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Distribution and superinfection of bovine leukemia virus genotypes in Japan

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Summary. A study to investigate the types and distribution of bovine leukemia virus (BLV) was conducted on about eight hundred cattle drawn from 53 farms found in 16 prefectures in Japan. Agar gel immunodiffusion (AGID) tests of serum samples and nested-PCR to detect BLV provirus, in peripheral blood leukocytes were performed. To identify genotypes, restriction fragment length polymorphism (RFLP) was performed with a PCR-amplified 444 bp fragment of the *env* gene using endonucleases. Three genotypes (1, 3, and 5) were dominant in Japan, and were found in 48.3%, 32.7%, and 16.9% of PCR positive cattle, respectively. Of the cattle infected with genotype 1, 84.7% were strongly positive in the AGID test. Similarly, in cattle with genotype 3, 78.9% were strongly positive. However, only 59.1% of cattle with genotype 5 were strong positive. Three cattle showed unusual RFLP patterns and they were found to be infected with more than one genotype. These results suggest that some BLV infected cattle can not induce effective immune reactions and suffer from superinfection by BLV in the field.

Introduction

Bovine leukemia virus (BLV) is the causative agent of enzootic bovine leukosis, a neoplasm of lymphatic tissue in bovine species [3, 9, 15, 17]. BLV is classified into the genus *Deltaretrovirus* family *Retroviridae* [33]. The majority of infected animals remain healthy and there are no negative economic effects, but some BLV

carriers develop a form of the disease known as persistent lymphocytosis (PL) and a few percent of BLV-infected animals develop lymphoid tumors [9, 13, 17]. BLV infection has a worldwide distribution [3] and seroepidemiological studies have indicated higher prevalences in some countries [1, 5]. BLV infection was found to be high on some farms in Japan [21].

Most cattle develop a strong permanent antibody response to the BLV envelope glycoprotein antigen after BLV infection and maintain the provirus in their lymphocytes [25]. Diagnosis of BLV infection using the agar gel immunodiffusion (AGID) test for gp 51 is widely used [3]. In the last several years, BLV infections with low or transient levels, or even the absence, of detectable BLV antibodies, even if the provirus is integrated, have been described [4, 7, 8, 11, 14]. This makes elimination of BLV infection difficult using only serological tests. Thus, it can be complemented by genomic diagnosis, like the polymerase chain reaction (PCR). The use of PCR has increased in recent years for the diagnosis of BLV infection [2, 11, 24].

The BLV envelope is comprised of two glycoproteins: gp 35 [32], a transmembrane protein, and gp 51, an associated surface molecule. Both are derived by posttranslational proteolytic cleavage of a common precursor, gp 72, encoded by the *env* gene [22, 30]. Glycoproteins contain the recognition site of the cell surface receptor required for virus adsorption and entry, and gp 51 elicits an immune response [17]. Based on restriction enzyme analysis and nucleotide and amino acid sequence comparisons, it was demonstrated that different BLV variants are found in different geographical regions [6, 16, 22, 26]. The previously sequenced BLV isolates have demonstrated that *Bam*H I, *Bcl* I, *Hae* III, *Bgl* I, and *Pvu* II restriction sites on BLV fragments are good markers for differentiated BLV variants [6, 22, 29, 30]. The objectives of this study were to investigate the distribution of BLV genotypes and their relationships with their BLV antibody development. The epidemiology of BLV infection is also discussed using the BLV genotype as a marker.

Materials and methods

Study area and animals

The study was carried on a total of 808 cattle drawn from 53 farms found in 16 prefectures in Japan from June 2002 to December 2003. The sixteen prefectures selected in this study adequately represented the geography of all Japan (Fig. 1). The blood samples were collected from the cattle on the farms where BLV infection was prevalent, with the help of livestock hygiene service stations. Serum separation for the AGID test, leukocyte count, and DNA extraction from peripheral blood leukocytes (PBL) were performed upon receipt without delay.

Ager gel immunodiffusion

AGID was performed on 808 serum samples collected from cattle. The test was conducted as described by Kono et al. [18]. The gel consisted of 1% noble agar, 8.5% NaCl, and 0.6% Tris (hydroxymethyl) aminomethane. The pH was adjusted to 8.6. The wells were 5 mm in diameter and six peripheral wells were placed at a distance of 3 mm from the central well. The antigen

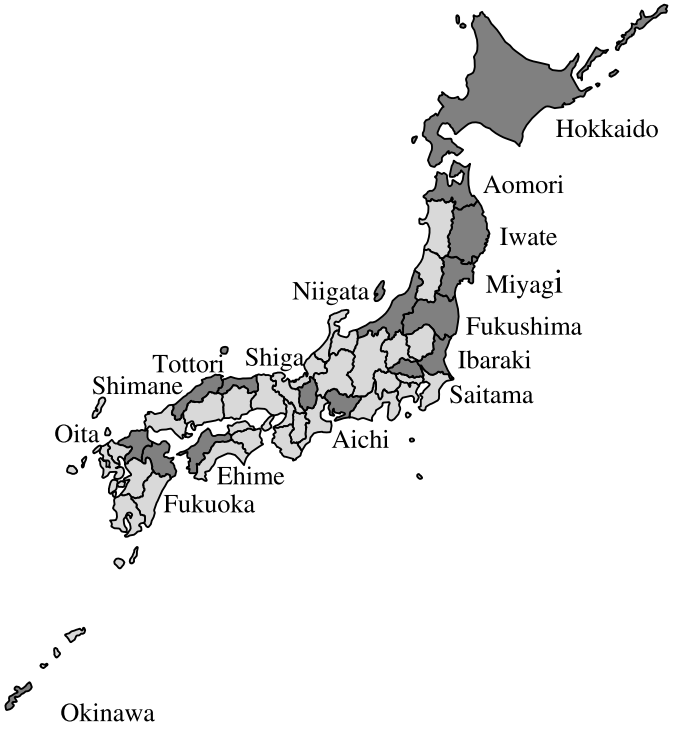

Fig. 1. Map of Japan showing prefectures where serum samples from cattle were collected (shaded prefectures)

was prepared from culture fluid of fetal lamb kidney cells persistently infected with BLV [31] and the procedure of antigen preparation was reported previously [18, 19]. The antigen contained gp 51 of BLV predominantly. The antigen was placed in the central well. Each of the reference positive sera and test samples were put in the six peripheral wells alternatively, reference positive sera in three wells and test samples in the remaining three. The gel plate was allowed to stand at room temperature for 48 h before reading formed precipitation lines. If the sample formed a complete precipitation line, it was judged to be strongly positive and indicated as ++. If the sample did not form a precipitation line but bent the precipitation line formed by control positive sera inside, it was judged to be weakly positive and indicated as +.

Leukocyte count

Leukocyte counting was done using a Coulter Counter (Model ZF, Coulter Electronics Inc., Hialeh, FL, USA). Cattle that had PBL numbers of more than 20,000/μl were considered to have PL, though this included granulocytes and monocytes.

DNA preparation

To 5 ml of EDTA-treated blood taken in a test tube, two volumes of 0.83% ammonium chloride was added to induce hemolysis of red blood cells. Washing 3 times with phosphate-buffered saline (PBS) followed by centrifugation at 2000 rpm for 5 min yielded leukocytes. DNA was extracted from these leukocytes using a commercial nucleic acid extractant kit (SepaGene; Sankyo Junyaku, Tokyo, Japan) following the manufacturer's instructions. The extracted DNA was stored at −20 °C until required for PCR.

PCR amplification

Nested polymerase chain reaction (N-PCR) was carried out using the following forward and reverse primers.

Forward primers:

env_{5032}(5′-TCTGTGCCAAGTCTCCCAGATA-3′)
env_{5099}(5′-CCCACAAGGGCGGCGCCGGTTT-3′)

Reverse primers:

env_{5521r}(5′-GCGAGGCCGGGTCCAGAGCTGG-3′)
env_{5608r}(5′-AACAACAACCTCTGGGAAGGGT-3′)

Primers chosen for this study were designed on the basis of published sequence data [30]. The primers were reported previously for BLV provirus detection by PCR [12] and Amplitaq Gold (Applied Biosystems, Foster City, CA, USA) was used as the DNA polymerase. The reaction mixture was prepared in 50 μl aliquots (1 μl of the sample and 49 μl of the reaction mixture). External primers – env_{5032}/env_{5608r} – resulted in the amplification of a 598 bp DNA fragment, and internal primers – env_{5099}/env_{5521r} – amplified a 444 bp DNA fragment in the gp 51 region of the *env* gene. The amplification reactions were performed in a PE 9700 DNA Thermal Cycler (Applied Biosystems). At first, an initial incubation at 94 °C for 9 min was carried out, followed by 40 cycles, each consisting of denaturation at 95 °C for 30 sec, annealing at 62 °C (external primers) or at 70 °C (internal primers) in both cases for 30 sec and extension at 72 °C for 60 sec. This was followed by final extension at 72 °C for 4 min. For the second round of PCR, 1 μl of the product was taken from the first amplification and reamplified. The PCR products were placed on a gel plate prepared using Tris EDTA buffer containing 1.5% agarose. After electrophoresis at 100 V, the gel plate was stained by ethidium bromide. The amplified PCR products were observed using ultraviolet (UV) light.

All PCR negative but AGID positive samples were subjected to β-actin PCR to make sure the sufficient DNA collection from peripheral blood leukocytes, and the samples that did not show clear positive reactions were excluded from the group.

Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) was performed on PCR products from 391 BLV-infected bovine leukocytes. The samples were selected as follows. If the farm had less than 20 PCR-positive cattle, all samples were subjected to RFLP. If the farm had more than 21 of PCR-positive cattle, 20 samples were selected randomly. Each sample containing 10 μg of DNA was digested with 10 U of restriction endonucleases (*Bam* HI, *Bgl* I, *Hae* III, *Bcl* I or *Pvu* II) at 37 °C overnight (about for 14 h). All restriction endonucleases were obtained from Toyobo Biochemicals (Tokyo, Japan). The digested products were subjected to electrophoresis at 50 V in 2% agarose gels. They were stained with ethidium bromide and the bands were observed using UV light. The sizes of the resulting bands were compared with a 100 bp DNA ladder. Since unusual sizes of DNAs were produced from some samples after digestion with a restriction enzyme, such digested products were subjected to electrophoresis at 50 V in 12% polyacrylamide gel with control samples of genotypes 1, 3, 4 and 5, and stained in ethidium bromide to confirm the size.

Nucleotide and amino acid sequencing

The PCR-amplified fragments were purified using a QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The sequencing reactions

were performed with a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Ca, USA), purified using a DyeEx Spin Kit (QIAGEN) and analyzed with an ABI model 373 DNA Auto Sequencer (Applied Biosystems). The nucleotide and amino acid sequences were analyzed using the GENETYX-MAC sequence analysis program (Software Development Co. Ltd., Tokyo, Japan).

Relationship between BLV genotypes and antibody reaction to BLV in ELISA

To quantify the positive antibody reaction to BLV in each serum, antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) using commercial bovine leukemia virus antibody test kit (HerdChek; IDEXX Laboratories Inc., Westbrook, Me, USA), following the manufacturer's instructions. Serum samples were selected randomly from each genotype (75 samples from genotype 1, 69 samples from genotype 3 and 42 samples from genotype 5), diluted 1:100 in diluent buffer that provided in the kit, and used for assay. Their optical density (OD) values were determined with an ImmunoMini NJ-2300 ELISA reader (InterMed, Tokyo, Japan). The sample to positive (S/P) ratio for each sample was calculated as follows.

$$\mathrm{S/P} = (\text{Sample OD value} - \text{Negative control sample OD value})/ (\text{Positive control sample OD values} - \text{Negative control sample OD value})$$

Both positive and negative control samples were provided in the kit. The degree of antibody to BLV was determined by the S/P ratio.

Results

BLV infection as tested by AGID and PCR

The results of the AGID serological test for detection of BLV antibodies in serum samples and PCR for proviral DNA in corresponding PBL samples are summarized in Table 1. About 60% of samples were positive in the AGID test and 54.4% of samples were positive in the PCR. Total 38.87% of samples were positive in both tests. One hundred and thirty two samples were PCR negative but AGID positive, and they were all subjected to β-actin PCR. Among them, 12 samples did not produce clear positive reactions. They were judged to fail in sufficient DNA extraction and treated as "AGID positive (++ or +) but PCR was not done" in Table 1.

BLV genotypes in Japan

The 444 bp DNA fragment of the *env* gene of the BLV provirus was amplified by N-PCR. The amplified products were digested with five different restriction endonucleases as described in the Materials and Method section. Restriction sites for *Bam* HI and *Bgl* I were common and those for *Pvu* II were not found among the DNA products. *Bam* HI cleaved the 444 bp DNA product into two fragments of 315 and 130 bp in all animals. Similarly, *Bgl* I cleaved it into two fragments of 330 and 115 bp in all animals. Thus, these enzymes did not help in the differentiation

Table 1. Detection of BLV infected cattle by AGID and PCR

Prefecture	Number of farms investigated	Number of samples tested	AGID++ PCR			AGID+ PCR			AGID− PCR		
			+	−	Not done	+	−	Not done	+	−	Not done
Hokkaido	9	57	40	10		3	1		1	2	
Aomori	10	151	11	9	1	6	32	2	8	82	
Iwate	1	54	2	0		0	2		3	47	
Miyagi	2	35	19	6	1	1	0		3	4	1
Fukushima	1	7	5	0		2	0		0	0	
Ibaraki	1	24	10	1		1	1		4	7	
Saitama	3	17	8	3	1	2	0		1	2	
Niigata	2	21	18	3		0	0		0	0	
Aichi	6	119	58	25	5	0	9		5	17	
Shiga	2	40	30	5	1	2	0		2	0	
Shimane	1	42	15	2		5	1	1	2	16	
Tottori	1	26	20	1		1	0		1	1	2
Ehime	2	20	11	0		1	0		0	8	
Ohita	2	20	15	5		0	0		0	0	
Fukuoka	2	139	37	0		14	4		13	5	66
Okinawa	8	36	15	0		1	0		6	14	
Total	53	808	314	70	9	39	50	3	49	205	69

of BLV genotypes in the present study. However, other endonucleases, namely *Bcl* I and *Hae* III, could be used for the differentiation of BLV genotypes.

Six different RFLP patterns were identified (Tables 2 and 3). These were genotypes 1, 2, 3, 5 and two unusual types. The samples that showed unusual digested products were electrophoresed in polyacrylamide gel. One unusual genotype sample contained DNA fragments of both genotypes 3 and 4. Two other samples contained DNA fragments of both genotypes 2 and 3 (Fig. 2). They were detected on farms where genotype 3 was prevalent (Table 2).

Usually, one genotype predominated on each farm. However, multiple genotypes were found on some farms. No special genotypes were detected in cattle with PL and weakly positive reactors in AGID tests. PL was observed in 7% of cattle that were positive by the AGID test. Genotype 1 was recorded in 48.3% of the samples. Genotypes 3 and 5 were recorded in 32.7% and 16.9% of the samples, respectively. Namely, genotypes 1, 3, and 5 were the most widely distributed, and unusual genotypes were very rare (Table 3).

PCR products from genotype 1, 3 and 5 were sequenced and compared with reported nucleotide sequences of each genotype. The nucleotide sequences and deduced amino acid sequences showed high homology among each genotype. The divergence was less than 3 in 444 nucleotides and less than 2 in 148 amino acids within each genotype (Date is not shown).

Table 2. Relationship between BLV genotypes and AGID serological status, and distribution of BLV genotypes by geographic region

Prefecture	Samples tested by RFLP	Genotype identity		Results of AGID test		
		Genotype	Sample number	++	+	−
Hokaido	44	1	44	40	3	1
Aomori	25	3	25	9	8	8
Iwate	5	3	4	2		2
		5	1			1
Miyagi	23	3	22	18	1	3
		2 + 3	1	1		
Fukushima	7	1	7	5	2	
Ibaraki	15	1	15	10	1	4
Saitama	11	1	11	8	2	1
Niigata	18	1	1	1		
		3	17	17		
Aichi	54	1	46	45		1
		2	2	2		
		3	5	5		
		5	1	1		
Shiga	34	3	32	28	2	2
		2 + 3	2	2		
Tottori	21	1	18	17	1	
		5	3	3		
Shimane	22	1	13	9	4	
		2	2			2
		3	6	5	1	
		3 + 4	1	1		
Ehime	12	1	12	11	1	
Ohita	15	3	15	15		
Fukuoka	64	1	1			1
		3	2	2		
		5	61	35	14	12
Okinawa	22	1	1	15	1	6
Total	391	1	189	160	14	15
		2	4	2		2
		3	128	101	12	15
		5	66	39	14	13
		2 + 3	3	3		
		3 + 4	1	1		
		Total	391	306	40	45

Relationship between BLV genotypes and serological status

The relationship between BLV genotypes and AGID serological status is shown in Table 2. Strongly positive BLV antibodies were observed in 78.3% (306 of 391)

Table 3. Ratios of the different genotypes found in Japan

Genotype	Number of prefectures found	Number of farms found	Number of animals infected with (%)
1	11	31	189 (48.3)
2	2	2	4 (1.0)
3	9	18	128 (32.7)
5	4	5	66 (16.9)
2 + 3	2	2	3 (0.8)
3 + 4	1	1	1 (0.3)

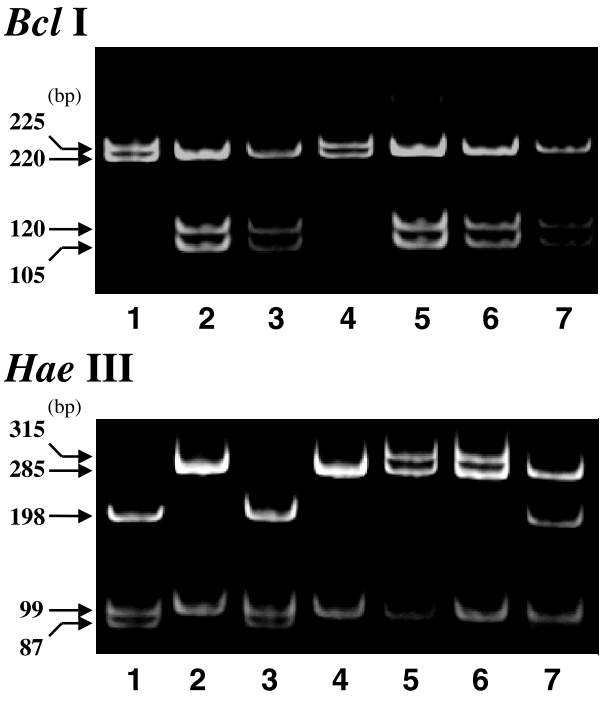

Fig. 2. PCR-RFLP analysis of PCR products. Upper panel: *Bcl* I digestion of PCR products. Lower panel: *Hae* III digestion of PCR products. *1*: genotype 1, *2*: genotype 3, *3*: genotype 4, *4*: genotype 5, *5*: a sample collected from a bovine in Miyagi prefecture infected with both genotypes 2 and 3. *6*: A sample collected from a bovine in Shiga prefecture infected with both genotypes 2 and 3. *7*: A sample collected from a bovine in Shimane prefecture infected with both genotypes 3 and 4. After the enzyme digestion, PCR products were electrophoresed in polyacrylamide gel and stained with ethidium bromide

of the samples. In those cattle infected with genotype 1, 84.7% were strongly positive in the AGID test. Similarly, in cattle infected with genotype 3, 78.9% were strongly positive. However, only 59.1% were strongly positive in cattle infected with genotype 5.

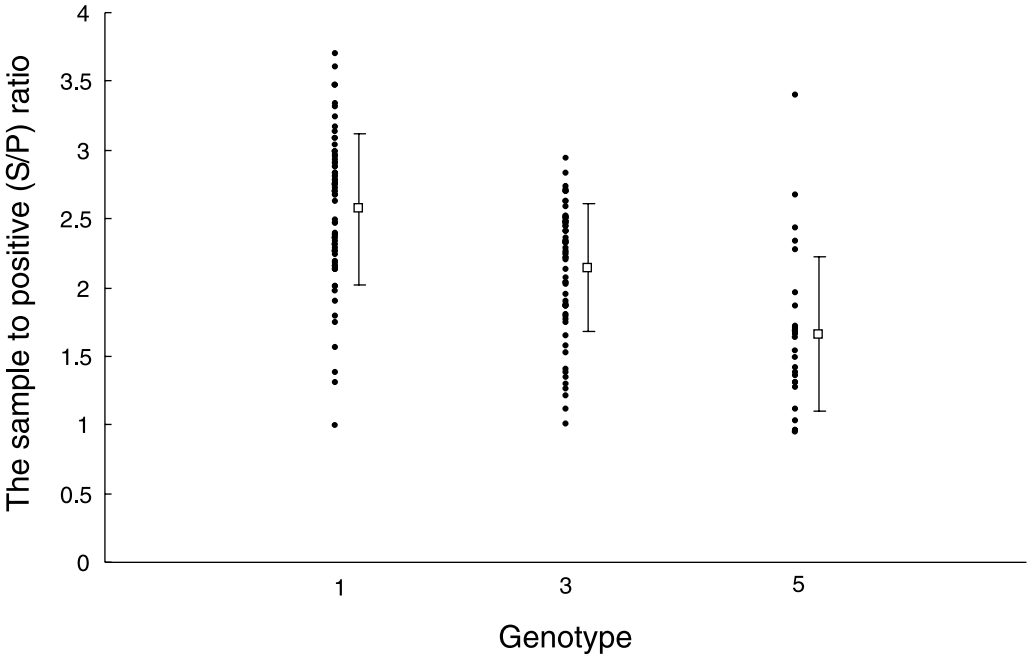

Fig. 3. Antibody reactions of sera from cattle infected with different BLV genotypes in ELISA. Serum samples were classified according to the infected BLV genotypes and their antibody titers were determined in ELISA. The S/P ratio = (Sample OD value–Negative control sample OD value)/(Positive control sample OD values – Negative control sample OD value)

Relationship between BLV genotype and antibody reaction in ELISA

The serum samples that were diagnosed as positive in AGID test were all positive in ELISA. As serum samples were classified into the genotypes, the degree of their antibody reactions were a little different each other. The average of SP ratio and standard deviation (SD) for each genotype was shown in Fig. 3.

Discussion

Generally, the diagnostic test employed limits the reliability of a diagnosis. A reliable diagnosis in turn is crucial for correct interpretation [23]. The AGID test is a widely used serological test for the diagnosis of viral diseases. However, BLV infections with lack of BLV antibodies detectable by the AGID test have been observed in recent years [4, 7, 8, 11, 14]. This makes eradication of BLV infection difficult using the serological test alone. PCR is being increasingly used in the diagnosis of BLV infections [2, 8, 11, 14, 24, 27, 28]. Fechner et al. detected almost 17.0% more positive animals by PCR than by the AGID test and 10% more by PCR than with ELISA [11]. In this study, however, more positive samples were recorded with the AGID test than with the PCR. This could be due to the difference of the PCR procedure and collected blood samples. Since PCR detects the BLV

genome and the AGID test detects antibodies to BLV in samples, it is impossible to obtain equal results from these two tests. If the number of infected leukocytes is low in PBL, the sensitivity of PCR could be reduced [8, 20]. If there are cattle in the early stage of BLV infection and prior to production of a detectable immune response, the sensitivity of the AGID test could be decreased. We have to use both tests complementarily to detect BLV-infected cattle correctly.

The prevalence of BLV infection in cattle was quite high on some farms in Japan. It would be due to the movement of cattle among farms. For instance, cows were brought to farms even outside of each prefecture. In addition, cattle from different farms (herds) are mixed when they are taken to public pastures for breeding. BLV infection was also found on some farm where animals are not introduced from outside for a long time. Ferrer and Piper [10] indicated that 3–20% of calves from BLV-infected dams were infected with BLV at birth, thereby maintaining the BLV infection from one generation to the next. This would be one reason why BLV infection is still high even in those herds.

Four types of genomic heterogeneity of BLV in the gp 51 gene were identified in this study. Genotype 1 was recorded in half of the cattle tested in 11 of the 16 prefectures. Thus, this was the predominant genotype in Japan. Genotypes 3 and 5 are also common in Japan. Single infection of genotypes 4 and 6 was not found in present study, but confirmed to exist in Japan [21]. Hence, a total of six BLV genotypes have so far been identified in Japan. On most farms, cattle were infected with one of the genotypes. However, multiple genotypes were recorded on some. On these farms, introduction of cattle from distant places occurred.

In this study, a difference in the degree of detecting strongly positive AGID reactions among different BLV genotypes was observed as shown in Table 2. Similar phenomena were also observed in ELISA that can express the antibody titers more quantitatively (Fig. 3). The S/P ratio was highest in genotype 1 (Average 2.569; SD 0.547) and it decreased in genotype 3 (Average 2.142; SD 0.465) and genotype 5 (Average 1.622; SD 0.583). The BLV antigen used for the AGID test and ELISA was prepared from tissue culture fluid of fetal lamb kidney cells [31] persistently infected with BLV genotype 1[21]. About half of the samples in this study were not genotype 1. Hence, there might have been a difference between homologous and heterologous strains of BLV in the reactions of the AGID test ELISA, though there were no genotypes that could not react at all with the antigens used for the present both test. Further study is required to determine the degree of cross reactions between heterologous strains in the AGID test and ELISA.

Two unusual genotypes were identified. At present we do not know the explanation for this observation. However, the following hypotheses may hold true: that one animal may be infected by two genotypes due to mutations or superinfection with a different BLV type. There is evidence that the BLV genome is quite susceptible to genetic mutation [32]. The genetic variation of the BLV gene appears to be minimal [22, 26]. Thus, it is not likely that they originated from persistently infected BLV. However, it is possible that they were infected with a different BLV genotype after the first infection. They were found on farms where BLV genotype 3 was prevalent and they were also infected with BLV genotype 3. Thus, they

could already have been infected with one BLV genotype outside of the farms and then infected again with BLV genotype 3 on the farms. Protection against BLV infection is established by the BLV antibody, but it requires high antibody titers [19]. AGID antibody titers of more than 1:64 are required to prevent BLV infection mediated by infected leukocytes. These phenomena may indicate the difficulty of BLV prevention by vaccination with the general procedure. Further experiments on maintaining high antibody titers for long periods are required for it.

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