Virology 537 (2019) 45–52

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

A point mutation to the long terminal repeat of bovine leukemia virus related to viral productivity and transmissibility

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ARTICLE INFO

Keywords:
Bovine leukemia virus
Genetic variation
Transmission
Molecular clone
Proviral load
Reverse genetics

ABSTRACT

It is important to establish the molecular basis of the high transmissibility of bovine leukemia virus (BLV) to develop new methods of preventing viral transmission. Hence, the aim of this study was to determine whether some strains had transmission advantages. First, we determined the whole BLV genome sequences of all 34 BLV-infected cows from one farm. Phylogenetic analysis divided strains into 26 major and 8 minor strains. The major strains dominantly spread independent of host factor, bovine leucocyte antigen. Further analysis, with molecular clones, associated transmissibility with viral productivity *in vitro*. In addition, the two groups could be classified by group-specific mutations. The reverse genetic approach demonstrated that a spontaneous mutation at nucleotide 175 of the BLV genome, which is located in the viral promoter region, could alter viral productivity by changing viral transactivation, suggesting that BLV transmissibility is affected by a spontaneous mutation associated with viral productivity.

1. Introduction

Bovine leukemia virus (BLV) is the etiological agent of enzootic bovine leukosis and a lethal infectious disease leading to significant economic losses in the livestock industry through lower lifetime milk production, reproductive efficiency, and lifespan (Brenner et al., 1989; Nekouei et al., 2016; Polat et al., 2017b; Schwartz and Levy, 1994). BLV infection is prevalent in several regions worldwide and controlling it is difficult (LaDronka et al., 2018; Liang et al., 2017; Murakami et al., 2011b). BLV cannot be technically eliminated from infected cows because the virus integrates into the host genomic DNA of peripheral blood cells as a provirus (Murakami et al., 2011a). Therefore, preventing BLV spread is the most efficient countermeasure for reducing the economic losses due to BLV infection.

BLV transmission to the uninfected host occurs through few lymphocytes (the least infectious dose: 926 lymphocytes) (Burny et al., 1987). The lymphocyte/white blood cell (WBC) count is closely associated with proviral load (PVL) in blood (Alvarez et al., 2013; Nakada et al., 2018) and previous studies have indicated that BLV transmissibility is related to PVL (Jimba et al., 2010; Juliarena et al., 2016; Ohno et al., 2015; Somura et al., 2014). In addition, The PVL was associated with host factors such as differences in the immune response and single nucleotide polymorphism in the host genome (Abdalla et al., 2016; Farias et al., 2016). Thus, analyses of host factors would be one of determinant to presume BLV transmissibility. On the other hand, our previous study showed that PVLs were static in the cow infected with a BLV strain with a partial deletion of the *G4* gene (Murakami et al., 2016). Hence, prediction of BLV transmissibility would be needed to understand not only host factors but also viral factors.

The BLV provirus consists of two long terminal repeats (LTRs) at both the 5' and 3' termini, and the *gag-pro-pol*, *env*, and pX region, which is located between the *env* gene and 3' LTR. The pX region encodes five micro RNAs (miRNAs) and nonstructural genes (*AS1*, *R3*, *G4*, *tax*, and *rex*) (Aida et al., 2013; Durkin et al., 2016; Kincaid et al., 2012; Rosewick et al., 2013). The BLV genome is highly conserved and the genetic variations are small (Mansky and Temin, 1994; Polat et al., 2017a). On the other hand, our recent study results suggest that the viral properties dependent on genetic variations in wild-type (WT) BLV strains may be associated with PVL transition (Murakami et al., 2018). It is possible that the BLV transmissibility is associated with specific

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https://doi.org/10.1016/j.virol.2019.08.015
Received 16 May 2019; Received in revised form 15 August 2019; Accepted 16 August 2019
Available online 17 August 2019

mutations in the BLV proviral genome. In addition, a previous study demonstrated that inducing recombination of miRNA regions down-regulated PVL (Gillet et al., 2016). These previous studies suggest that the BLV transmissibility is impacted by mutations; thus, specific strains would have an advantage/disadvantage for transmission in the field. Based on these factors, we hypothesized that strains with high transmissibility can quickly spread and become major strains in a farm. Therefore, the present study was to estimate whether BLV transmissibility in WT strains is affected by a specific mutation.

2. Results and discussion

2.1. Two genetically distinct viral groups were prevalent among cows from a single farm

To determine the genetic diversity of BLV WT strains circulating in the study farm, blood samples were collected from all 70 cows in December 2017. Sequence analysis of the PCR products revealed 34 cows (cows AJ001 − 0034) were infected with BLV. Next, the whole BLV genomes of the samples collected from all 34 cows were sequenced (Suppl. Table. 1) and compared. Consequently, some unique INDEL mutations were detected. The provirus AJ020 and AJ031 (pvAJ020 and pvAJ031, respectively) strains, which infected cows AJ020 and AJ031, harbored 1136-bp and 1-bp deletions in the *gag-pro-pol* gene sequence, respectively, which resulted in protein truncation (Suppl. Fig. 1). The putative receptor recognition sites in ENV protein (A97, S98, H115, N170, W127, and E128), which are important for the recognition of putative receptor bovine adaptor protein 3 complex subunit delta-1 (Corredor et al., 2018), were highly conserved in the strains except for pvAJ002. In contrast, the pvAJ002 had a unique 1-bp and 18-bp insertion mutation in the *env* gene and the miRNA coding region BLV-miR-B2-5p, respectively. The insertion mutation of *env* gene resulted in a frameshift and truncation of the ENV protein (Suppl. Fig. 2A). In addition, there was also an incomplete duplication mutation to an adjacent upstream sequence, possibly resulting in an abnormal structure of BLV miRNA (Suppl. Fig. 2B and C). These data implied that the three strains were spread-deficient viruses while the other virus strains were believed to infect cells and replicate. Comparing data from all strains revealed nucleotide substitutions in all regions and genes (Suppl. Fig. 3), but the maximum substitution rate among the strains was 0.762%. Thus, the genetic variation was rather limited.

Because our previous study found the BLV strains could be divided into three groups based on limited genetic variation (Murakami et al., 2018), we investigated whether the 34 strains could be similarly categorized. To reveal genetic differences among the 34 strains obtained in the present study and the 28 strains in our previous study, all 62 strains were analyzed using MEGA software. The results showed that the 62 strains could indeed be classified into 3 groups (A, B, and C), as reported in our previous study (Fig. 1) and in accordance with common genotype strains in Japan (genotype 1). All but two strains could be classified into the same groups reported in our previous study; pvAF245 and pvAF982 were classified as group C. It was thought that these two strains could not be accurately classified in our previous study because of the small sample size.

Phylogenetic tree analysis showed that group A and C strains were circulating among the cows from the study farm. Among the 34 BLV-infected cows on the farm, more than three times as many cows were infected with group A strains than with group C strains (26 (76.5%) vs. 8 (23.5%), respectively). The results showed that the group A and C strains were major and minor strains, respectively (Fig. 1). It is speculated that major strains would have a greater potential for spread than minor strains.

2.2. Difference of spread in the major and minor strains

To estimate whether the major strains were dominantly spread in

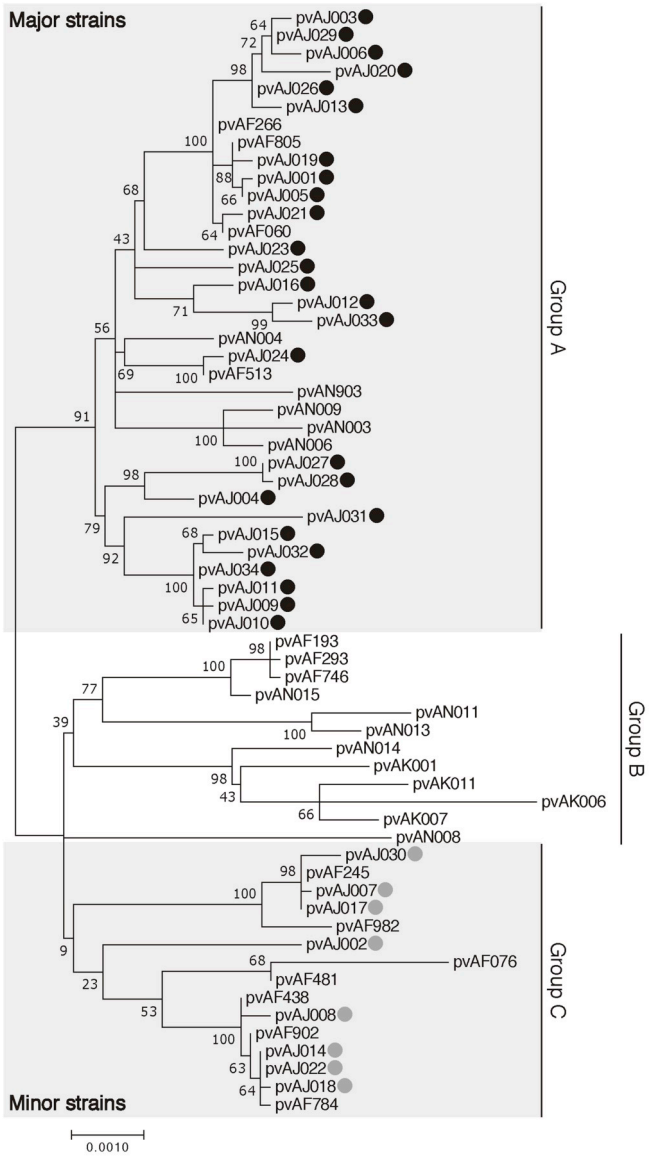

Fig. 1. Phylogenetic tree of WT BLV strains identified in this study and a past study. A phylogenetic tree was generated with the maximum-likelihood method using the whole BLV genome of 62 strain sequence identified in this as well as a past study. The dots show strains identified in this study while the other strains were isolated in the past study, which have been deposited to the GenBank database. The black and gray dots represent major and minor strains, respectively.

the farm, we analyzed host traits, the locations of the cows on the farm, and network analysis based on viral genome. Previous studies demonstrated that bovine leucocyte antigen (BoLA)-DRB3, which is the most highly polymorphic BoLA class II locus in cattle, is well associated with PVL (Aida, 2001; Carignano et al., 2018; Juliarena et al., 2007, 2016; Miyasaka et al., 2013; Takeshima et al., 2017, 2019). Thus, to analyze whether the major strains were dominantly spread according to host traits, we analyzed the BoLA-DRB3 allele using a PCR sequence-based typing method. We identified alleles including resistant (**0201* and **14011*), susceptible (**1201* and **1501*), and other neutral alleles against BLV spread and pathogenesis (Suppl. Table. 2). Major strains did not dominantly infect cows carrying the susceptible trait, although cows carrying the susceptible trait have high transmissibility of BLV infection (Suppl. Fig. 4). Therefore, the major strains can spread independent of host traits.

Next, we analyzed the association between the positions of cows and

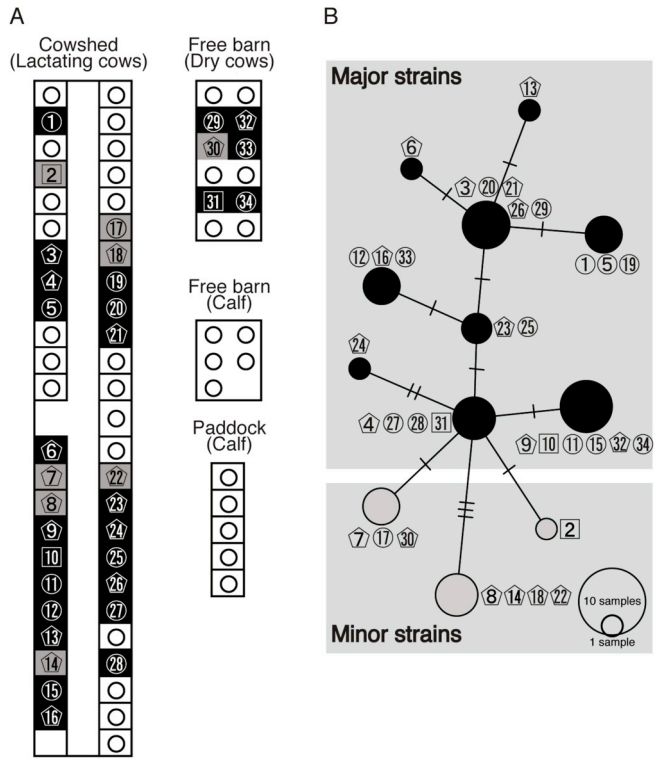

Fig. 2. The arrangement of cows on the farm and the strain types. (A) The squares, circles, and numbers show cow pens, cows, and pvAJ strains, respectively. The black, gray, and white squares represent cows infected with the major strain, the minor strains, and uninfected cows, respectively. (B) The median joining network of 34 strains identified in this study. The strain numbers are included. A mutation is shown as a hatch mark. The numbers surrounded by squares, pentagons, and circles indicate resistant, susceptible, neutral traits based on BoLA-DRB3 allele.

viral spread. The positions of individual cows and the groups of BLV strains are shown in Fig. 2A. The lactating cows were kept tied in stalls, while the dry cows were free in a barn. Hence, the cows were classified into one of the three groups: infected with a major strain, infected with a minor strain, or uninfected. In contrast, calves were kept in paddocks or free in a barn and BLV infection was not detected in them. All cows except for a cow infected with pvAJ015 were born on the farm, and each lactating cow had been tied in a pen during lactation period. Thus, it was thought that the almost cows were infected with BLV in the study farm and new infections in the farm were associated with the cows' positions. Many lactating cows infected with the major strains were adjacent to other cows also infected with the major strains, while most cows infected with the minor strains were not (Fig. 2A). Next, to estimate a derivation of the major and minor strains, a network analysis was estimated using a median joining network, described by Alexandersen et al. (2017). A representative network is shown in Fig. 2B. The results showed that the major strains formed a network, while the minor strains did not, suggesting that the major strains would be spread from a particular origin strain, while three haploid strains in minor strains would have three different origin strains. In addition, adjacent cows in the cowshed were related to some same haploid strains such as pvAJ009 – 011 and some network forming haploid strains such as pvAJ019 – 021. In contrast, the BLV-infected cows' positions in the cowshed were not completely associated with the network analysis. These results imply that the BLV-infected cows were moved to the cowshed after infection. It seems that the haploid strains such as pvAJ009 – 011 and pvAJ019 – 021, which are major strains, would be spread in the cowshed but minor strains did not. The result suggested that the major strains can more efficiently infect adjacent hosts than the minor strains. Next, we analyzed relationship between network analysis and host traits. It appeared that the haploid minor strains, such as pvAJ007, 008, 014, 018, 022, and 030, circulated in cows carrying the susceptible trait, while the major strain formed a network independent of the traits produced by BoLA. These data suggested that minor strain transmission is dependent on host traits, while the major strain could spread independently of host traits.

2.3. *The transmissibility is associated with viral productivity in vitro*

It is important to identify differences between major and minor strains. First, the WBC counts were calculated for all cows. Although the WBC counts of the BLV-infected cows were significantly higher than those of the uninfected cows, there was no significant difference in WBC counts between those infected with the major strains vs. the minor strains (Fig. 3A). Next, differences in PVL between the two groups were investigated. However, there were no significant differences in PVLs between the two groups (Fig. 3B). The PVLs were not reflected to difference of transmission in the major and minor strains. The result might be affected by host traits based on the BoLA-DRB3 allele because the almost cow infected with the minor strain carried a susceptible trait. It was thought that the minor strains could not be efficient spread in the study farm despite high PVL. On the other hand, our previous study revealed changes to the PVL in response to BLV infection (Murakami et al., 2016, 2018). Therefore, it was also thought that one-time PVL measurements may have been unsuitable for identifying strains with high/low transmissibility and be required to be measured at least twice

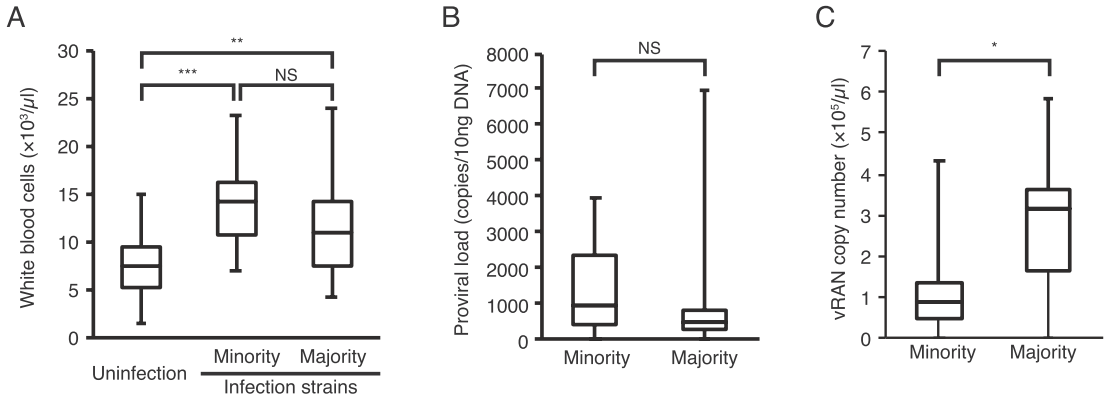

Fig. 3. Factors associated with BLV strains. (A) The relationship between WBC counts and BLV strains. (B) The relationship between PVL and BLV strains. (C) The relationship between viral productivity and BLV strains. Uninfection, minority, and majority show BLV-uninfected, minor strain-infected, and major strain-infected cows, respectively. The measurements of PVL and vRNA copy number were performed in triplicate. Significant differences are indicated by single, double, and triple asterisks ($P < 0.05$, $P < 0.01$, and $P < 0.001$, analysis of variance or Student's *t*-test). NS indicates no significant difference.

to definitely ascertain the real PVL.

Our previous studies showed that the high and low PVL transitions in BLV-infected cows were related to viral production *in vitro* (Murakami et al., 2016, 2018). Hence, we proposed the hypothesis that viral productivity *in vitro* may be related to transmissibility. To investigate this hypothesis, we examined the *in vitro* viral productivity of 34 BLV molecular clones that were prepared from each BLV-infected cow. Briefly, 293T cells were transfected with the molecular clones, and viral RNA (vRNA) copy number in the supernatants were measured by quantitative PCR (qPCR). Consequently, the viral productivity of the major strains was significantly greater than that of the minor strains (Fig. 3C). Our previous studies demonstrated that infection with the group A (major) and group C (minor) strains resulted in high and low transitions in PVL levels, respectively, although not consistently (Murakami et al., 2016, 2018). Thus, the spread of major and minor strains cannot be estimated by a one-time PVL measurement. In contrast, viral productivity *in vitro* may help distinguish major from minor strains. The results implied that high viral productivity would be advantageous to transmission by producing more viral particles in the early phase of infection showing viremia. In addition, viral productivity *in vitro* was only dependent on genetic variations of the BLV genomes, suggesting that the genetic characteristics related to viral productivity would determine the spread of BLV infection within a herd.

2.4. The genetic difference between major and minor strains

We further analyzed critical nucleotide positions that differed between the major and minor strains. As indicated by the summary of group-specific nucleotide substitutions, presented in Fig. 4A, there were several differences in the nucleotide sequences between the major and minor strains throughout the genome. Differences in amino acid between the major and minor strains are shown in Fig. 4B. Notably, most of the group-specific nucleotide substitutions were nonsense mutations; however, several of them were useful for distinguishing the major strains (group A) from the group B and minor strains (group C). In addition, the nucleotide substitutions at positions 175 and 4908 were induced alterations to the TATA box sequence of the U3 region within the 5' LTR, which is a promoter region in the BLV genome (Pluta et al., 2018; Tajima et al., 2003), and the amino acid sequences of the ENV protein, respectively. These findings suggest that specific nucleotide substitutions affect viral productivity.

2.5. One nucleotide substitution in the LTR region was associated with viral productivity

To identify the region responsible for viral productivity *in vitro*, we selected molecular clones from among the 62 WT strains analyzed in this study. Previously our study showed that the PVL was repeatedly measured in samples from the pvAF481 (minor group) and pvAF513 (major group)-infected cows, which revealed low and high PVL transitions, respectively (Murakami et al., 2018). In addition, viral productivity significantly differed between the clones pBLV-AF481 and -AF513, which were cloned pvAF481 and pvAF513, respectively. Therefore, transmissibility differed between pBLV-AF481 and -AF513, suggesting these clones would be suitable to use in further analysis.

Next, to identify the region responsible for viral productivity in WT strains, we constructed chimera viruses by recombination of the LTR, *gag-pro-pol*, *env*, and pX regions. The chimera virus schematic is shown in Suppl. Fig. 5. Briefly, 293T cells were transfected with either the WT or chimera viruses, and vRNA copy number was measured by qPCR. The results showed that the LTR was recombined in the chimera viruses, which changed viral productivity, while recombination events in the other chimera viruses had no effect (Fig. 5A). The nucleotide sequences of the LTR in the two molecular clones were nearly identical, except for only two nucleotides at positions 175 and 8384 in the 5' and 3' LTRs, respectively. Since nucleotide 175 (nt 175) was located within TATA box in the U3 region of the 5' LTR, we speculated that the mutation at nt 175 had induced changes to the promoter activity.

Next, to determine whether the substitutions at nt 175 in the U3 region affected viral productivity, substitutions from thymidine to cytosine (T175C) and vice versa (C175T) were induced in pBLV-AF481 and -AF513, respectively. Predictably, the T175C mutation had significantly increased viral productivity (Fig. 5B), while C175T mutation had the opposite effect (Fig. 5C). Together, these data implied that a spontaneous mutation at nt 175 was associated with viral productivity by inducing a change of TATA box function.

2.6. The substitution at nt 175 in 5' LTR was associated with viral transactivation

A reporter assay was performed to determine whether the mutation at nt 175 in the U3 region of the 5′ LTR affected viral transactivation. First, the U3 region and *tax* gene, which transactivate the 5′ LTR (Willems et al., 1987), were cloned into plasmid vectors. Briefly, the firefly luciferase gene was cloned downstream of the U3 regions of pBLV-AF481 and -AF513. In addition, the T175C and C175T mutations were induced into the U3 regions of pBLV-AF481 and -AF513, respectively. In the two WT molecular clones, the Tax amino acid sequences were completely identical. Thus, the *tax* gene of pBLV-AF513 was cloned into a plasmid vector. The cloned vectors, together with pRL-TK, which carried the *Renilla* luciferase gene downstream from the herpes simplex virus thymidine kinase promoter, were used to measure transfection efficiency in 293T cells. The U3 regions' transcriptional activities were estimated by normalizing firefly luciferase activity to *Renilla* luciferase activity. The results showed that the transcriptional activities could be significantly increased or decreased by inducing the T175C or C175T mutation, respectively (Suppl. Fig. 6A and B). In addition, to estimate the transcriptional activities of the mutation-induced U3 in bovine cells, we performed the same reporter assay using bovine cell line, CKT-1. Consequently, it was revealed that the mutations could change transcriptional activity (Fig. 6A and B). These results showed that the nt 175 within 5' LTR could affect transcriptional activity.

2.7. Advantages of transmissibility of specific strains

The present study showed that two BLV strain groups isolated from the same farm have different biological properties, i.e., viral productivity and transcription activity (Figs. 1, 3 and 6). These differences may be associated with virus transmissibility, as speculated previously (Fig. 2) (Murakami et al., 2016). The unique substitutions to each group detected in this study present useful markers for classification of each group (Fig. 4). In particular, the spontaneous mutation at nt 175 of the BLV genome associated with viral productivity would be the most important among the specific substitutions. Moreover, the unique major group 2 strains (pvAJ012 and pvAJ033), which contained nt 175T (Fig. 4), showed low viral productivity (1.5×10^5 and 0.8×10^5 copies/μl, respectively), and PVL was also low in the infected cows (18.5 and 47.3 copies/10 ng DNA, respectively), which did not have a resistant trait. These data suggest that a mutation at nt 175 of the BLV genome is one of determinant to predict BLV transmissibility. In addition, previous reports have demonstrated that nucleotide mutations are closely related to transmissibility of other viruses (Stein, 2011; Wong et al., 2015), we speculated that several mutations to the BLV genome would also have a great impact on transmission. In support of this, previous study demonstrated that cows with low and high PVL were isolated two strains, which did not harbor 175C mutation (Dube et al., 2000, 2009) and our previous study suggested that the small deletion mutation on the *G4* gene associated with static PVL (Murakami et al., 2016). Therefore, it is speculated that BLV transmissibility would be changed by not only a point mutation at nt 175 but also limited mutations in viral genome.

In most BLV genotypes, nt 175T is conserved (Pluta et al., 2018;

A

Region		5'LTR	gag-pro-pol	gag-pro-pol	gag-pro-pol	gag-pro-pol	gag-pro-pol	gag-pro-pol AS2	gag-pro-pol AS2	gag-pro-pol AS2	gag-pro-pol	gag-pro-pol	gag-pro-pol	gag-pro-pol	gag-pro-pol	gag-pro-pol	gag-pro-pol	env	env	env	env			AS1	tax	tax	3'LTR
Nucleotide position		175	950	1189	1498	1513	1848	2031	2313	2594	2843	3317	3368	3452	3652	3680	3923	4181	4908	5298	5441	5948	6465	6486	7010	7987	8014	8384
Group	Consensus	C	G	T	T	A	C	G	T	T	G	C	G	T	C	A	T	C	C	C	A	A	G	C	G	C	T	C
A (Majority)	pvAF060	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAF266	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAF513	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.
	pvAF805	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAJ001	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAJ003	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAJ004	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	pvAJ005	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAJ006	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAJ009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	pvAJ010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	pvAJ011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	pvAJ012	T	A	A	.	G	.	.	.	C	.	.	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.
	pvAJ013	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAJ015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	pvAJ016	.	.	.	.	.	.	.	.	C	.	.	.	.	A	G	C	.	.	T	.	.	.	.	.	.	.	.
	pvAJ019	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAJ020	.	.	.	.	.	-	-	-	-	-	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAJ021	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAJ023	.	.	.	.	.	.	.	.	C	.	.	.	.	A	G	C	.	.	T	.	.	.	.	.	.	.	.
	pvAJ024	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.
	pvAJ025	.	.	.	.	.	.	.	.	C	.	.	.	.	A	G	C	.	.	T	.	.	.	.	.	.	.	.
	pvAJ026	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAJ027	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	pvAJ028	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	pvAJ029	.	.	.	.	.	.	.	.	C	.	.	A	.	A	G	C	.	.	T	C	.	.	.	.	.	.	.
	pvAJ031	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	pvAJ032	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	pvAJ033	T	A	A	.	G	.	.	.	C	.	.	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.
	pvAJ034	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	pvAN003	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.
	pvAN004	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	C	.	.	.	G	.	.	.	.	.	.	.
	pvAN006	.	A	.	.	.	.	.	.	C	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.
	pvAN009	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.
	pvAN903	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.
B	pvAF193	T	.	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAF293	T	.	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAF746	T	.	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAK001	T	.	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAK006	T	.	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAK007	T	.	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAK011	T	.	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAN011	T	.	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAN013	T	.	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAN014	T	.	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAN015	T	.	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
C (Minority)	pvAF076	T	A	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAF245	T	A	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	.	A	T	C	T
	pvAF438	T	A	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	A	T	C	T
	pvAF481	T	A	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	.	T	C	T
	pvAF784	T	A	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	A	T	C	T
	pvAF902	T	A	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	A	T	C	T
	pvAF982	T	A	.	.	.	.	.	.	.	.	T	.	C	.	.	.	T	G	.	.	G	A	.	A	T	C	T
	pvAJ002	T	A	A	.	G	.	.	.	C	.	.	.	.	A	.	C	.	G	.	.	G	A	T	.	T	C	T
	pvAJ007	T	A	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	.	A	T	C	T
	pvAJ008	T	A	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	A	T	C	T
	pvAJ014	T	A	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	A	T	C	T
	pvAJ017	T	A	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	.	A	T	C	T
	pvAJ018	T	A	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	A	T	C	T
	pvAJ022	T	A	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	A	T	C	T
	pvAJ030	T	A	A	C	G	T	A	C	.	A	T	.	C	.	.	.	T	G	.	.	G	A	.	A	T	C	T
Singleton	pvAN008	T	.	A	.	G	T	A	C	.	.	T	.	C	.	.	.	T	G	.	.	G	A	T	A	T	C	T

B

Gene		gag-pro-pol					env		
Protein		MA	MA	MA	CA	RT-IN	SP	SU	AS1
Amino acid position		41	87	108	282	1009	28	235	21
Group	Consensus	K	E	V	S	T	S	S	E
A (Marority)	pvAF060	R	.	.	G	K	.	.	.
	pvAF266	R	.	.	G	K	.	.	.
	pvAF513	.	.	.	.	K	.	.	.
	pvAF805	R	.	.	G	K	.	.	.
	pvAJ001	R	.	.	G	K	.	.	.
	pvAJ003	R	.	.	G	K	.	.	.
	pvAJ004	.	.	.	.	.	.	.	.
	pvAJ005	R	.	.	G	K	.	.	.
	pvAJ006	R	.	.	G	K	.	.	.
	pvAJ009	.	.	.	.	.	.	.	.
	pvAJ010	.	.	.	.	.	.	.	.
	pvAJ011	.	.	.	.	.	.	.	.
	pvAJ012	.	.	I	.	K	.	.	.
	pvAJ013	R	.	.	G	K	.	.	.
	pvAJ015	.	.	.	.	.	.	.	.
	pvAJ016	.	.	.	.	K	.	.	.
	pvAJ019	R	.	.	G	K	.	.	.
	pvAJ020	R	.	.	G	-	.	.	.
	pvAJ021	R	.	.	G	K	.	.	.
	pvAJ023	.	.	.	.	K	.	.	.
	pvAJ024	.	.	.	.	K	.	.	.
	pvAJ025	.	.	.	.	K	.	.	.
	pvAJ026	R	.	.	G	K	.	.	.
	pvAJ027	.	.	I	.	.	.	.	.
	pvAJ028	.	.	I	.	.	.	.	.
	pvAJ029	R	.	.	G	K	.	.	.
	pvAJ031	.	.	.	.	-	.	.	.
	pvAJ032	.	.	.	.	.	.	.	.
	pvAJ033	.	.	I	.	K	.	.	.
	pvAJ034	.	.	.	.	.	.	.	.
	pvAN003	.	.	.	.	K	.	.	.
	pvAN004	.	.	.	.	K	.	.	.
	pvAN006	.	.	I	.	K	.	.	.
	pvAN009	.	.	.	.	K	.	.	.
	pvAN903	.	.	.	.	K	.	.	.
B	pvAF193	.	D	.	.	.	C	.	D
	pvAF293	.	D	.	.	.	C	.	D
	pvAF746	.	D	.	.	.	C	.	D
	pvAK001	.	.	.	.	.	C	.	D
	pvAK006	.	D	.	.	.	C	.	D
	pvAK007	.	.	.	.	.	C	.	D
	pvAK011	.	.	.	.	.	C	.	D
	pvAN011	.	D	.	.	.	C	.	D
	pvAN013	.	D	.	.	.	C	.	D
	pvAN014	.	.	.	.	.	C	.	D
	pvAN015	.	D	.	.	.	C	.	D
C (Minority)	pvAF076	.	D	I	.	.	C	G	D
	pvAF245	.	D	I	.	.	C	.	.
	pvAF438	.	.	I	.	.	C	G	.
	pvAF481	.	.	I	.	.	C	G	D
	pvAF784	.	.	I	.	.	C	G	.
	pvAF902	.	.	I	.	.	C	G	.
	pvAF982	.	D	I	.	.	C	.	.
	pvAJ002	.	.	I	.	K	C	-	.
	pvAJ007	.	D	I	.	.	C	.	.
	pvAJ008	.	.	I	.	.	C	G	.
	pvAJ014	.	.	I	.	.	C	G	.
	pvAJ017	.	D	I	.	.	C	.	.
	pvAJ018	.	.	I	.	.	C	G	.
	pvAJ022	.	.	I	.	.	C	G	.
	pvAJ030	.	D	I	.	.	C	.	.
Singleton	pvAN008	.	.	.	.	.	C	.	.

Fig. 4. A summary of the group-specific substitutions in 62 WT BLV genomes. (A) Group-specific nucleotide substitutions. The number indicates the location in the BLV genome. (B) Group-specific amino acid substitutions. The numbers indicate the locations of coding genes. The dots indicate the same nucleotide or amino acid as the consensus sequence. The dash ("-") indicates a nucleotide deletion.

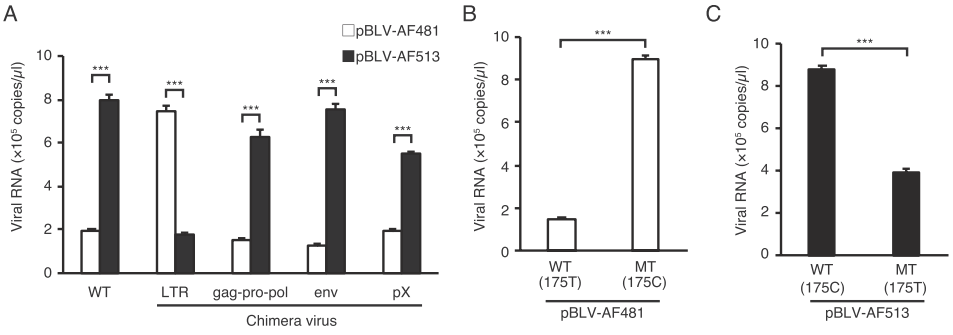

Fig. 5. Analysis of mutations affecting viral productivity using WT molecular clones, pBLV-AF481 and -AF513. (A) The viral productivity of the WT and chimera molecular clones constructed from the WT clones. The LTR, *gag-pro-pol*, *env*, pX are BLV regions, and chimera clones which with recombination of the same region between pBLV-AF481 and -AF513. Viral productivity of the induced point mutation (MT) from thymidine to cytosine and vice versa at nt 175 of the BLV genome (T175C and C175T, respectively). (B) WT (175T) and MT (175C) show pBLV-AF481 and T175C mutation-induced pBLV-AF481, respectively. (C) WT (175C) and MT (175T) show pBLV-AF513 and C175T mutation-induced pBLV-AF513. The experiments were performed in triplicate. The bar graph with an error bar indicates the mean value and standard error. Significant differences are indicated by triple asterisks ($P < 0.001$, Student's *t*-test).

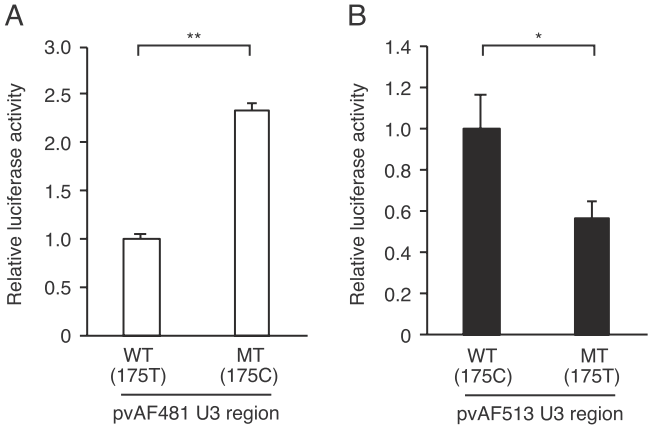

Fig. 6. Viral transactivation of mutation-induced U3 region using CKT-1 cells. (A) WT (175T) and MT (175C) show pBLV-AF481 and T175C mutation-induced pBLV-AF481 U3 regions, respectively. (C) WT (175C) and MT (175T) show pBLV-AF513 and C175T mutation-induced pBLV-AF513 U3 regions. The experiments were performed in triplicate. The bar graph with an error bar indicates the mean value and standard error. Significant differences are indicated by single and double asterisks ($P < 0.05$ and $P < 0.01$, Student's *t*-test).

Polat et al., 2016), while nt 175C may be minor (Murakami et al., 2018; Zhao et al., 2007). To date, 10 and 2 strains with the 175C nucleotide have been reported in Japan and Vietnam, respectively (GenBank accession numbers: DQ288199–288203, DQ288207, DQ288208, AB987702, MH170027, MH170028, LC005615, and LC005616). BLV with the nt 175C may have recently emerged and spread quickly among the cow at the study farm. Therefore, strains harboring the mutation should be monitored because they are high transmissible and will be obstacles to controlling BLV infection.

The present study also suggests that transmission in the minor strains was dependent on a susceptible trait (Fig. 2B), but it is also thought that other host factors would be associated with BLV transmissibility because previous studies have suggested that genetic polymorphism in the other gene and difference in immune response affect PVL (Abdalla et al., 2016; Farias et al., 2016). In addition, programmed death-1/programmed death-ligand 1 (Ikebuchi et al., 2011; Konnai et al., 2017; Nishimori et al., 2017), are associated with transmissibility and leukemogenesis in BLV-infected cows. In contrast, our results showed that important characteristics of BLV can be easily changed by a point mutation, which is a general concept for RNA virology (Domingo et al., 2012). In addition, a point mutation relating to transactivation may enhance *cis*-activation, which related to pathogenesis (Safari et al., 2017). These data imply that BLV strains, isolated from enzootic bovine leukosis-developed cows, may harbor many virulent markers affecting viral properties. Taken together, further studies understanding interaction between host and viral factors would be useful for effective prevention of economic losses caused by BLV infection.

3. Materials and methods

3.1. Samples

Blood samples were collected from 70 Holstein-Friesian cows on a single dairy farm in Japan. Whole blood samples were used for WBC counts and genomic DNA extraction, which were performed using a Celltac hematology analyzer (Nihon Kohden, Tokyo, Japan) and the Wizard genomic DNA purification kit (Promega Corporation, Madison, WI, USA), respectively, in accordance with the manufacturers' instructions.

3.2. Sequencing

The BLV genomes were sequenced according to previous report (Murakami et al., 2016). The whole BLV genome was amplified using PrimeSTAR GXL polymerase (Takara Bio, Inc., Shiga, Japan). The PCR products from each sample were electrophoresed in 1% agarose gels and purified from the gels using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation). The purified PCR products were used as sequencing templates. Sequences of the products and BLV molecular clones were determined using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), in accordance with the manufacturers' instructions. The whole BLV genome sequences of the 34 strains identified in this study were deposited in the GenBank database under the accession numbers AP019565 to AP019598 (Suppl. Table 1).

3.3. Genetic analysis

To perform phylogenetic analysis, the genome sequences were aligned using the ClustalW algorithm and maximum-likelihood trees were constructed using MEGA 7.0 software (Kumar et al., 2016), as described previously (Polat et al., 2016). The reliability of the phylogenetic relationships was evaluated using nonparametric bootstrap analysis with 1000 replicates. The haplotypes were analyzed with PopART software version 1.7 (Bandelt et al., 1999). To reveal the haplotype of the virus, 400-bp *env* sequences, which show a genetic characteristic of BLV (Rola-Luszczak et al., 2013), were used.

3.4. Measurement of PVL

Quantification of proviral BLV genomes was performed for all samples using qPCR, as described previously (Murakami et al., 2016) with GoTaq Probe qPCR Master Mix (Promega Corporation) and the 7500 Real-Time PCR system (Applied Biosystems).

3.5. Typing of the BoLA-DRB3 gene

Polymorphisms of the *BoLA-DRB3* gene in BLV-infected cows were identified using a PCR sequence-based typing method as described previously (Takeshima et al., 2011). The cows harboring the three alleles were identified by different collected samples.

3.6. Cells

Human embryonic kidney 293T cells were maintained in Dulbecco's Modified Eagle's Medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum. Bovine kidney cell line, CKT-1, which was purchased from the Japanese Cancer Research Resources Bank, was maintained in Eagle's Medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum.

3.7. Molecular clones

Molecular clones were constructed from the blood samples of 34 BLV-infected cows according to the method described by Murakami et al. (2016).

3.8. Measurement of viral productivity

vRNA copy number of viruses produced from molecular clone-transfected 293T cells were measured using qPCR. Briefly, 293T cells were transfected with the molecular clones together with the pTK-Luc vector using FuGENE HD Transfection Reagent (Promega Corporation). At 24 h post-transfection, the cells were washed twice with Dulbecco's Modified Eagle's Medium and further cultured in fresh growth medium. At 48 h post-transfection, luciferase activity and vRNA copy number were measured, using the cells and cell supernatants after removal of cell debris by centrifugation, respectively. To determine transfection efficiency, the luciferase activity of the cells was measured using the Pikka Gene Kit (Toyo Ink America LLC, Wood Dale, IL, USA), according to the instruction manual. For qPCR, vRNA was first extracted from the supernatant using the High Pure Viral Nucleic Acid Kit (Roche, Penzberg, Germany), according to the manufacturer's instructions. cDNA was then synthesized from the vRNA using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan), according to the manufacturer's instructions. qPCR was performed using GoTaq Probe qPCR Master Mix (Promega Corporation) and a 7500 Real-Time PCR system (Applied Biosystems), as previously reported (Murakami et al., 2016).

3.9. Measurement of viral transactivation

The pBLV-AF481 and -AF513 U3 regions, the pBLV-AF513 *tax* gene sequence, and the firefly luciferase gene of the pCMV-Luc plasmid vector (Promega Corporation) were amplified by PCR. The U3 regions' amplified PCR products were cloned into in the pBApo-EF1α-Neo plasmid (Takara Bio, Inc.), and then the firefly luciferase gene was inserted downstream of the U3 region (pU3-Luc). The amplified PCR products of the *tax* gene sequence were cloned into the plasmid pCAGGS (Tax-pCAGGS). Each pU3-Luc together with Tax-pCAGGS and pRL-TK (Toyo Ink, Tokyo, Japan) were transfected into the 293T and CKT-1 cells using FuGENE HD Transfection Reagent (Promega Corporation) and Lipofectamine LTX with Plus Reagent (ThermoFisher Scientific, Waltham, MA, USA). The pU3-Luc and pRL-TK plasmids were used to measure viral transactivation and transfection efficiency, respectively. At 48 h post-transfection, the cells were analyzed for firefly and *Renilla* luciferase activities using the Pikka Gene Dual Assay Kit (Toyo Ink), as previously reported (Murakami et al., 2016).

3.10. Induction of recombination and point mutations

The chimeric BLV molecular clones were constructed based on the BLV molecular clones pBLV-AF481 and -AF513. Each region (LTR, *gag-pro-pol*, *env*, and pX) was amplified by PCR using the specific primers listed in Suppl. Table 3. The chimeric clones were constructed from a combination of each PCR product using the In-Fusion HD Cloning Kit (Takara bio, Inc.). The point mutation at nt 175 of the BLV genome and the U3 region were generated using mismatched primers (Suppl. Table 4). PCR amplification was performed using mismatched primers and molecular clones or pU3-Luc as a template. The amplified PCR products were ligated. The molecular clones that induced a recombination/point mutation and pU3-Luc, which induced a point mutation, were amplified in competent *E. coli* Stbl3 and DH5α cells, respectively.

3.11. Statistical analysis

Statistical analyses were performed using Student's *t*-test, one-way analysis of variance with the Tukey's post hoc test. All the statistical analyses were performed using R version 3.5.2 statistical software (Team, 2018). A probability (P) value of < 0.05 was considered statistically significant.

Conflicts of interest

The authors have no conflicts of interest to declare.

Ethics statement

This study was conducted in accordance with the Guidelines for Laboratory Animal Welfare and Animal Experiment Control set out by the School of Veterinary Medicine of Azabu University (approval No. 17113-3).

Acknowledgements

This study was supported by a research project grant awarded by the Azabu University Research Services Division, Ministry of Education, Culture, Sports, Science and Technology (MEXT) Supported Program for the Private University Research Branding Project, 2016–2019, and JSPS KAKENHI [grant number 17K15385 and 19K06410].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.08.015.

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