The Journal of Veterinary Medical Science

NOTE

Virology

Broadly applicable PCR restriction fragment length polymorphism method for genotyping bovine leukemia virus

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ABSTRACT. Bovine leukemia virus (BLV) is a causative agent of enzootic bovine lymphoma (EBL). BLV is prevalent worldwide, and ten genotypes have been classified based on the sequence of the envelope glycoprotein (gp51) gene. In this study, we present a simple and generally applicable PCR restriction fragment length polymorphism (PCR-RFLP) method to identify BLV genotypes. While the genotyping results obtained by previously described PCR-RFLP methods matched only 78.96% to the results of phylogenetic analysis, we demonstrated that our PCR-RFLP method can identify 90.4% of the sequences available in the database *in silico*. The method was validated with 20 BLV sequences from EBL tumor tissues and 3 BLV sequences from blood of BLV infected cattle, and was found to show high specificity. We utilized this method to determine genotypes of blood samples from 18 BLV seropositive cattle in Kanagawa and Niigata, as well as 12 EBL cattle in Chiba, Japan. Our analysis with the modified PCR-RFLP detected two genotypes, Genotypes 1 and 3. Genotype 1 was detected as the main genotype, while Genotype 3 was sporadically observed. This technique can be used as a reliable system for screening a large number of epidemiological samples.

KEY WORDS: bovine leukemia virus, genotyping, PCR restriction fragment length polymorphism

J. Vet. Med. Sci.
81(8): 1157–1161, 2019
doi: 10.1292/jvms.18-0603

Received: 12 October 2018
Accepted: 22 May 2019
Advanced Epub: 12 June 2019

Bovine leukemia virus (BLV), belongs to the family *Retroviridae* and the genus *deltaretrovirus*, is a causative agent of enzootic bovine lymphoma (EBL) [17]. Approximately 30% of BLV infected cattle develop a lifelong lymphocytosis, while <5% of BLV infected cattle develop EBL, which has severe financial implications on the cattle industry [2]. BLV is prevalent worldwide, and ten genotypes have been classified based on the sequence of the envelope glycoprotein (gp51) gene [7, 13–16, 18]. Previous reports demonstrated that different BLV genotypes have been found across the world. Genotype 1 is the most prevalent genotype [6], Genotypes 2, 5, 6 and 9 have been found in South America, while Genotypes 4, 7 and 8 have been found in Russia and Europe [16, 18]. Genotype 10 is prevalent in relatively restricted areas such as Thailand and Myanmar [7]. Genotype 3 has been reported in the US, Japan and Korea [6, 12]. The origin of this geographical distribution would probably be linked to the cattle trade routes in the past. Since BLV sequences are genetically stable [11], the low sequence variations of BLV can be used as a molecular tool for tracking the migration of infected cattle populations as well as for gaining new insights into the origin, evolution, and mode of transmission of BLV and its hosts.

The PCR-restriction fragment length polymorphism (PCR-RFLP) analysis has been widely used for BLV genotyping because the analysis is relatively simple [3–5, 8, 10]. A previous study suggested that RFLP of 444 bp of partial BLV*env* gene (nt5099–5542) amplicon and the combination of five restriction endonucleases (*Bam*H I, *Bcl* I, *Bgl* I, *Hae* III, *Pvu* II) are good markers to differentiate BLV variants [4]. Another study showed that RFLP with 3 out of 5 restriction endonucleases (*Bcl* I, *Hae* III, *Pvu* II) are sufficient for BLV genotyping [8]. Although the genotyping results obtained by these two PCR-RFLP methods matched only 78.96% [20], the protocol with 3 endonucleases is now widely used in the field of BLV epidemiology [1]. In Japan, by applying this method, six different genotypes, Genotype 1–5 and 6 (defined by Licursi *et al.* [8]), have been identified. In previous studies, however, all BLV sequences originated in Japan were found to be clustered with Genotypes 1 and 3 by phylogenetic analysis [7].

In this study, in order to resolve the discrepancy between PCR-RFLP-based genotyping and sequence-based phylogenetic

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(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

clustering, we present a new protocol based on PCR-RFLP of 444 bp BLV*env* genes for differentiation of 10 BLV genotypes with a number of endonucleases. All the experimental protocols have been approved by the committee at Tokyo University of Agriculture, Japan (Permit Number: 300107).

Tumor tissue samples (n=20 from 20 farms) and blood samples (n=12 from 12 farms) were obtained in 2015 from EBL cattle diagnosed by veterinary officers at 2 local abattoirs (Meat Inspection Station of Kanagawa Prefectural Government, Kanagawa, Japan and Chiba Prefectural Nanso Meat Inspection Office, Chiba, Japan). Blood samples from BLV infected cattle were taken in 2016 from the following anti-BLV antibody positive cattle: Holstein cattle in Kanagawa (n=15 from 7 farms), Japanese black cattle in Niigata (n=3 from 3 farms) of Japan. Genomic DNA was extracted from the samples above using a commercially available kit (DNeasy Blood & Tissue Kit: QIAGEN, Hiden, Germany). Plasmid DNAs containing sequences of Genotypes 6, 9 and 10 (Accession numbers: LC080658, LC080665 and LC154848) [16] were used for verification of our PCR-RFLP protocol.

The partial BLV*env*-gp51 sequences from GenBank/EMBL/DDBJ database (n=396) were aligned by MEGA7 software. The maximum-likelihood (ML) method was used to obtain phylogenetic trees of partial BLV*env* (444 bp) sequence. ML trees were constructed using MEGA7. The Kimura 2-parameter model plus gamma distribution (K2+G) was chosen as the best fit model for nucleotide substitution. Genetyx 5.1 software was used for *in silico* digestion of the BLV*env* PCR products with the 229 restriction endonucleases; namely, all the commercially available endonucleases from Takara Bio Inc. (Kusatsu, Japan), Toyobo (Osaka, Japan) and New England Biolabs, Inc. (Ipswich, MA, U.S.A.).

Partial BLV*env* gene was amplified by nested PCR using ExTaq (Takara Bio Inc.) and the following primers: Forward env_{5032} (5′-TCTGTGCCAAGTCTCCCAGATA-3′) and Reverse env_{5608} (5′-AACAACAACCTCTGGGAAGGGT-3′) for 1st PCR, Forward PCR env_{5099} (5′-CCCACAAGGGCGGCGCCGGTTT-3′) and Reverse env $_{5521}$ (5′-GCGAGGCCGGGTCCAGAGCTGG-3) for 2nd PCR, described previously [4]. The reaction mixture contained 400 *n*g of template DNA, 5 μl of 10 × Ex Taq Buffer, 4 μl of 2.5 mM dNTP mix, 0.25 μl of Ex Taq polymerase, and 1 μl of each primer (10 μM) in a total of 50 μl of aliquots. PCR was carried out with primer pairs env_{5032} and env_{5608} (external primer) resulting in the amplification of 598 bp fragment, and with env_{5099} and env_{5521} (internal primers) resulting in a 444 bp fragment. Conditions for PCR amplification were as follows: 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C (external primers) or at 70°C (internal primers) for 30 sec, and extension at 72°C for 1 min. Amplified products were visualized on 1% agarose gels (Takara Bio Inc.) by staining with ethidium bromide. The bands were identified according to their size using a 100 bp DNA ladder as a marker. The PCR amplified fragments (444 bp) were purified by a gel extraction kit (QIAquick gel extraction kit: QIAGEN) with the DNA recovered in 40 μl of an elution buffer. RFLP reactions (20 μl) contained 2.5 μl of purified PCR products, 15 U of *Bmr*I, *Alw* I and *Hph* I (New England Biolabs, Inc.), 20 U of *Taq* I, *Pvu* II, *Bam*H I and *Mse* I (Takara Bio Inc.), with an appropriate buffer for each restriction endonuclease. The reaction mixtures were incubated at 37°C for 2 hr (*Bmr* I, *Hph* I, *Pvu* II and *Alw* I), 30°C for 2 hr (*Bam*H I), 60°C for 2 hr (*Mse* I) and 65°C for 2 hr (*Taq* I) respectively. RFLP digestion products, along with a 20 bp DNA ladder and a 100 bp DNA ladder (Takara Bio Inc.), were separated with electrophoresis on 3% TAE agarose gel (Agarose KANTO HC, Kanto Chemical Co., Inc., Tokyo, Japan) at 100 V for 30 min. The genotyping results obtained from this protocol were highly consistent with that by phylogenetic analysis.

To design a broadly applicable PCR-RFLP method, we first conducted genotyping of publicly available 396 sequences of BLV*env* (nt5099–5542, 444 bp) along with sequences obtained from 20 EBL tumor samples by constructing the ML Phylogenetic tree. As consistent with the findings of previous studies, a phylogenetic tree classified BLV into 10 genotypes (Supplementary Fig. 1). Among the 20 sequences from the EBL cattle, 18 sequences were clustered with Genotype 1 (deposited to DDBJ: accession no. LC361248-LC361258, LC361260-LC361266), while 2 sequences were clustered with Genotype 3 (deposited to DDBJ: accession no. LC361259, LC361267).

To discriminate 10 BLV genotypes by RFLP, 396 amplicons of partial BLV*env* sequences (444 bp) genotyped by the ML tree (Supplementary Fig. 1) were digested by 229 restriction endonucleases *in silico*. The theoretical restriction profiles obtained were manually analyzed to find unique RFLP profiles specific to each genotype (Fig. 1). Because fragments smaller than 50 bp are not

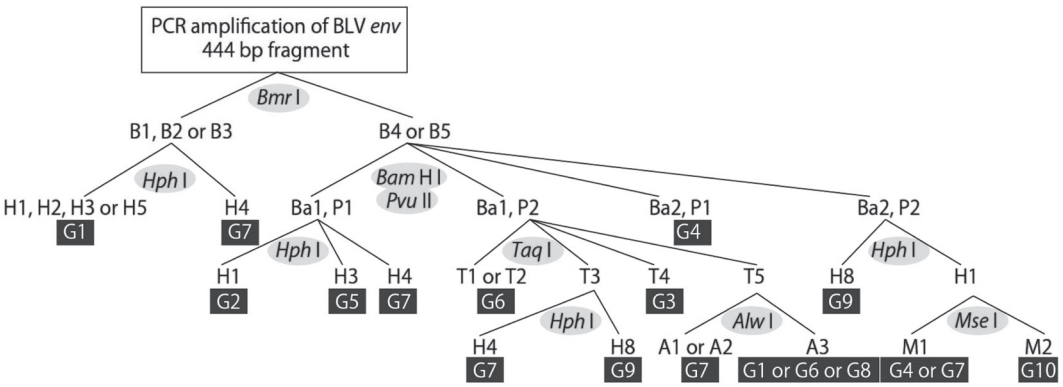

Fig. 1. Schematic representation of the restriction fragment length polymorphism (RFLP) method for identification of bovine leukemia virus (BLV) genotypes. The endonucleases are indicated by grey circles with RFLP profiles. BLV genotypes are indicated by solid black rectangle.

always clearly visible on an agarose gel, such small fragments were not taken into consideration in genotype discrimination. Each RFLP profile of each endonuclease was labelled by its own code (Table 1): all RFLP profiles for genotyping were listed in Table 2.

Since a majority of the sequences in the database have been classified as Genotype 1, we first designed the PCR-RFLP method to discriminate Genotype 1 from other genotypes. *Bmr* I led to five different RFLP patterns and 97.9% (143/146) of Genotype 1 sequences fell into the profile B1 defined in Table 1 with a single sequence of Genotype 7 from Russia (JQ353638). Within this group, the digestion using a second endonuclease (*Hph* I) could be used to confirm the distinction between Genotypes 1 and 7. For Genotype 1, *Hph* I cleaved the amplicon into 3 very similar fragments: profile H1 (Fragment sizes: 224 and 220 bp), profile H2 (217, 196 and 31 bp) and profile H3 (217, 181 and 46 bp). For Genotype 7, the amplicon was cleaved into 3 different fragments: profile H4 (217, 137 and 90 bp) can be allowed as the distinction of Genotype 7 from Genotype 1 (Table 2).

*Bam*H I and *Pvu* II digestion were useful to differentiate between Genotypes 2 to 10. Because 3 out of 4 RFLP profiles of combination of *Bam*H I and *Pvu* II digestion, namely Ba1- P1, Ba1- P2, Ba2- P2, included two or more genotypes, successive digestion with two or more endonucleases such as *Hph* I, *Taq* I, *Alw* I and *Mse* I was deemed to be necessary. After the RFLP analysis with the total of 2 to 5 endonucleases, 90.4% (358/396) of the BLV sequences were genotyped corresponding to the ML phylogenetic tree (Supplementary Fig. 1, Table 2).

To test our proposed PCR-RFLP method, we applied this system to the analysis of the BLV sequences from tumor tissue samples of the 20 EBL cattle and plasmids DNA which harbor BLV sequences from asymptomatic cattle in South America (Paraguay and Bolivia) and Southeast Asia (Myanmar). The ML phylogenetic tree of BLV*env* 444 bp revealed that the sequences from these samples were classified into Genotypes 1 (n=18), 3 (n=2), 4 (n=1), 6 (n=1) and 10 (n=1) (Supplementary Fig. 1). For *in vitro* analysis, a PCR amplicon of BLV*env* 444 bp was cleaved by *Bmr* I. For 18 PCR amplicons, *Bmr* I cleaved most amplicons into 4 fragments (Profile B1: 109, 143, 162 bp with 30 bp fragment which is not always clearly visible on an agarose gel), while other 5 PCR amplicons into 3 fragments (Profile B4: 192, 143 and 109 bp) (Fig. 2A). These amplicons were subsequently digested by *Hph* I for profile B1, or by combination of *Bam*H I, *Pvu* II, *Taq* I, *Hph* I, *Alw* I and *Mse* I for profile B4 (Fig. 2B–D). The PCR-RFLP profile showed that PCR amplicons from 18 tumor samples were all identified as Genotype 1, while PCR amplicons from the other 2 tumor samples were identified as Genotype 3, which corresponded to the genotypes analyzed by the ML phylogenetic tree. Moreover, 2 PCR amplicons from BLV plasmids were identified as Genotypes 9 and 10 by both PCR-RFLP method and ML phylogenetic tree. On the other hand, PCR amplicons from the other BLV plasmid was clustered as Genotype 6 by the ML phylogenetic tree and fell into the profile including 3 genotypes (Profile A3; Genotypes 1, 6 or 8) which is indistinguishable by this PCR-RFLP method because of cutting pattern similarity.

Table 1. PCR-restriction fragment length polymorphism (PCR-RFLP) profile types of BLV*env* gene fragment

Restriction endonuclease	RFLP profile type	Restriction fragment size (bp)
Bmr I	B1	162, 143, 109, 30
	B2	173, 162, 109
	B3	301, 143
	B4	192, 143, 109
	B5	335, 109
Hph I	H1	224, 220
	H2	217, 196, 31
	H3	217, 181, 46
	H4	217, 137, 90
	H5	220, 196, 28
	H6	220, 153, 71
	H7	437, 7
	H8	224, 171, 49
	H9	444
*Bam*H I	Ba1	316, 128
	Ba2	444
Pvu II	P1	280,164
	P2	444
Taq I	T1	357, 82, 5
	T2	357, 58, 29
	T3	324, 87, 33
	T4	303, 87, 54
	T5	357, 87
Mse I	M1	326, 118
	M2	168, 118, 113, 45
Alw I	A1	321, 123
	A2	196, 125, 123
	A3	196, 123, 89, 36

The proposed PCR-RFLP method was also applied to identify genotypes obtained from 15 BLV seropositive dairy cattle in 7 different farms in Kanagawa, and 3 BLV seropositive beef cattle in 3 farms in Niigata, and 12 EBL cattle in Chiba, Japan. The PCR-RFLP results of BLV*env* 444 bp showed that all of these BLV amplicons were classified as Genotype 1 (data not shown).

In this study, we have established a broadly applicable PCR-RFLP method to differentiate BLV genotypes using BLV*env* partial sequences. Although phylogenetical analysis based on DNA sequencing is the most reliable analysis for genotyping, it is more costly and equipment dependent. PCR-RFLP is a simple method that requires just basic molecular laboratory capabilities (PCR amplification and gel electrophoresis), and can provide a reliable system to screen large numbers of epidemiological samples.

In the previous studies, PCR-RFLP was frequently used to identify BLV genotypes [1, 8, 9, 20]. However, some results of recent molecular studies demonstrated that several genotypes identified with PCR-RFLP might be inconsistent with those identified with phylogenetical clustering [20]. For example, a predicted RFLP pattern of BLV*env* sequence of AY185360 using three endonucleases, *Bcl* I, *Hae* III, *Pvu* II [8], indicated that this BLV sequence was identified as Genotype 1. On the other hand, phylogenetic analysis in this study and other previous studies demonstrated that this sequence fell in cluster of Genotype 6 [7, 16, 19]. In spite of these misidentifications, identifications of new genotypes such as Genotype 9 in Bolivia and Genotype 10 in Thailand and Myanmar [7, 16] led to the development of a modified, broadly applicable PCR-RFLP method. Although certain RFLP profiles need further phylogenetic analysis because of the same cutting pattern (Profile A3; Genotype 1, 6 or 8), and we have not shown all cutting pattern displayed in Table 1 *in vitro*, we demonstrated that our PCR-RFLP method can identify 90.4% of the sequences registered in database *in silico* (Table 2). Previous studies with PCR-RFLP have demonstrated that Genotypes 1–5

Table 2. The bovine leukemia virus (BLV)*env* PCR-restriction fragment length polymorphism (PCR-RFLP) profiles *in silico*

Bmr I	*Hph* I	*Bam*H I	*Pvu* II	*Hph* I	*Taq* I	*Alw* I	*Mse* I	Geno-type[a)]	No[b)]	A/N[c)]	Country of isolation
B1	H1	-	-	-	-	-	-	G1	141/146		
B1	H2	-	-	-	-	-	-	G1	1/146	LC075548	Peru
B1	H3	-	-	-	-	-	-	G1	1/146	M35239	U.S.A.
B1	H4	-	-	-	-	-	-	G7	1/51	JQ353638	Russia
B2	H1	-	-	-	-	-	-	G1	1/146	KP201467	Korea
B3	H5	-	-	-	-	-	-	G1	1/146	EU266062	Iran
B4 or B5	-	Ba1	P1	H1	-	-	-	G2	24/24		
B4 or B5	-	Ba1	P1	H3	-	-	-	G5	8/8		
B4 or B5	-	Ba1	P1	H4	-	-	-	G7	3/51		
B4 or B5	-	Ba1	P2	-	T1	-	-	G6	1/28	AY185360	Brazil
B4 or B5	-	Ba1	P2	-	T2	-	-	G6	1/28	LC075574	Bolivia
B4 or B5	H4	Ba1	P2	-	T3	-	-	G7	1/51	JQ353656	Russia
B4 or B5	H8	Ba1	P2	-	T3	-	-	G9	19/22		
B4 or B5	-	Ba1	P2	-	T4	-	-	G3	10/10		
B4 or B5	-	Ba1	P2	-	T5	A1	-	G7	1/51	JF720352	Russia
B4 or B5	-	Ba1	P2	-	T5	A2	-	G7	44/51		
B4 or B5	-	Ba1	P2	-	T5	A3	-	G1	1/146	AY151262	Brazil
B4 or B5	-	Ba1	P2	-	T5	A3	-	G6	26/28		
B4 or B5	-	Ba1	P2	-	T5	A3	-	G8	6/6		
B4 or B5	-	Ba2	P1	-	-	-	-	G4	73/75		
B4 or B5	-	Ba2	P2	H1	-	-	M1	G4	2/75		
B4 or B5	-	Ba2	P2	H1	-	-	M1	G7	1/51	JQ353649	Russia
B4 or B5	-	Ba2	P2	H1	-	-	M2	G10	26/26		
B4 or B5	-	Ba2	P2	H8	-	-	-	G9	3/22		

a) BLV genotyping according to the phylogenetic classification, b) The number of analyzed BLV sequences with the respective restriction endonuclease/total number of the sequence of respective genotype. c) The database accession number of each sequence. -: Non-relevant profile. Underlined BLV genotypes indicate that these sequences are indistinguishable by this method.

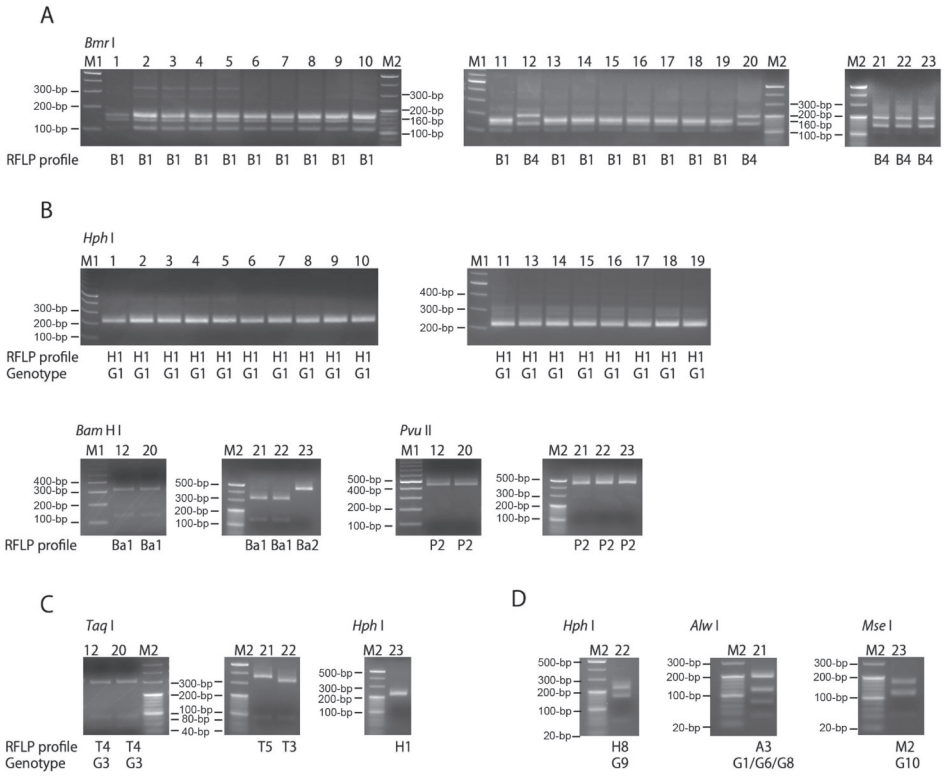

Fig. 2. Differentiation of the bovine leukemia virus (BLV) genotypes of 20 enzootic bovine lymphoma (EBL) tumor samples (samples 1–20) and three plasmids which harbor BLV sequences from BLV infected cattle (samples 21–23) identified by PCR-restriction fragment length polymorphism (PCR-RFLP) method. *Bmr* I (A), *Hph* I, *Bam*H I and *Pvu* II (B), *Taq* I and *Hph* I (C), *Hph* I, *Alw* I and *Mse* I (D) RFLP profiles of the BLV*env* gene. M1: 100 bp ladder, M2: 20 bp ladder. Each representative RFLP profile is the types identified in Table 2.

and 6 (defined by Licursi *et al*., [8]) were prevalent in Japan [8]. However, among the 444 bp of BLV*env* sequences available in databases, all the sequences from Japan clustered in Genotype 1 or 3, and 99% of the sequences were Genotype 1. Our PCR-RFLP system is a simple method that can be used for BLV genotyping. A further advantage of our RFLP method is that this method can easily detect the most prevalent Genotype 1 with just 2 restriction endonucleases. This method has proved to be an effective method for routine clustering of BLV genotypes, and it may thereby be an important tool in BLV epidemiology.

ACKNOWLEDGMENTS. This study was supported in part by The Science Research Promotion Fund from Japan Private School Promotion Foundation. We would like to thank the staff of Kanagawa Shonan Livestock Hygiene Service Center and Chiba Nansou Meat Inspection Station for blood sample collection, the staff of Meat Inspection Station of Kanagawa Prefectural Government for EBL tumor tissue sample collection, and Dr. Kimura and Dr. Takizawa for proofreading the manuscript.

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