaction amplicon. The sequences are aligned, and the differences between the *Bcl* I cleavage site in genotype 1 and the unclassified genotypes are shown. G1, genotype 1; U1, LC640097; U2, LC640098.

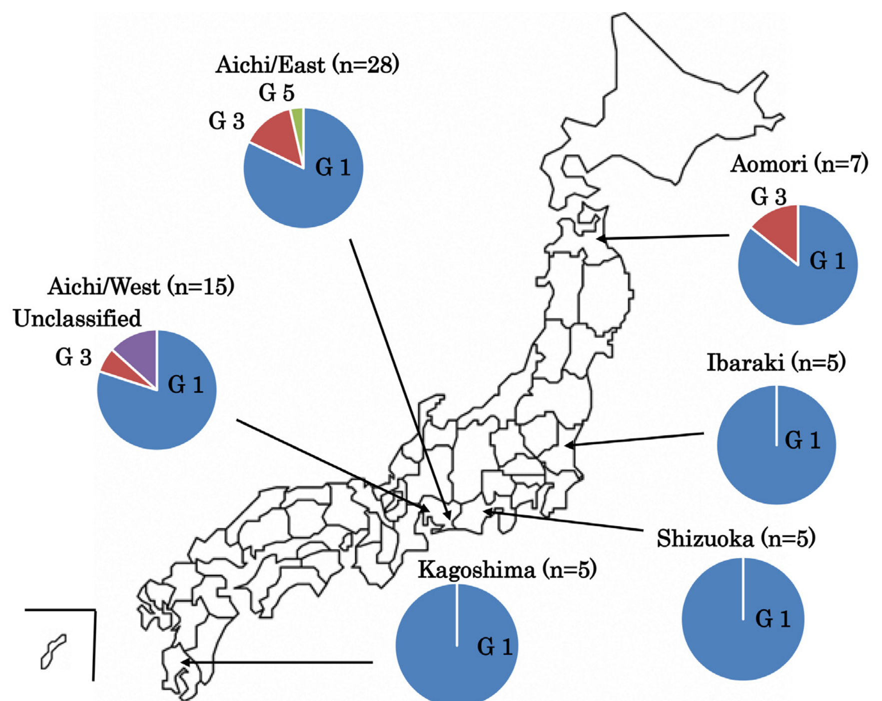

Fig. 3. Prevalence and distribution of bovine leukemia virus (BLV) genotypes. Map of the distribution of BLV genotypes used in this study. The pie charts show the proportion of each BLV genotype in the different regions from which the samples in this study originally came from. G1, genotype 1; G3, genotype 3; G5, genotype 5.

Table 3. Bovine leukemia virus genotypes in breed

Breed	Number of samples tested	Number of BLV-positive	Prevalence (%)	Genotype			
				1	3	5	Unclassified
Japanese Black	84	30	35.7	24	4		2
Japanese Black × Holstein	60	29	48.3	27	2		
Holstein	26	6	23.1	5		1	
Total	170	65	38.2	56	6	1	2

BLV, bovine leukemia virus.

The BLV positive rate in this study was 38.2%, which was higher than the 28.7% BLV positive rate observed in beef breeding cattle in a previous national survey [10]. It is possible that the 17 farms used in this study were selected from the farms in which BLV positive cattle were found between 2015 and 2018. In this survey, BLV genotype 1 was the most prevalent, followed by genotypes 3 and 5. Previous studies assessing nationwide BLV genotype distribution have shown that genotypes 1 and 3 are the most prevalent, followed by genotypes 5, 2, 4, and 6 [2, 8]. The distribution of BLV genotypes observed in this study was similar to that reported previously [2], indicating that the genotype distribution has been maintained for almost 20 years in Japan. Apart from that, previous local reports showed breed associated differences in the genotype distribution [7]. However, this study did not find any significant differences between dairy and beef breeding cattle, and we did not find an association between genotypes and regional characteristics. This discrepancy could be explained by the following reason: local reports with limited survey (prefecture level) tended to affect the genotype distribution in terms of calf origin and distance between farms. However, in this survey, the aforementioned factors had small effects, and it may be considered that there was no association between cattle breed or regional characteristics and genotype distribution. On the other hand, the two unclassified BLV genotypes belonged to a farm that introduced calves from the same prefecture outside Aichi Prefecture, suggesting that these genotypes could have been introduced by cattle outside the prefecture.

BLV is prevalent worldwide, and phylogenetic analyses have identified ten clusters based on the sequence of the viral *env* gene [12]. However, almost all 444 bp BLV *env* fragments available in databases in Japan belonged to the same phylogenetic cluster [11]. Our results demonstrate that the PCR-RFLP method is useful to genetically characterize the circulating BLV in Japan. Furthermore, PCR-RFLP is a simple, accessible, and cost-effective method that requires just basic molecular biology techniques. We suggest that BLV epidemiology in Japan can be easily assessed and analyzed through routine identification of BLV genotype clusters using PCR-RFLP, and that DNA sequencing should be performed only if greater detail is required or when PCR-RFLP displays an atypical pattern. Furthermore, it is expected that further nationwide epidemiological analyses of BLV could be performed by sequencing these types of genotypes and investigating their trends.

CONFLICT OF INTEREST. There is no conflict of interest.

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