Genomic characterization and distribution of bovine foamy virus in Japan

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ABSTRACT. Bovine foamy virus (BFV) is distributed through worldwide cattle herds. Although the biological features of BFV are not well understood, appearance of clinical manifestation by superinfection with other microorganisms is inferred. In Japan, reports of genomic characterizations and epidemiology of this virus are limited. In this study, we performed whole genomic sequencing of BFV strains Ibaraki and No.43, which were isolated in this country. Additionally, we investigated BFV in geographically distant four daily farms in Japan, to estimate the distribution of BFV and its correlation to bovine leukemia virus (BLV). BFV was distributed throughout Japan; the average positive rate was 12.7%. The nucleotide sequence identities of the isolates were 99.6% when compared with BFV strain isolated in the USA. The phylogenetic tree using *env* gene sequence showed strains Ibaraki, No.43 and Kagoshima were sorted in the same cluster including the USA and Chinese strains, while Hokkaido strain was in the other cluster including European strains. Although no clear correlation between BFV and BLV could be found, BFV and BLV infections were likely to increase with ages. Our data on epidemiology and characteristics of BFV will provide important information to reveal biological features of BFV.

KEY WORDS: bovine foamy virus, bovine leukemia virus, epidemiology

Foamy viruses (FVs) belong to the subfamily *Spumaretrovirinae* which is a member of the family *Retroviridae* [19]. The length of viral genome is about 12 kb. FV was first isolated and identified using rhesus monkey kidney cells in 1955 [33]. FVs have been identified in many vertebrates, including mammals [1, 16, 24, 30, 35, 36], amphibians [2] and fish [2, 9, 10, 32]. FVs infect several kinds of primary and lined cells and form multinucleated giant cells. The foamy aspect is confirmed by microscopic observation of cultured cells, and this feature became the root of the name of FV [34]. The FV genome encodes three structural, *gag*, *pol*, *env*, and two nonstructural, *bel-1* and *bel-2*, genes. Tas proteins, the *bel-1* products, play a role in trans activator, while the function of Bet proteins, complex of the *bel-2* products and a part of *bel-1* products, has not yet been determined [23, 34].

BFVs have been isolated in Western countries and China, and considered to be common in cows [5, 12, 14, 15, 29]. This implies they may be spreading over a wide region. In Japan, BFV strain No.43 was isolated from peripheral blood leukocytes (PBLs) of a clinically healthy cow in 2018 [8], by co-culturing BFV-infected PBLs with fetal bovine muscle (FBM) cells. In that report, the prevalence of BFV infection was shown to be 8.8% (five out of 57 cattle) in Kanagawa prefecture, Japan, and a part of the long terminal repeat (LTR), *env* and *bel-1-bel-2* sequences were identified. Another recent study showed that BFV was detected among cattle in the Kanto region in Japan showing that the average BFV-positive rate was 16.7% (91 out of 545 cattle) [13]. However, the full-length genome sequence of strain No.43 was undetermined. Moreover, the information on the prevalence of BFV infection was limited to the Kanto region. Consequently, epidemiology and genomic characteristics of BFV in Japan is still limited.

FVs are considered to be nonpathogenic alone [34]. However, there are some reports indicating the possibility of FV

pathogenicity. Prototype FV activates human immunodeficiency virus type 1 (HIV-1) via LTR activation [18]. Correlation of feline foamy virus (FFV) and feline leukemia virus (FeLV) infection has been described, in particular, cats infected with progressive FeLV are more susceptible to FFV [6, 28]. Also, buccal FFV viral load measured by quantitative PCR of FFV/FeLV co-infected cats was higher than among FFV mono-infected cats [6]. In addition, the cats infected experimentally appeared clinically normal but histopathological changes were observed in their lungs and kidneys [7]. Considering the possibility that FVs affect the disease manifestation with other retroviruses, in the present study, we investigated the relationship between BFV infection and bovine leukemia virus (BLV). BLV Tax protein was shown to activate HIV-1 transcription in a previous study [18], suggesting that BLV infection may have a potential to alter BFV infection. The seropositive rate of BLV in Japan recently reached about 40% among dairy cattle [26], and infection control of BLV is a matter of utmost concern in Japan. However, there are few reports focusing on relationship between BFV and BLV.

In this study, to further understand the characteristics of BFV in Japan, detection, isolation, and genomic characterization of BFV were conducted. In addition, the association between the BLV infection and BFV prevalence among dairy cattle was examined.

MATERIALS AND METHODS

Animals

The whole blood samples analyzed in this study were obtained from a total of 181 Holstein-Friesian or F1 (crossbreed of purebreds) cattle kept in the respective farms of Ibaraki, Kagoshima, Hokkaido, and Miyagi prefectures (Supplementary Fig. 1) aged between 0 to 10 years old (Table 1). The whole blood was collected from the jugular vein of each cattle with EDTA-coated capillary tubes. In addition to the whole blood, serum was isolated from the cows kept in Ibaraki.

BFV surveillance

The prevalence of BFV in Ibaraki, Kagoshima, Hokkaido and Miyagi was determined by conventional PCR. Genomic DNA was directly extracted from 181 cattle blood samples (Table 1) with QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. A primer pair amplifying a 6,781 bp fragment from *pol* to *bel-1* region referring to the BFV reference sequence (GenBank accession number: U94514) was designed and applied. The sequences of the primer pair were 5′-AATACCAAG CTCTATGGCAATGTT -3′ (8F) and 5′- ACCAGGGCTGTACTGGTTCT -3′ (8R). PCR was performed using DNA polymerase, KOD Fx Neo (TOYOBO, Osaka, Japan). The cycling parameters were: initial denaturation was at 94°C for 2 min, and 45 cycles of the following 2 steps: at 98°C for 10 sec, 68°C for 3.5 min. The PCR products were electrophoresed on 1% agarose gels to confirm the presence of 6,781 bp band.

BFV isolation and preparation

Thirty-four cattle serum samples collected from a farm in Ibaraki prefecture were tested by agarose gel immunodiffusion (AGID) test to determine the BFV infection. AGID tests were performed according to the methods described elsewhere [8]. Positive serum and BFV antigen were kindly provided by Prof. Sentsui (Nihon University). Cows showing a clear precipitation line in AGID test were selected, and three strains (No. 5266, 5270, and 7800) were isolated from the cattle in Ibaraki. The full genome sequences of the isolated viruses were determined.

Blood was collected with EDTA tubes from the jugular vein of three cattle showing AGID test-positive. To isolate BFV, the peripheral blood mononuclear cells (PBMC) from three AGID-positive cases were separated from the blood stored at 4°C by Lymphopep (Cosmo Bio, Carlsbad, CA, USA) within 24 hr from the blood collection. PBMC derived from cows reared in Ibaraki were co-cultured with BHK21 cells in 6-well plates at 37°C in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich Co., LLC, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS). After 48 hr, the cells were washed with DMEM and fresh medium was added. Two days after, cells were subcultured by trypsinization. Blind passage was carried out at intervals of two or three days until cytopathic effect (CPE) was observed. Two negative controls were prepared as: BHK21 cells only and BHK21 cells cocultured with PBMC of BFV-negative cows. In addition to the three AGID-positive BFV samples, strain No.43, which was preciously isolated and kindly provided by Prof. Sentsui, was co-cultured with BHK21 cells and subcultured at passage five. When CPE was observed, the cells were stained with Giemsa after being fixed with methanol to observe the multinucleated giant cells.

Whole and partial genome sequence analysis

DNA was extracted from BHK21 cells cocultured with BFV-infected PBMC derived from cows reared in Ibaraki at passage

Table 1. Sample information

Prefecture	Breed	Age (years)	Number of cows tested	BFV prevalence
Ibaraki	Holstein-Friesian/ F1	0–10	34	11.8% (4/34)
Kagoshima	Holstein-Friesian	0–2	29	13.8% (4/29)
Hokkaido	Holstein-Friesian	0–6	98	15.4% (15/98)
Miyagi	Holstein-Friesian	0–1	20	0.0% (0/20)
Total		0–10	181	12.7% (23/181)

BFV: bovine foamy virus. F1: crossbreed of purebreds.

12 and strain No.43 at passage five. Prior to the DNA extraction, cells were washed with PBS twice and peeled off with trypsin-EDTA. The suspension was processed using QIAamp DNA Mini Kit (QIAGEN), following the manufacturer's instructions. Three pairs of primers described in Fig. 1 were used for detecting each gene. All primer sets were designed referred to the BFV reference sequence (GenBank accession number: U94514). The primer sequence used for amplifying an 1,869 bp fragment of *gag* to *pol* region were as follows: 5′- GGCAACCCTTGCCTATTCCT -3′ (gagpolF) and 5′- CTCAATACGCCGATGTCCGA -3′ (gagpolR). A 6,781-bp fragment comprising of *pol*, *env* and part of *bel-1* was amplified using the 8F/R primers. The other 3,665-bp fragment was amplified with primer named ipcrF 5′- GAACACCCGGACAGCA-TACACTC -3′ and ipcrR 5′- CCATCGGAGCAGAGACCACT -3′ by inverse PCR method. The PCR cycle to amplify from *bel-1* to *gag* region was carried as follows: initial denaturation was at 94°C for 2 min, and 45 cycles of the following 2 steps: at 98°C for 10 sec, 68°C 4.5 min. The PCR condition of 8F/R primers was the same protocol as described above in the BFV surveillance section. The other PCR condition with gagpolF/R primers was performed as follows: 94°C for 2 min; followed by 45 cycles of 98°C for 10 sec, 56°C for 30 sec and 68°C for 1 min. The PCR products were electrophoresed on 1% agarose gels and purified using NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL, Düren, Germany). The sequence except *pol* to *bel-1* region of Ibaraki and the whole sequence of the strain No.43 were determined by direct sequencing. With regard to *pol* to *bel-1* (*env*) region, purified PCR products of strain Ibaraki, BH47 and K7 were cloned using TOPO XL PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA) or Zero Blunt TOPO PCR cloning Kit (Thermo Fisher Scientific). The identity of the direct and cloned sequence of *env* region of Ibaraki strain was confirmed. The other purified PCR products, corresponding to *gag* to *pol* region and *bel-1* to *gag* region of strain Ibaraki and strain No.43 were cloned using TOPO XL PCR Cloning Kit (Thermo Fisher Scientific) or Zero Blunt TOPO PCR cloning Kit (Thermo Fisher Scientific). Primer sets for PCR and sequence analysis were shown in Supplementary Table 1. Sequences were analyzed with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific). The obtained sequences were registered in GenBank with the following accession numbers noted in parenthesis: Ibaraki (LC510606), No.43 (LC510607), K7 (LC510608), BH47 (LC510609).

Phylogenetic analysis

Multiple alignment and phylogenetic analysis were performed using Clustal W in MEGA7 software [21]. Phylogenetic trees based on the nucleotide sequences of each gene or genome were generated by the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (for *gag*, *pol*, *env* and whole genome) and Kimura 2-parameter model (for *bel-1* and *bel-2*) with bootstrap replicates of 1,000 [11, 20]. The analysis was performed including the sequences registered in GenBank, including named BFVbta_BSV11 (USA, U94514), BFVbta_100 (Poland, JX307861), BFVbta_Riems (Germany, JX307862) and BFV3026 (China, AY134750).
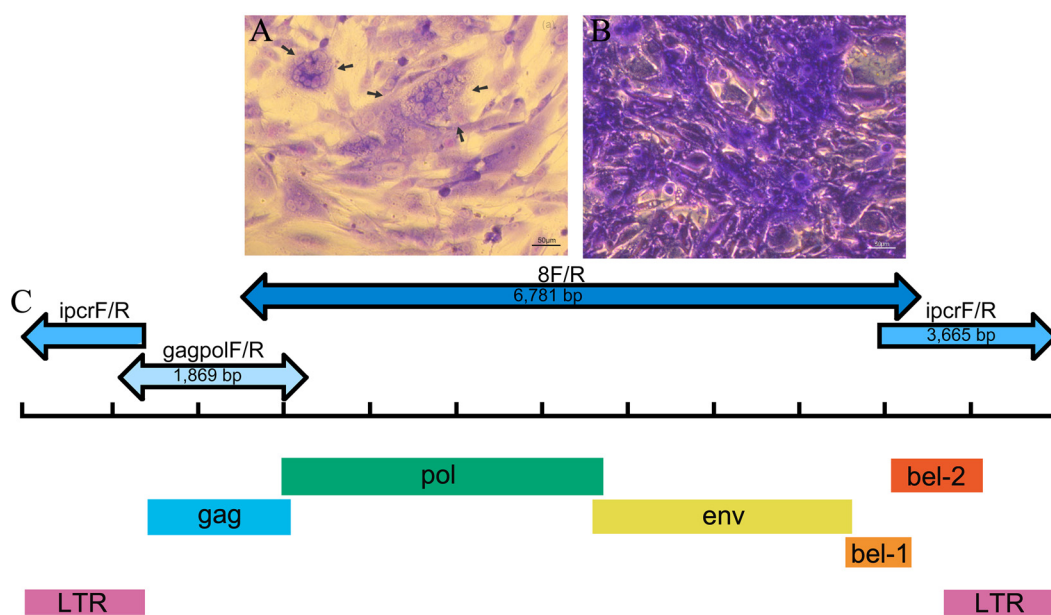

Fig. 1. Bovine foamy virus (BFV) isolation and primer design for full-length sequence analysis. (A) BHK21 cells cocultured with peripheral blood mononuclear cells (PBMC) from BFV positive cow (No. 5266). Blind passage was performed nine times and then stained with Gimza. Characteristic multinucleated giant cells were formed (arrows). (B) Control BHK21 cells. Control BHK21 cells were cocultured with PBMC from BFV-negative cow. Cells were stained with Gimza at nine passages. (C) BFV genomic structure and primer target. BFV has the three structural genes and two regulatory genes at the 3′ end of the provirus. The positions of each primer target and expected PCR amplicon size referred to the BFV reference sequence (GenBank accession number: U94514) are shown.

Quantitation of bovine leukemia virus

Quantitation of BLV by real time PCR was performed according to the methods as described previously [27]. The standard plasmid samples for quantitation containing both BLV *pol* and beta-actin (ACTB) at an accurately equivalent molecular ratio were shown in the previous study [27]. The standard plasmids were diluted by 10-fold serial dilution using EASY Dilution (Takara Bio, Kusatsu, Japan). To quantitate BLV proviral load, Taqman probe assay using Applied Biosystems Step One Plus (Thermo Fisher Scientific) and Thunderbird Probe qPCR Mix (TOYOBO) was carried out.

Statistics in cows tested for BFV PCR and BLV qPCR

A total of 153 samples identified in Japan were applied in the phylogenetic tree. Pearson's χ^2 test was done to investigate association between BFV infection and BLV infection. Other statistics to find out the relationship between BFV infection and BLV *pol* copy number or age, or between BLV infection and age were performed using Mann–Whitney *U* test.

RESULTS

Prevalence of BFV

The prevalence of BFV infection in the tested cattle are shown in Table 1. The prevalence ranged from 0% to 15.4%. In total, 23 out of 181 (12.7%) cattle were BFV positive.

Isolation of BFV

BHK21 cells were cocultured with BFV-infected PBMC and passed until passage 12. At passage nine, BHK21 cells cocultured with PBMC from three AGID-positive cattle samples (No.5266, 5270, 7800) represented characteristic of multinucleated giant cells. CPE was not observed in the control wells co-cultured with PBMC derived from BFV-negative cow (Fig. 1).

Comparison and phylogenetic analysis

The PCR results were confirmed by electrophoresis showing the expected amplicon size noted in Fig. 1. The full-length sequence homology of each isolate was determined using the National Center for Biotechnology Information (NCBI) BLASTN from the NCBI BLAST software distribution and MEGA7 software. The isolates derived from the cattle in Ibaraki (No.5266, 5270, and 7800) were the most identical to the reference sequence of BFV (GenBank accession number: U94514) showing 99.6% sequence identity (11,949/12,001 nt). The sequence identity of strain No.43 (LC510607) was 99.5% (11,945/12,001 nt) when compared with the reference sequence. The three nucleotide substitutions among the isolates derived from the cattle in Ibaraki (No.5266, 5270, and 7800) were observed, located in LTR (1,099 nt in 5′ LTR corresponding to 11,792 nt in 3′ LTR) or *gag* (1,699 nt) region referred to the BFV reference sequence. These substitutions did not affect amino acid sequences. The phylogenetic tree showed two clusters, including the USA and Chinese cluster and European cluster (Fig. 2). The isolates identified in the present study were the closest to the BFV reference strain of the viral structural genes, *gag*, *pol*, and *env*, while the accessory genes were closely related to strain BFV3026.

Phylogenetic analysis of env region

The Japanese strains were named "strain Ibaraki" derived from cattle No.5266, "strain K7" detected in Kagoshima, and "strain BH47" detected in Hokkaido.

The phylogenetic analysis using whole *env* sequence showed that strains Ibaraki, No.43, K7, Reference U94514, and BFV3026 were grouped in the same branch, while strain BH47 clustered with European isolates to form a separate lineage (Fig. 3). Three Japanese strains excluding BH47 were the most similar to each other based on the whole *env* sequence.

Relationship between BFV and BLV

Fifty-one cattle were found to be positive for BLV among the tested 153 cattle. The range of BLV copy number was from 2 to 1,188 copies per 100 cells. The infection status of BFV and BLV is shown in Table 2. No correlation was found between the two viral infections by Pearson's χ^2 test (*P*>0.05, data not shown). There was no difference of BLV copy number regardless of BFV positive or not (*P*=0.597 >0.05). Regarding positivity of BFV and BLV, there was a significant difference of age, and uninfected cattle were younger than the infected ones (Fig. 4).

DISCUSSION

BFV prevalence

BFV was distributed throughout Japan; the average positive rate was 12.7%. In previous study, BFV seroprevalence was 8.8% in Kanagawa prefecture [8] and 16.7% in the Kanto region [13], respectively. The rate of BFV infection differed between farms [13]. Our results were compatible with previous studies in Japan. The prevalence of BFV in Japan is much lower than that of Poland (41.5%) [31] and Canada (39.6%) [14]. It may be due to the difference of rearing style of the cattle. High prevalence in BFV infection in Poland is considered to be caused by a close contact between calves and dams, in other words, maternal-fetal transmission via milk [31]. The farm in Ibaraki gave calves colostrum which had been frozen beforehand. BFV infection is tightly cell-associated [4] as well as bovine leukemia virus (BLV). Therefore, as demonstrated in case of BLV [17], freeze-thaw process
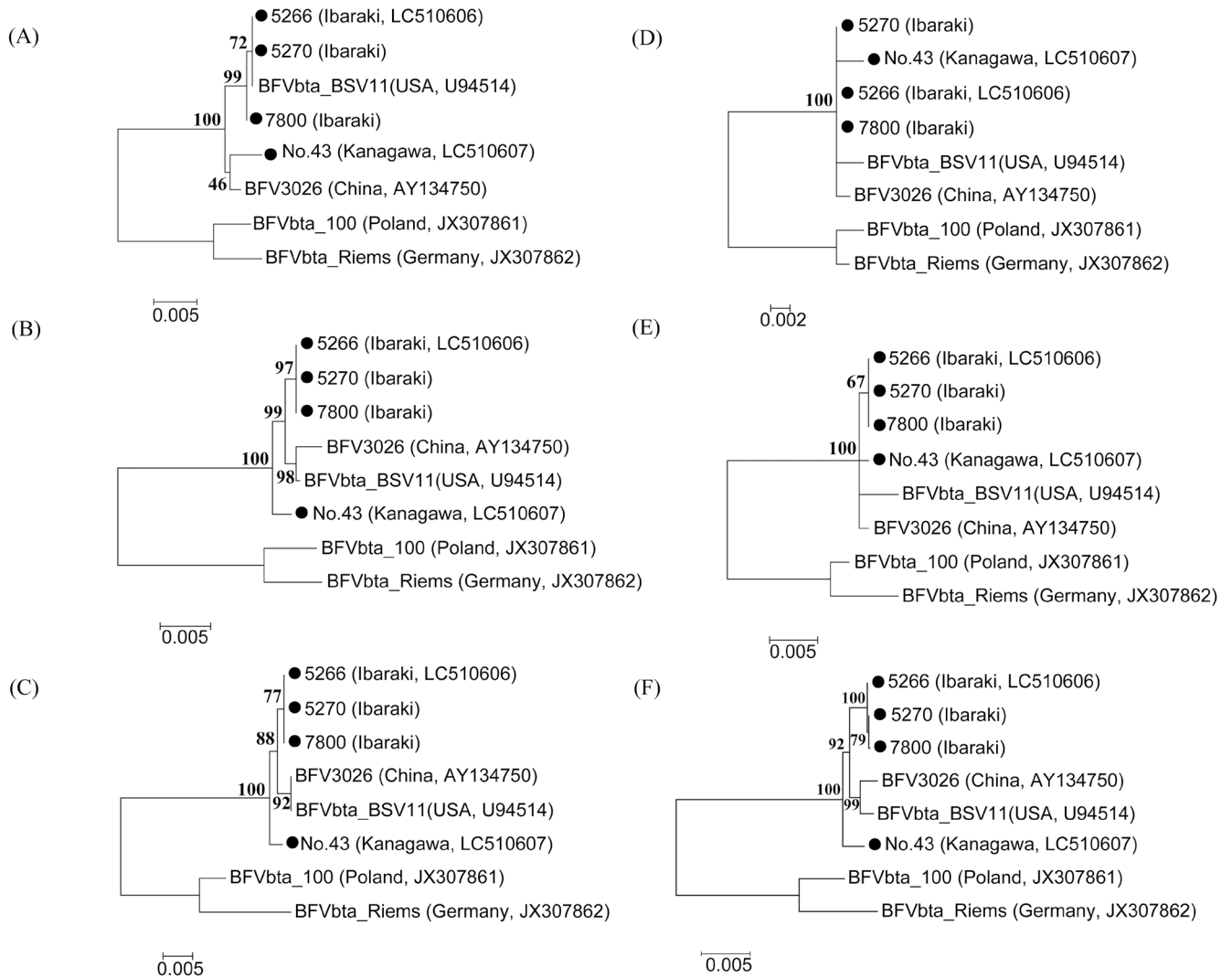
Fig. 2. Phylogenetic analysis for the isolates based on each region. The isolates identified in the present study are noted by closed circles. Phylogenetic tree based on *gag* (A), *pol* (B), *env* (C), *bel-1* (D), *bel-2* (E) and whole genome (F).
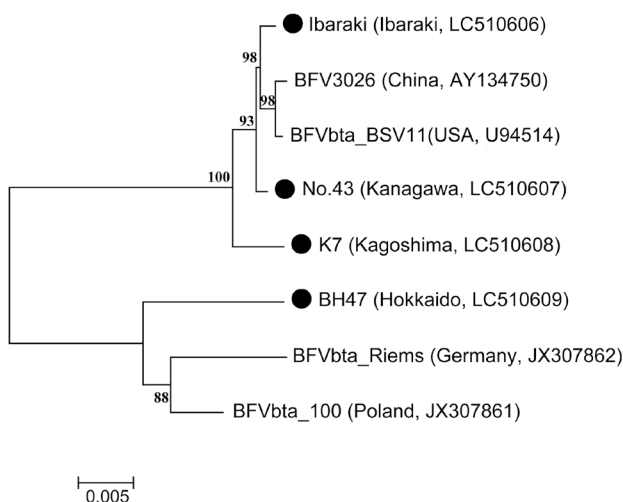

Fig. 3. Phylogenetic analysis of *env* region among bovine foamy virus (BFV) strains including the Japanese strains. Phylogenetic tree was generated using whole *env* sequence. The isolates identified in the present study are noted by closed circles.

Table 2. Summary of bovine foamy virus (BFV) and bovine leukemia virus (BLV) PCR results

		BLV		Total
		+	−	
BFV	+	6	16	22
	−	45	86	131
Total		51	102	153

One hundred fifty three samples were used for BFV prevalence test. PCR and real time PCR were used to detect BFV and BLV respectively.
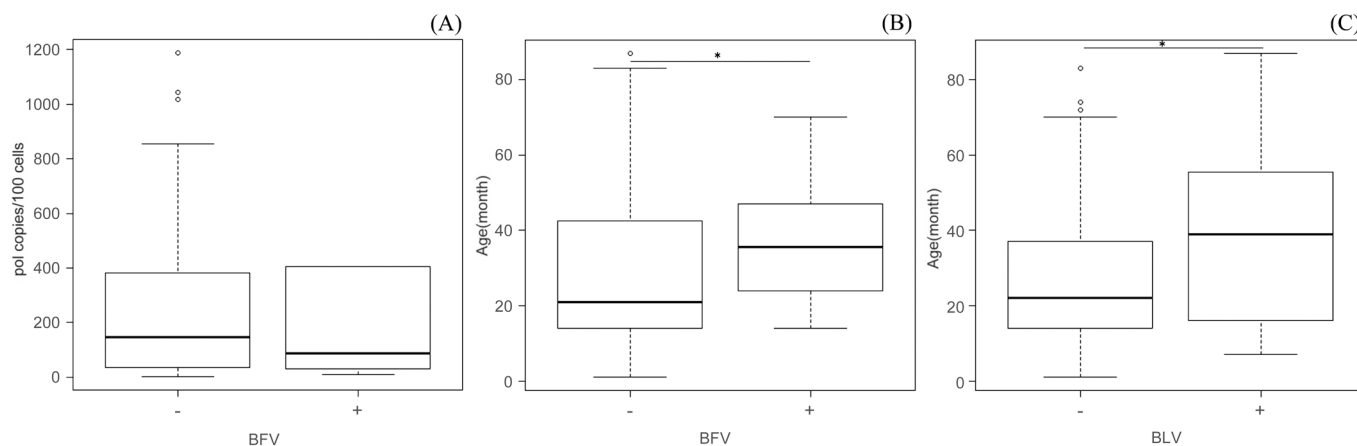
Fig. 4. Relationship among bovine foamy virus (BFV), bovine leukemia virus (BLV) and age. (A) BLV *pol* copy number of BFV infected or uninfected cattle. The y-axis shows the BLV copy number targeting pol in 100 cells by qPCR method. There was not significant difference between BLV copy number and BFV infection (*P*=0.597 >0.05). (B) Age of BFV infected or uninfected cattle. (C) Age of BLV infected or uninfected cattle. The y-axis shows the age of cattle in month. Significant difference of age on BLV positivity (*P*=0.011 <0.05) as well as on BFV positivity (*P*=0.0332 <0.05).

may reduce the risk of BFV transmission through milk. In addition, separation of a newborn calf from its mother immediately after birth may also reduce a risk.

Phylogenetic analysis

Phylogenetic analysis based on partial and whole sequence indicated that there were clearly two BFV clusters. The isolates identified in this study were the closest to the reference data of BFV, indicating that the isolates from Ibaraki (No.5266, 5270, and 7800) were closer to the USA and Chinese isolates than that of another Japanese strain No.43 when compared with *pol*, *env* and whole sequences. According to the recent review on the taxonomy and nomenclature, an alignment comprising the *pol* and *env* gene sequences can be utilized for FV classification [19]. Our results also showed that the phylogenetic tree of whole genome, *pol* and *env* were similar to each other. Therefore, the analysis based on BFV *pol* and *env* nucleotide sequences may reflect the BFV full length relationship. In our study, the phylogenetic tree using *env* gene sequence showed strains Kagoshima and No.43 were sorted in the same cluster including the USA and Chinese strains, while Hokkaido strain was in the other cluster including European strains. These results may suggest that there are several routs of the introduction of BFV into Japan. Further studies are needed to evaluate how BFV was introduced and spread in Japan.

BFV isolates identified in Ibaraki farm showed three nucleotide mutations in *gag* and LTR region, but amino acid sequence was identical. This result indicated that BFV sequence in the farm level is likely to be conserved. In the present study, the nucleotide similarity of *gag* region (96.0%) was higher than that of the *env* region (94.6%). This result suggests that *env* region of BFV is more variable than its *gag* region, which is similar to other retroviruses. Regarding amino acid level sequence identity of each protein, Gag was the most conserved (98.2%) among the viral proteins while Tas (94.8%) was the least. Gag was reported to be the most conserved protein in retroviruses [25, 31]. Although all FVs produce Tas-Bel2 fusion proteins [22], Tas was found to be less conserved in this study. Further studies are needed to find out whether these substitutions affect viral replication, infectivity and pathogenicity.

Relationship among BFV, BLV and age

In this study, the number of BFV-infected cows was less than BLV-infected cows, in accordance with the previous report [13]. No correlation was found between the BFV and BLV infections, suggesting that BFV and BLV infection routes were independent. In addition, no correlation was found between BFV infection and BLV copy number (*P*=0.597 >0.05). Significant difference was found between the age of cattle and BFV or BLV positivity. BFV or BLV positive cattle were older than negative animals, suggesting horizontal transmission of both viruses. Vertical transmission from mothers to calves was reported to be the main cause of BFV transmission [3, 14, 31], and BFV infection was considered to occur in young cattle [3] or age-independent [31]. However, our results show that BFV infection is more common in older cows. This may suggest that horizontal transmission is important as a BFV transmission route in Japan.

Taken together, BFVs are supposed to be widely distributed in Japan from north to south with each region harboring various genomic characteristics. Although no clear correlation between BFV and BLV infection was shown, the present study showed that the infection rate increased with age. This is the first study to clarify the full-length information of BFV in Japan. Our study contributes to the accumulation of BFV genome information and the characteristics of BFV in Japan.

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