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Bovine leukemia virus genotype 1 and 6 are circulating among dairy and beef cattle of small and medium holding farms in northern Vietnam

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Abstract

The aims of this study were to determine the prevalence of bovine leukemia virus (BLV) infection among cattle in northern Vietnam, and to molecularly characterize the detected strains. Ninety-three blood samples (45 dairy and 48 beef cattle) were randomly collected from 464 cattle from 22 farms in four districts in northern Vietnam. The samples were tested for the presence of BLV by using two real-time PCR detection systems that targeted the BLV *pol* gene and *tax* gene. The samples were also tested for BLV antibody, using an ELISA kit. Nested PCR was designed to obtain the full-length nucleotide sequence of the BLV *env* gp51 gene. From the BLV *pol* gene real-time PCR and ELISA results, 33 of the 93 (35.48%) samples consistently tested positive for BLV infection. Phylogenetic analysis of the BLV *env* gp51 gene showed that the Vietnam strains were clustered into genotypes 1 and 6 (G1 and G6), of which G1 was dominant. Of three G6 strains detected, two were clearly distinguished from the known subgenotypes G6a–G6e and were therefore designated as a new subgenotype, G6f. Results of the phylogenetic analyses highlighted the requirement of the full-length *env* gp51 gene for reliable identification of the G6 strains. It was also noted that the commercial real-time PCR kit for the BLV *tax* gene failed to detect the Vietnam G6 strains, as was likewise previously reported for Myanmar BLV G10 strains.

Key Words: Bovine leukemia virus, Genotype, Gp51, Subgenotype, Vietnam

Introduction

Bovine leukemia virus (BLV) is an oncogenic virus belonging to the family *Retroviridae*, genus *Deltaretrovirus*. The virus causes a persistent infection of B lymphocytes in cattle, which can proceed to lymphosarcoma (i.e., enzootic bovine leukosis) in the infected animal[8,27]. BLV infections have been reported worldwide and have financial impacts on the cattle industry, not only because of the neoplastic feature but also due to the harmful influences of reduced fertility and decreased milk production in dairy cattle, increased heifer replacement costs, loss of income

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resulting from the culling of premature animals, and international trade restrictions[2,6,27]. Transmission of BLV occurs primarily via blood during insect bites and animal manipulation procedures, such as ear tattooing, dehorning, rectal palpation, and continuous injection. Moreover, colostrum and milk are described as one of the major materials of virus transmission[27]. The transmission via placental has been recognized as a main route in the infected dam with high proviral load[26].

BLV is composed of the viral *gag*, *pro*, *pol*, and *env* genes, which are required for virus particle synthesis and are flanked by two identical long terminal repeats (LTR_S). A pX region located between the *env* gene and the 3′ LTR encodes the Tax, Rex, R3, and R4 proteins[8]. The envelope protein of BLV is cleaved into two subunits: the extracellular surface subunit gp51 protein, and the transmembrane (TM) gp30 protein. The envelope glycoprotein gp51 plays an essential role in the virus life cycle and is required for cell entry; it is also the target of neutralizing antibodies[3,10,14,22]. In addition, the BLV gp51 protein induces a massive expression of specific antibodies in infected animals[13] and is therefore normally used for both diagnostic and genotyping purposes[8]. Many BLV surveillance studies have been conducted and described on all continents. Based on the analyses of BLV gp51 *env* gene sequences from strains isolated in different countries worldwide, this virus has been classified into 10 genotypes (G1–G10) to date[1,4,11,15–21,23,24,29].

Vietnam is located in Southeast Asia, and its economy depends mainly on agricultural industries. According to statistics from Vietnam's Husbandry Office (http://www.channuoivietnam.com) and the General Statistics Office of Vietnam (http://www.gso.gov.vn), the local population of cattle in 2016 was approximately 6.1 million, made up of 3.1 million beef cattle, 2.5 million water buffaloes, and 463,000 dairy cattle. Vietnam cattle produce approximately 308,000 tons of beef and 795,000 tons of milk each year. The cattle populations are distributed prominently in northern Vietnam, including the Hanoi region. The Vietnamese government has been strict in controlling the spread of many cattle diseases in the country, with a primary focus on foot-and-mouth disease, bovine anthrax, bovine pasteurellosis, and so on. However, there have been no studies focusing on the surveillance and molecular epidemiology of BLV infection in Vietnam. The aims of this study were therefore to determine the prevalence of BLV infection among cattle in Vietnam and to molecularly characterize the strains detected.

Materials and Methods

Ethics statement: The cattle blood samples used in this study were collected by the National Institute of Veterinary Research (NIVR) in accordance with the National technical regulation on Animal diseases- General requirements for samples collection, storage and shipment (QCVN 01-83:2011/BNNPTNT) of Vietnam. All samples were used for the study under the permission of the owners of the cattle farms.

Samples: In February 2017, a total of 93 blood samples were obtained from cattle farms in four districts in northern Vietnam (VinhTuong, BaVi, ChuongMy, and MyDuc). The samples were from 45 dairy and 48 beef cattle that had been randomly selected among 464 individuals from 22 farms. These cattle farms were differentiated into two groups according to the number of animals present on the farm. Those holding 20 or fewer cattle were considered as small farms, whereas those holding 20–100 cattle were considered as medium-sized farms. In this study, the blood samples were obtained from 17 small and 5 medium holding farms and accounted for approximately 20% of the total cattle population on each farm. All the cattle involved in this study ranged from 12 to 36 months of age and appeared healthy. The beef cattle were either

Table 1. Primers and probes used for real-time PCRs targeting the BLV *pol* and *tax* genes, and the nested PCR targeting the *env* gene

Name	Sequence (5′ – 3′)	Position*	Reference
BLV-pol-F	CCCTGGCCTACTTCCAGACC	3304–3323	
BLV-pol-R	CTTGGCATACGAGCTTAAGGCC	3395–3416	(Heenemann *et al.*, 2012)
BLV-pol-probe	FAM- TTGACTGACAACCAAGCCTCACCT-BHQ1	3327–3350	
BLV 525 F	GACCCTAGGGCCATCATCC	3126–3144	
BLV 525 R	AGCCCCGTCACTAAAGAGG	3632–3650	
BLV Outer F	TCAGAGGGCGGAGAAACAC	4650–4668	Designed for this study
BLV Outer R	GGTCAAGCATTTTATCAGG	6190–6208	
BLV Inner F	TGGGTTCCCTGGCGTTT	4794–4810	
BLV Inner R	AAAAAGGGCTAATAGGAACAGG	6148–6169	

*Nucleotide positions according to the GenBank accession No. LC164086.

native to Vietnam (Lai Sind) or of F1 BBB breed (Vietnam native cattle × Blanc Bleu Belge), whereas the dairy cattle were predominately Lai HF (Vietnam native cattle × Holstein Friesian).

Detection of BLV provirus DNA by real-time PCR: Genomic DNA was extracted from 200 µl of the whole blood, using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) and stored at 4°C. Serum samples obtained were stored at −20°C. The extracted genomic DNA samples were tested by an in-house real-time PCR targeting the BLV *pol* gene (BLV *pol* real-time PCR), as reported by Heenemann *et al*.[9] but with slight modifications. The sequences of the primers (BLV-pol-F and BLV-pol-R) and probe (BLV-pol-probe) used are listed in Table 1. The reaction mixture contained 12.5 µl of Eagle Taq Master Mix (Roche), 0.6 µl of each primer (10 µM each), 0.3 µl of probe (5 µM), 6.0 µl of distilled water, and 5.0 µl of DNA sample as the template. The reaction was conducted in a LightCycler Nano Instrument (Roche) under the following conditions: initial denaturation for 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of denaturation for 15 sec at 95°C and 1 min of annealing and extension at 58°C. Fluorescence data were collected during the annealing step. A control plasmid for the BLV *pol* real-time PCR was constructed by ligating a 525-bp fragment of the BLV *pol* gene into the pGEM-T Easy Vector (Promega Corporation, Madison, WI, USA). The partial BLV *pol* gene was amplified from genomic DNA of the BLV-infected cattle using primers BLV 525 F and BLV 525 R (Table 1). In order to confirm the reliability of the BLV *pol* real-time PCR, a real-time PCR targeting the BLV *tax* gene (BLV *tax* real-time PCR) was also conducted using the primers and probe provided by Takara Bio Inc. (Shiga, Japan) and the Takara Cycleave PCR Reaction Mix (Takara Bio Inc.).

Detection of anti-BLV antibody by ELISA: Antibodies against the BLV gp51 protein in the serum samples were detected using a commercially available BLV ELISA kit (JNC Corporation, Tokyo, Japan) according to the manufacturer's instructions. Color development was measured by reading the optical density (OD) at 450 nm with a spectrophotometer (TECAN Genios Pro; Tecan Group Ltd, Männedorf, Switzerland). The antibody-positive sample was determined by calculating the sample-to-positive (S/P) ratio from the OD values obtained for the tested serum, and the positive and negative control sera provided with the kit. The sample was regarded as positive if the value of the S/P ratio was greater than 0.3 and the result of the positive control was greater than 0.6.

Nucleotide sequence of the complete BLV env gp51 gene: Two sets of primers for the nested PCR

(BLV Outer F/R and BLV Inner F/R; Table 1) were designed to sequence the full-length BLV *env* gp51 gene (903 bp) for this study. Nested PCR was performed using the GoTaq Green Master Mix (Promega) under the following two sets of conditions. The first PCR for a 1,558-bp sequence of the BLV *env* gp51 gene involved an initial denaturation at 95°C for 5 min; 25 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 3 min; and one cycle of 72°C for 10 min. The second PCR for a 1,375-bp sequence of the BLV *env* gp51 gene involved 95°C for 5 min; 40 cycles of 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min; and a final cycle of 72°C for 10 min. The final PCR products were separated on 1.5% agarose gels, purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and then subjected to sequencing analysis. Approximately 250 ng of the purified DNA obtained from the nested PCR for each sample was used for the sequencing reaction, which was performed using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosciences, Foster City, CA, USA) and the primers BLV Inner F and BLV Inner R. The resultant products were then purified using the BigDye XTerminator Purification Kit (Applied Biosciences). The nucleotide sequences were analyzed on the 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA), and the sequence data obtained were analyzed with GENETYX v.13.0.3 software (GENETYX Corp., Tokyo, Japan). The consensus sequences were generated from two bidirectional repeats of each sample.

Phylogenetic analysis: The BLV *env* gp51 nucleotide sequences obtained in this study were aligned with sequences from worldwide BLV reference strains of all 10 genotypes (G1–G10), using the CLUSTALW multiple alignment tool in BioEdit v.7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit). First, a 444-bp fragment of the partial *env* gp51 gene at the 277–720 nucleotide (nt) position, which was previously identified by restriction fragment length polymorphism and has often been used for genotyping of BLV[7,12], was applied for the alignment of the 33 Vietnam strains with 66 worldwide strains. Second, the complete BLV *env* gp51 gene sequences (903 bp) of all 33 Vietnam strains were aligned with those of 56 BLV strains available in GenBank. Phylogenetic analyses based on the two datasets (444 bp and 903 bp of the BLV *env* gp51 gene) were conducted using two methods: maximum-likelihood (ML) analysis in MEGA 6[28], applying 1,000 bootstrap replicates and the best-fit model K2+G; and Bayesian inference (BI) analysis in MrBayes v.3.2.6[25], applying the best evolutionary model (l set nst = 2, rates = gamma [corresponding to model HKY+G]), as estimated by MrModeltest2 in PAUP4 (http://paup.sc.fsu.edu/index.html). In the BI analysis, two runs and four Markov chains were used for 10,000,000 generations, and the trees were sampled at every at 1,000 generations. The first 25% of the BI trees were discarded as "burn-in." A consensus tree was constructed from the output file using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/). The phylogenetic trees were then rooted at G5 for ease of comparison[1,11].

Mean nucleotide and amino acid distances and amino acid substitutions in BLVs: The average percentages of nt and amino acid (aa) distances for the complete BLV *env* gp51 genes were estimated within (intra-subgenotype) and between (inter-subgenotype) G6 subgenotype groups, using the Kimura 2-parameter model with a gamma distribution and the Poisson model in MEGA 6[28], respectively. In addition, 301 aa of the BLV gp51 *env* protein sequences of the 33 Vietnam strains and 56 worldwide strains (representing 10 BLV genotypes) were observed and compared throughout the functional regions as described in previous studies[21,30].

Table 2. Positive rates of BLV infection in cattle farms in northern Vietnam, as determined by ELISA and real-time PCR (*pol* and *tax* genes)

District	% ELISA-positive in total cattle tested	% *pol* gene-positive in total cattle tested	% *tax* gene-positive in total cattle tested	Number of farms by size Positive*/Total		Number of farms by type Positive/Total	
				Small	Medium	Beef	Dairy
VinhTuong	14.29 (3/21)	14.29 (3/21)	9.52 (2/21)	2/7	None	0/2	2/5
BaVi	58.54 (24/41)	58.54 (24/41)	53.66 (22/41)	1/2	4/4	2/2	3/4
ChuongMy	30.00 (6/20)	30.00 (6/20)	30.00 (6/20)	0/4	1/1	1/5	None
MyDuc	0.00 (0/11)	0.00 (0/11)	0.00 (0/11)	0/4	None	0/4	None
Total	35.48 (33/93)	35.48 (33/93)	32.26 (30/93)	3/17	5/5**	3/13	5/9

*: The farm holding the BLV-infected cattle.
**: Positive rate between Medium farm and Small farm was significantly different by Fisher's exact test ($P < 0.01$).
Small: small cattle farm holding 20 or fewer cattle.
Medium: medium cattle farm holding 20–100 cattle.

Results

Prevalence of BLV infection in northern Vietnam

The ELISA detected antibodies against BLV in the samples from three districts in northern Vietnam: VinhTuong, BaVi, and ChuongMy, with positive rates of 14.29% (3/21), 58.54% (24/41), and 30.00% (6/20), respectively. The BLV *pol* real-time PCR results corresponded with the ELISA results for these three districts. However, whereas both BLV *tax* and *pol* real-time PCRs yielded the same positive rates in the ChuongMy district (i.e., 6/20), the rates obtained by BLV *tax* real-time PCR in the VinhTuong and BaVi districts were different, being 9.52% (2/21) and 53.66% (22/41), respectively. Thus, a total of three samples (1 in VinhTuong and 2 in BaVi) which tested positive by BLV *pol* real-time PCR and ELISA were tested negative by the BLV *tax* real-time PCR. Overall, the prevalence of BLV infection in northern Vietnam was estimated to be 35.48% (33/93) according to the ELISA and BLV *pol* real-time PCR results. Prevalence of BLV infection in small farms and medium farms were 17.6% (3/17) and 100% (5/5), respectively. The prevalence between small and medium farms was significantly different ($P < 0.01$), although prevalence between beef and dairy cattle was not (Table 2).

Phylogenetic analysis and genotyping of the Vietnam BLV strains

The complete BLV *env* gp51 nt sequence (903 bp) was obtained for all the 33 samples found to be positive by ELISA and BLV *pol* real-time PCR. The nt sequences of nine representative strains were deposited to GenBank under the accession numbers MF817716–24. The phylogenetic relationships of the Vietnam strains to the 10 BLV genotypes (G1–G10) were analyzed by the ML and BI approaches on the basis of the complete BLV *env* gp51 nucleotide sequences. The high supporting values from both methods indicated that the 33 Vietnam strains and 56 worldwide strains were clearly classified into the 10 genotype groups. The 33 Vietnam strains were divided into two distinct genotypes: 30 strains belonged to G1 and three to G6, supported by high posterior probability value of 1 for each group in the BI analysis (Fig. 1). Similar results were obtained in the ML analysis with high bootstrap value ≥ 96 for each group (Supplementary fig. 1). The phylogenetic analysis of the 444-bp partial gp51 gene sequences using the ML and BI methods also divided the Vietnam strains into two clusters. The 30 Vietnam strains belonging to G1, along with the worldwide reference strains of this genotype, produced a high bootstrap value of 90 in the ML analysis (Supplementary fig. 2) and a high posterior probability value of 1 in the

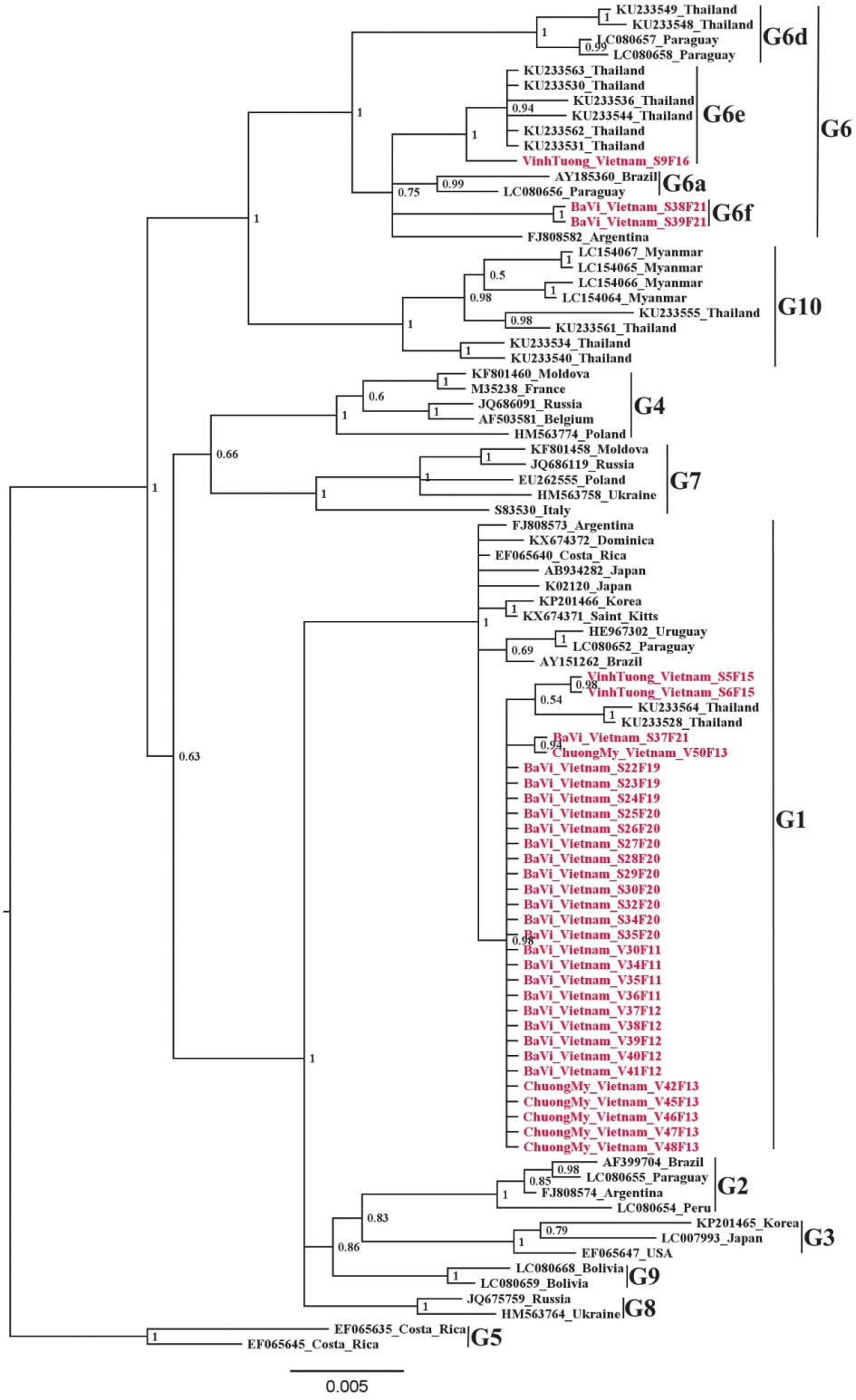


Fig. 1. Phylogenetic relationship of the complete nucleotide sequences of the BLV *env* gp51 gene (903 bp) from 33 Vietnam strains (shown in red) and 56 worldwide BLV strains of 10 genotypes, constructed by Bayesian inference. Numbers at the nodes indicate the posterior probabilities of sampling the node among 15,002 trees. Genotypes (G1–G10) are indicated by the numbers on the right of the figure. The bar at the bottom of the figure denotes the distance. The tree was rooted on G5.

Table 3. Average percentages of nucleotide and amino acid distances in the complete BLV *env* gp51 gene sequences within (intra-subgenotype) and between (inter-subgenotype) G6 subgenotype groups

Sub-genotype	G6a	G6b	G6d	G6e	G6f
G6a	**1.02 / 1.02**	1.19	1.11	1.34	**0.85**[b]
G6b	1.49	–* / –	1.27	1.51	1.02
G6d	2.36	2.54	**0.43 / 0.85**	1.13	0.93
G6e	1.48	1.56	1.94	**0.32 / 0.48**	**1.16**[a]
G6f	**1.79**[b]	1.85	**2.79**[a]	1.8	**0.00 / 0.00**

The intra-subgenotype and inter-subgenotype distances were calculated using the Kimura 2-parameter model and Poisson model, respectively.
Left lower matrix: mean nucleotide distances. Right upper matrix: mean amino acid distances.
The values on the left in bold along the diagonal are the intra-subgenotype nucleotide distances.
The values on the right in bold along the diagonal are the intra-subgenotype amino acid distances.
Numbers in bold marked by [a], [b] indicate the maximum and minimum nucleotide and amino acid distances of G6f from the other G6 subgenotypes, respectively.
* not calculated

BI analysis (Supplementary fig. 3). However, the worldwide G6 and G10 strains and the three Vietnam G6 strains were grouped into one cluster that included some polytomies, with the supporting value being low at 52 in the ML analysis (Supplementary fig. 2) but high at 0.99 in the BI analysis (Supplementary fig. 3). The BI phylogenetic analysis using the 444-bp sequence demonstrated that at least six subgenotypes existed in a topology of G6 supported by high posterior probability values ≥ 0.69 for all the subgenotype groups (Supplementary fig. 3). One of the three Vietnam G6 strains, VinhTuong_Vietnam_S9F16, which was detected from a dairy cow in the VinhTuong district, was grouped with Thailand BLV strains. The other two Vietnam G6 strains, BaVi_Vietnam_S38F21 and BaVi_Vietnam_S39F21, both of which were detected from dairy cows in the BaVi district, belonged to another group. Therefore, on the basis of these results, we propose the presence of a new G6 subgenotype, named G6f, in addition to the five known subgenotypes G6a–G6e (Fig. 1 and Supplementary fig. 3).

Mean nucleotide and amino acid distances and amino acid substitutions in Vietnam BLV strains

Analysis of the mean nt and aa distances, based on the complete BLV *env* gp51 gene sequences, in both G6 intra- and inter-subgenotypes indicated that the new subgenotype G6f was clearly distinguishable from the other G6 subgenotypes. The average percentages of nt distance showed that G6f was the most distant from G6d (with a value of 2.79%), and closest to G6a (1.79%). On the other hand, the average percentages of aa distance indicated that G6f was closest to G6a (0.85%) and farthest from G6e (1.16%) (Table 3).

By comparing Vietnamese BLV gp51 aa sequences with those of 10 known BLV genotypes, we observed a total of nine aa substitutions, among which three aa substitutions have never been reported before. One unique aa substitution, P179L (proline to leucine), located in the overlapping region of the CD8+ T-cell epitope and epitope E, was observed in a Vietnam G1 strain. Two other unique substitutions, Q51R (glutamine to arginine) at a "not-determined" region, and S189N (serine to asparagine) in the antigenic region E or E´ linear epitope, were observed in Vietnam G6 strains (data not shown).

Discussion

In this study, we provide the first report of the prevalence of BLV infection in beef and dairy cattle in Vietnam, along with the genetic characterization of the strains isolated. Among

93 cattle tested, 33 (35.48%) were determined by ELISA and BLV *pol* real-time PCR to be BLV-infected. One remarkable finding was that the TAKARA BLV detection kit for the BLV *tax* real-time PCR failed to detect three of the positive samples. It is interestingly note that phylogenetic analysis based on the complete BLV *env* gp51 gene clearly indicated that the three Vietnam strains not detected by BLV *tax* real-time PCR belonged to the genotype G6 (Fig. 1).

In a previous BLV surveillance study conducted in Myanmar, the TAKARA BLV detection kit also failed to detect three out of six samples that were found to be BLV-positive by real-time BLV-CoCoMo-qPCR2 targeting the BLV LTRs. Five of the six BLV strains were identified as G10[19]. Taken together, it would seem that genetic mutations in the *tax* gene of G6 and G10 BLVs could affect the sensitivity of the TAKARA BLV detection kit. Unfortunately, the detection site in the *tax* gene of the commercial kit is not currently available. Therefore, we applied the full-length *tax* gene sequences of the G6 Vietnamese strains, VinhTuong Vietnam S9F16 and BaVi Vietnam S38F21, which we obtained by the Next Generation Sequencing method, to the alignment with those of the G10 Myanmar strains and worldwide BLV strains to identify the specific mutations retained by both G6 Vietnam and G10 Myanmar strains. A total of seven specific nucleotide mutations were found exclusively in the BLV *tax* gene sequences of the G6 BLV strains including the Vietnam strains and G10 Myanmar strains[5]. However, further studies are needed to clarify whether the *tax* gene mutations in the G6 and G10 BLV strains possibly abrogate the gene detection by the commercial kit.

The highest prevalence of BLV infections (58.54%) was found in the BaVi district, which is one of the main livestock districts and holds 65% of the total cattle population in Hanoi, according to Vietnam's Husbandry Office and the General Statistics Office of Vietnam (November 2016) (http://www.channuoivietnam.com; http://www.gso.gov.vn). Considering the management of Vietnamese farming system, cattle replacement from different herds of farms, crossbreeding between native cattle with imported cattle, and extended calving interval may be the factors that caused the genetic diversification of this virus in this country.

The 33 Vietnam strains identified in this study were clustered into two genotypes, G1 and G6 (Fig. 1). G6 has five known subgenotypes, designated G6a through G6e[11,20]. We found two Vietnam G6 strains that were distinguishable from the other G6 subgenotypes and classified them into a new subgenotype, G6f. Another interesting finding was the clustering of two G1 strains and one G6 strain from the VinhTuong district with Thailand BLV strains (Fig. 1), suggesting that these strains may have the same origin. This finding may be related to the cattle trading between the two countries. Another important finding was that the 444-bp fragment of the BLV *env* gp51 gene that is widely used in BLV genotyping[1,7,11,17–20]) seemed to be limited in classifying G6 and G10 strains in both the ML and BI methods. In the phylogenetic trees based on the 444-bp sequences (Supplementary fig. 2 and Supplementary fig. 3), G6 and G10 could not be sufficiently separated. In order to differentiate these two BLV genotypes reliably, it is recommended that the complete BLV *env* gp51 gene sequence be used instead for the phylogenetic analysis.

In conclusion, we have reported the findings of the first BLV surveillance study in Vietnam. The prevalence of BLV infection among cattle in northern Vietnam was 35.48%, as determined by both serological and genetic analyses. The 33 detected Vietnam strains were of genotypes G1 and G6, one of which was a new subgenotype, G6f. One remarkable finding was that the commercial real-time PCR kit for the BLV *tax* gene failed to detect the Vietnam G6 strains, similar to a previous report on Myanmar G10 strains. The findings obtained herein will be useful for further studies on BLV infection not

only in Vietnam but also in other countries that require better cattle husbandry practices.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Supplemental data

Supplemental data associated with this article can be found, in the online version, at http://dx.doi.org/10.14943/jjvr.67.1.83

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