Viral RNA extraction using an automatic nucleic acid extractor with magnetic particles and genetic characterization of bovine viral diarrhea virus in Tokachi Province, Japan, in 2016–2017

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ABSTRACT. In this study, the viral genome extraction performance of automatic nucleic acid extractors and manual nucleic acid extraction kits was compared. We showed that compared with manual kits, the automatic extractors showed superior genome extraction performance using bovine viral diarrhea virus (BVDV) genome-positive cattle sera and bovine coronavirus/infectious bovine rhinotracheitis virus-spiked cattle nasal swabs. In addition, the subgenotyping of BVDV strains detected in Tokachi Province in Japan during 2016–2017 was performed. Results showed that most of these BVDV strains belonged to subgenotype 1b, while few strains belonged to subgenotypes 1a and 2a. This study showed the high applicability of automatic nucleic acid extractors in extracting multiple viral genomes and the dominant subgenotype of BVDV in Tokachi.

KEYWORDS: automatic nucleic acid extractor, bovine coronavirus, bovine viral diarrhea virus, genetic characterization, infectious bovine rhinotracheitis virus

Bovine viral diarrhea virus (BVDV) belongs to the genus Pestivirus of the Flaviviridae family, together with classical swine fever virus and border disease virus. The viral genome of BVDV comprises a positive-sense, ~12.3-kb-long single-stranded RNA containing one open reading frame and 5′ and 3′ untranslated regions (UTRs). The open reading frame encodes four structural (Core, E1, E2, and 5′) and eight nonstructural (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [22]. The 5′ UTR of the BVDV strains is highly conserved at the nucleotide level [22]; therefore, this region was used for the diagnosis and genetic characterization of BVDV strains.

Reverse transcription-polymerase chain reaction (RT-PCR) is a useful approach for detecting the viral genome in blood serum and identifying infection in cattle. However, extracting RNA from a large number of samples from herds for RT-PCR is laborious and time consuming, which is disadvantageous. One solution proposed to address this is the use of an automatic nucleic acid extractor. In this study, the viral genome extraction performance of an automatic extractor using magnetic particles was compared with that of commercially available manual RNA extraction kits using silica membrane for viral RNA extraction.

Tajima [20] investigated the prevalence of BVDV in Japan from 2000 to 2019. The author noted that the number of BVD cases increased from 161 in 2000 to 4,113 in 2019. Both BVDV 1 and 2 have recently been detected in Japan using phylogenetic analyses [1]. The genotype/subgenotype mismatch between the field and vaccine strains reduces the vaccination efficacy [5, 16]. Hence, understanding the genotype/subgenotype distribution pattern in each area and selecting the most suitable vaccine strain is crucial to
prevent the spread of BVDV infection. To understand the current BVDV genetic distribution in the Tokachi Province of Hokkaido Prefecture, Japan, this study conducted the genetic characterization of BVDV genome-positive cattle serum samples collected in this province in 2016–2017. A total of 57 BVDV genome-positive bovine serum samples were kindly provided by Hokkaido Tokachi Livestock Hygiene Service Center (Obihiro, Japan). Most of these samples were derived from cattle with persistent infection (PI), which was determined by performing RT-PCR twice at an interval of >3 weeks; however, samples for which RT-PCR had been performed just once could not be confirmed as originating from cattle with PI. All serum samples were collected from animals in the Tokachi Province from March 2016 to April 2017. A list of the samples used is shown (Table 1).

Overall, 24 BVDV genome-positive serum samples were subjected to total RNA extraction using an automatic nucleic acid extractor magLEAD 12gC (Precision System Science Co., Ltd., Matsudo, Japan) with two types of specific reagents: MagDEA Dx SV, which extracts both DNA and RNA, and MagDEA Dx SV RNA, which can extract only RNA from the samples. The same samples were subjected to total RNA extraction using a manual RNA extraction kit (QIAamp Viral RNA Kit; QIAGen, Venlo, Netherlands) following the manufacturer’s instructions. Herein, extraction was performed using the magLEAD 12gC with MagDEA Dx SV (LEAD DNA+RNA), magLEAD 12gC with MagDEA Dx SV RNA (LEAD RNA), and QIAamp Viral RNA Kit (QIA) under the same conditions (volumes of sample and elution). cDNA was reverse transcribed using FastGene cDNA Synthesis 5× ReadyMix OdT (NIPPON Genetics Co., Ltd., Tokyo, Japan). Real-time PCR for BVDV genome quantification was conducted using primers (BVD190-F and V326) and a probe TQ-Pest, as previously described [10] with slight modifications (Supplementary Table 1). T in TQ-Pest was changed to Y, which is highlighted in bold in Supplementary Table 1. Real-time PCR was performed under the following conditions: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 45 cycles at 95°C for 15 sec, and 60°C for 1 min. To prepare a control plasmid for BVDV genome quantification, the PCR product (380 bp) was amplified using a pair of primers, DH-BVDV-1F and DH-BVDV-1R (Supplementary Table 1) and inserted into a vector plasmid (pGEM-T Easy Vector System; Promega Co., Madison, WI, USA). The constructed plasmid was recovered from transformed competent Escherichia coli cells, serially diluted, and subjected to real-time PCR to obtain the relative standard curve. Known amounts of the plasmid DNA containing 2 × 10^5 copies/reaction were used as templates. The obtained standard curve demonstrated high accuracy (R^2 >0.99) and indicated that the assay can detect ≥20 copies of the BVDV genome/reaction with the Ct value of 35.95 (Supplementary Fig. 1). The number of BVDV genome copies/reaction in the nucleic acid extract obtained from the field samples using each extraction method (LEAD DNA+RNA, LEAD RNA, or QIA) was calculated using the standard curve. Based on these numbers, the viral RNA extraction performances were compared among the three different extraction methods. Friedman test with Dunn’s multiple comparison test was used to identify the significant differences in the copy numbers/reaction obtained through real-time RT-PCR for the three extraction methods. A P value <0.05 was considered statistically significant. Our results show that the number of copies/reaction was significantly higher in the LEAD DNA+RNA group than in the QIA group and in the LEAD RNA group than in the QIA group. No difference was observed between the LEAD DNA+RNA and the LEAD RNA groups (Fig. 1). Further, Ct values of BVDV genome-negative and genome-positive serum samples were compared. Nucleic acids were extracted from 10-fold serially diluted BVDV genome-negative field serum (previously obtained) and Tokachi-48-derived serum (a representative of BVDV genome-positive serum) using the three different nucleic acid extraction methods. Consequently, the Ct values of 1–5 log_{10}-times diluted BVDV genome-negative serum were >34.0 using all three methods. Alternatively, those of 1 log_{10}-times diluted BVDV genome-positive serum were <30.0, which increased in proportion to the serum dilution ratio in all methods. In 5 log_{10}-times diluted serum, the Ct values were >34.0, which were comparable to those of BVDV genome-negative serum (Supplementary Fig. 2). This result suggests that >34.0 Ct value represents the absence of BVDV genome (BVDV genome negative) using real-time RT-PCR system.

The viral genome extraction performance of LEAD was also evaluated for other viruses. The nasal swab samples obtained from four bovine coronavirus (BCoV)/infectious bovine rhinotracheitis virus (IBRV)-negative cows were spiked with the 2.25 log_{10} 50% tissue culture infective dose (TCID_{50})/mL of BCoV Mebus strain and 3.25 log_{10} TCID_{50}/mL of IBRV Los Angeles strain. Nonvirus-spiked nasal swabs were used as negative controls. The nasal swabs were suspended in a virus transport medium, which comprised Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 0.5% bovine serum albumin (FUJIFILM Wako Pure Chemical Co., Ltd., Osaka, Japan), 25 mM HEPES (FUJIFILM Wako Pure Chemical Co., Ltd., Tokyo, Japan), 1 mg/mL kanamycin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), 100 µg/mL gentamicin (MSD K.K., Tokyo, Japan), and 5 µg/mL amphotericin B (Bristol-Myers Squibb Co., New York, NY, USA). DNA+RNA extracts were obtained from the nasal swab samples using geneLEAD VIII (Precision System Science Co., Ltd.) with MagDEA Dx SV (LEAD DNA+RNA). The mechanism of nucleic acid extraction of geneLEAD VIII was the same as that of magLEAD 12gC. RNA or DNA extracts were also obtained from the nasal swab samples using QIA or High Pure PCR template preparation kit (HP-PCR kit; Roche, Basel, Switzerland), a manual DNA extraction kit, respectively. For real-time RT-PCR targeting BCoV, cDNA was synthesized from the DNA+RNA and RNA extracts and real-time PCR was performed using BCoV Mebus strain-specific primers and probe (Supplementary Table 2) under
### Table 1. Blood serum samples subjected to viral genome extraction and characterization

| Sample No. | Variety 1) | Sex 2) | Collection date | Age (months) | PI cattle or not | Conducted analysis 3) |
|------------|------------|--------|-----------------|--------------|------------------|-----------------------|
| Tokachi-1  | Hol        | F      | 2016/8/18       | 5            | PI               | R, G                  |
| Tokachi-2  | Hol        | F      | 2016/12/28      | 1            | PI               | R, G                  |
| Tokachi-3  | Hyb        | M      | 2016/11/15      | 2            | PI               | G                     |
| Tokachi-4  | Hol        | F      | 2016/11/15      | 2            | PI               | R, G                  |
| Tokachi-5  | Hol        | F      | 2016/12/7       | 3            | PI               | R, G                  |
| Tokachi-6  | Hol        | F      | 2017/1/27       | 5            | PI               | G                     |
| Tokachi-7  | Hol        | F      | 2017/2/2        | 6            | PI               | R, G                  |
| Tokachi-8  | Hol        | M      | 2016/8/2        | 1            | PI               | R, G                  |
| Tokachi-9  | Hyb        | M      | 2016/12/13      | 1            | PI               | G                     |
| Tokachi-10 | Hol        | F      | 2016/12/26      | 5            | PI               | G                     |
| Tokachi-11 | Hyb        | F      | 2016/12/21      | 1            | PI               | G                     |
| Tokachi-12 | Hol        | F      | 2016/11/25      | 1            | PI               | G                     |
| Tokachi-13 | Hol        | F      | 2016/12/6       | 9            | PI               | G                     |
| Tokachi-14 | Hol        | F      | 2016/10/5       | 12           | PI               | R, G                  |
| Tokachi-15 | JBl        | F      | 2016/10/5       | 2            | PI               | G                     |
| Tokachi-16 | Hyb        | F      | 2016/11/4       | 1            | PI               | R, G                  |
| Tokachi-17 | Hyb        | F      | 2016/11/8       | 2            | PI               | R, G                  |
| Tokachi-18 | Hol        | F      | 2016/12/12      | 1            | PI               | G                     |
| Tokachi-19 | Hol        | F      | 2016/6/24       | 10           | PI               | R, G                  |
| Tokachi-20 | Hyb        | F      | 2016/7/4        | 1            | PI               | G                     |
| Tokachi-21 | Hol        | F      | 2016/9/20       | 5            | PI               | G                     |
| Tokachi-22 | Hyb        | M      | 2016/9/27       | 1            | PI               | R, G                  |
| Tokachi-23 | Hol        | F      | 2016/8/8        | 2            | PI               | G                     |
| Tokachi-24 | Hol        | F      | 2016/9/27       | 5            | PI               | G                     |
| Tokachi-25 | Hyb        | F      | 2016/8/30       | 1            | Unknown          | G                     |
| Tokachi-26 | Hyb        | F      | 2016/9/26       | 1            | Unknown          | G                     |
| Tokachi-27 | Hyb        | F      | 2016/7/25       | 2            | PI               | G                     |
| Tokachi-28 | Hol        | F      | 2016/8/8        | 1            | PI               | G                     |
| Tokachi-29 | Hol        | F      | 2016/5/19       | 9            | PI               | G                     |
| Tokachi-30 | Hyb        | M      | 2016/5/26       | 2            | PI               | G                     |
| Tokachi-31 | Hol        | F      | 2016/5/24       | 4            | PI               | G                     |
| Tokachi-32 | Hol        | F      | 2016/5/23       | 9            | PI               | R, G                  |
| Tokachi-33 | Hol        | F      | 2016/7/12       | 2            | PI               | G                     |
| Tokachi-34 | Hol        | F      | 2016/5/24       | 3            | PI               | G                     |
| Tokachi-35 | Hol        | F      | 2016/5/31       | 2            | PI               | R, G                  |
| Tokachi-36 | Hol        | F      | 2016/9/21       | 3            | PI               | G                     |
| Tokachi-37 | Hol        | F      | 2016/5/11       | 9            | PI               | G                     |
| Tokachi-38 | Hol        | M      | 2016/9/8        | 1            | PI               | G                     |
| Tokachi-39 | Hyb        | M      | 2016/9/6        | 2            | PI               | G                     |
| Tokachi-40 | Hol        | F      | 2016/10/31      | 2            | Unknown          | G                     |
| Tokachi-41 | Hol        | F      | 2016/12/8       | 9            | PI               | G                     |
| Tokachi-42 | Hol        | F      | 2016/12/7       | 11           | PI               | G                     |
| Tokachi-43 | Hol        | F      | 2017/1/26       | 12           | PI               | R, G                  |
| Tokachi-44 | Hol        | F      | 2017/2/2        | 12           | PI               | G                     |
| Tokachi-45 | Hol        | F      | 2017/2/2        | 11           | PI               | G                     |
| Tokachi-46 | Hol        | F      | 2017/2/1        | 7            | PI               | G                     |
| Tokachi-47 | Hol        | F      | 2017/3/24       | 8            | PI               | R, G                  |
| Tokachi-48 | Hyb        | F      | 2017/3/6        | 5            | Unknown          | R, G                  |
| Tokachi-49 | Hol        | F      | 2017/2/23       | 8            | PI               | R, G                  |
| Tokachi-50 | Hol        | F      | 2017/2/7        | 1            | PI               | G                     |
| Tokachi-51 | Hyb        | M      | 2016/4/15       | 1            | PI               | R, G                  |
| Tokachi-52 | Hyb        | M      | 2016/12/1       | 2            | PI               | R                     |
| Tokachi-53 | Hol        | F      | 2017/1/26       | 8            | PI               | R                     |
| Tokachi-54 | Hol        | F      | 2016/5/11       | 12           | PI               | R                     |
| Tokachi-55 | Hol        | F      | 2017/2/3        | 6            | PI               | R                     |
| Tokachi-56 | Hol        | F      | 2016/12/7       | 4            | PI               | R                     |
| Tokachi-57 | Hyb        | M      | 2016/12/29      | 1            | PI               | R                     |

1) Hol: Holstein; Hyb: Hybrid; JBl: Japanese Black, 2) F: female; M: male, 3) R: real-time RT-PCR; G: genetic analysis; PI: persistent infection.
the following conditions: 1 cycle at 95°C for 10 min, 45 cycles at 95°C for 15 sec, and 60°C for 1 min. Student’s t-test was used to identify the significant differences in Ct values for the LEAD DNA+RNA and QIA groups. Conventional PCR for IBRV was performed using DNA+RNA and DNA extracts as well as IBRV-specific primers (Supplementary Table 3) [11] under the following conditions: 1 cycle at 96°C for 2 min, 35 or 40 cycles at 96°C for 20 sec, 55°C for 30 sec, and 72°C for 2 min; and 1 cycle at 72°C for 3 min. The results of real-time RT-PCR targeting BCoV showed that the Ct values of virus-spiked nasal swab samples were significantly lower in the LEAD DNA+RNA group than in the QIA group. Conversely, the value was not obtained in the nonvirus-spiked samples in both LEAD DNA+RNA and QIA groups (Fig. 2A). Conventional PCR targeting IBRV revealed that the intensity of IBRV-specific bands in the virus-spiked nasal swab samples was stronger in the LEAD DNA+RNA group than in the HP-PCR kit group, and no bands were detected in the nonvirus-spiked samples in both LEAD DNA+RNA and HP-PCR kit groups (Fig. 2B).

Total RNA was extracted from 51 BVDV genome-positive serum samples using LEAD DNA+RNA. The 5′ UTR region (288 bp) of the BVDV genome was amplified through PCR using a pair of primers (324F and 326R) [24]. These 51 samples were selected based on the different sampling times and wide geographical distribution. Further, 288-bp-long PCR products were separated on 1.5% agarose gels and purified by GeneClean II Kit (MP Biomedicals LLC, Santa Ana, CA, USA). Nucleotide sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific Inc.). Seven representative BVDV strains (Bovine/Japan/Hokkaido/Tokachi-8, 13, 15, 22, 27, 31, and 47) obtained in this study were selected for BVDV genome sequencing using next-generation sequencing (NGS). Briefly, a NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) was used to construct cDNA libraries from RNA. For the Tokachi-31 strain, rRNA was removed using a NEBNext rRNA Depletion Kit (New England Biolabs) before constructing a cDNA library. A pool of cDNA libraries was applied into a reagent cartridge using a MiSeq Reagent Kit v3 (150 cycles) (Illumina, San Diego, CA, USA) after the quantification of libraries on a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). A MiSeq bench-top sequencer (Illumina) was used to perform deep sequencing. A MiSeq Reporter program was used to construct FASTQ formatted sequence data. Trimmed sequences were generated from 76 nucleotide length of raw paired-end reads to trim low-quality sequence reads using the Trim Sequences command with default parameters in NGS Core tools in CLC Genomics Workbench. Further, contigs were generated from the trimmed sequence reads with default parameter settings using the de novo assembly command with Map reads back to contigs option. In some strains, the trimmed sequences were mapped to reference data comprising known representative BVDV genome sequences, and sequencing gaps between contigs were subsequently filled by sequencing the amplicons obtained through RT-PCR with specific primers.

The alignment and analyses of nucleotide sequences with ~235 bp at 5′ UTR (position: 131st–365th at genome of NADL strain [AJ133738.1], used as reference strain) were conducted using Clustal W multiple alignment tool [8, 23] in BioEdit v.7.2.5 for the 51
Japanese strains detected in this study and the BVDV strains available in GenBank. Furthermore, the genome sequence (~12,100 bp) and full-length E2 gene sequence (1,122 bp) of the seven representative Japanese BVDV strains were analyzed.

Nucleotide homology was compared among these strains using the BLASTn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). A maximum likelihood method using the Hasegawa–Kishino–Yano model of nucleotide substitutions was used to construct the phylogenetic tree. The confidence values of the phylogenetic trees were assessed by bootstrapping with 1,000 replicates using MEGA6 software [21]. The sequences of 5′UTR region obtained in this study have been deposited with GenBank under accession numbers OM238223 to OM238273, and the virus genome sequences of seven representative strains have been deposited with DNA Data Bank of Japan under accession numbers LC727644 to LC727650.

Based on the sequence of 5′UTR, genetic characterization revealed that the 47 strains obtained in this study belonged to subgenotype 1b, two strains (Bovine/Japan/Hokkaido/Tokachi-22 and Bovine/Japan/Hokkaido/Tokachi-27) belonged to subgenotype 1a, and two strains (Bovine/Japan/Hokkaido/Tokachi-31 and Bovine/Japan/Hokkaido/Tokachi-47) belonged to subgenotype 2a. Phylogenetic analysis indicated that these 47 subgenotype 1b strains could be divided into several clusters in the phylogenetic tree (Fig. 3). A phylogenetic tree based on the genome sequences revealed that five strains (Bovine/Japan/Hokkaido/Tokachi-8, 13, 15, 22, and 27) belonged to genotype 1, whereas two strains (Bovine/Japan/Hokkaido/Tokachi-31, and 47) belonged to genotype 2 (Fig. 4A). Phylogenetic analysis based on full-length E2 gene sequences indicated that six of the seven BVDV strains belonged to the following subgenotypes: 1b (Bovine/Japan/Hokkaido/Tokachi-8, 13, and 15), 1a (Bovine/Japan/Hokkaido/Tokachi-22), and 2a (Bovine/Japan/Hokkaido/Tokachi-31 and 47) (Fig. 4B). These subgenotypes were similar to those based on 5′UTR region sequences. The remaining one strain (Bovine/Japan/Hokkaido/Tokachi-27) was indicated to differ from the genetic analyses between 5′UTR (subgenotype 1a) and full-length E2 gene (subgenotype 1b) sequences.

LEAD has been used to extract the RNA of severe acute respiratory syndrome coronavirus 2 [2, 3]. In general, manual nucleic acid extraction is time intensive and requires human intervention for every step, whereas automated DNA/RNA extraction offers simplicity and convenience. In the present study, we found that compared with the manual extraction kit (at least 35 min for one round), the LEAD system was not only quick (26 min at the shortest for one round depending on LEAD series variations) and easy to use but also generated high copy numbers of the BVDV genome obtained from serum-derived nucleic acid extracts. In addition, the LEAD system’s superior performance for extracting BCoV and IBRV genomes from nasal swab samples was shown. The LEAD system could assess more than eight samples (depending on BVDV series variations) easily at once. As nucleic acid extraction using LEAD is completed in a shorter time and fewer steps than the manual system, the chances for samples to be exposed to RNase and DNase in the environment may be low, resulting in a higher quality of nucleic acid extraction. This study indicated the high applicability of the LEAD system for detecting the genomes of BVDV and other viruses in the field.

In this study, most BVDV-positive cows were confirmed to be PI. Cattle with PI tend to have a higher viral RNA copy number in serum than acute infected animals [4]. Goto et al. [7] performed RNA extraction using QIA from acutely infected cows, and the viral RNA copy numbers in the sera were evaluated using real-time RT-PCR. Notably, 72,000, 69,000, 2,200, 290, 280, 3, and 1 copies/μL of serum were obtained. In our study, the lowest copy number among the tested BVDV-positive samples was 100 copies/μL of serum, which was obtained using QIA from the serum of Tokachi-53. This result suggests that our real-time RT-PCR system after RNA extraction using QIA can possibly detect viral genome in the sera of many acutely infected cows, except for sera with extremely low viral genome copy numbers. Moreover, the copy numbers were 5,012 and 1,995 copies/μL of serum when RNAs were extracted using LEAD RNA and LEAD DNA, respectively, from the serum of Tokachi-53. This result suggests that the efficiency of viral genome detection in the serum of PI animals and acutely infected animals can be further improved using the LEAD system.

BVDV was first identified in Japan in the late 1960s, and infection has been subsequently reported by various researchers [1, 9, 12–14, 18, 20]. Genetic detection and characterization of BVDV may provide valuable information on the diversity of viral strains for disease control and eradication. Abe et al. [11] reported that cattle in Hokkaido in 2006–2014 were infected with BVDV subgenotypes 1a, 1b, 1c, and 2a. Among those strains, the predominant subgenotype was 1b [1]. In our study, subgenotype 1b was also identified to be predominant in cattle in Tokachi Province in 2016–2017. Hirose et al. [9] recently reported that the subgenotypes of BVDV detected in Tokachi Province in 2018–2020 were 1a, 1b, and 2a, with 1b being the predominant subgenotype. This subgenotyping was performed based on the nucleotide sequence of the entire E2 gene. Nishimori et al. [15] reported that BVDV 1b and 2a were predominant and subdominant strains, respectively, circulating in some prefectures, including Hokkaido in 2014–2020. This subgenotyping was performed based on the nucleotide sequences of both the 5′UTR and E gene. The distribution pattern reported by Hirose et al. [9] and Nishimori et al. [15] are in accordance with that in our study and further confirms that subgenotype 1b is the most important BVDV subgenotype endemic in Tokachi Province. Commercial inactivated and live-attenuated vaccines based on BVDV subgenotypes 1a and 2a strains and commercial inactivated vaccines containing BVDV 1b and 2a are available in Japan [20]. Cattle vaccinated for BVDV 1a produced low antibody titers against the heterologous BVDV 1b virus [6, 17]. Subgenotyping of 51 samples was performed based on the 5′UTR sequence region in this study, but the E2 protein was the major target of neutralizing antibodies. However, Nishimori et al. [15] showed that the result of subgenotyping based on 5′UTR region was consistent with that based on the E2 gene region. In contrast, another study reported the mismatch in the results of subgenotyping of 5′UTR- and E2 gene-based analysis [19]. This result was similar to that of the present study, where genetic analysis based on 5′UTR and full-length E2 gene sequences provided different results in Bovine/Japan/Hokkaido/Tokachi-27 strains. These findings show that although the results of subgenotyping based on 5′UTR and full-length E2 gene sequences were consistent in most BVDV strains, these were not matched in some strains. Therefore, genetic analysis based on 5′UTR and other regions, such as full-length E2 gene and full genome sequences, are necessary to obtain more accurate genetic information of BVDV. Furthermore, antigenic analysis is necessary to evaluate vaccine efficacy. Abe et al. [11] suggested that antigenicity differences were detected based on cross-neutralization tests and genetic characterization of E gene
Fig. 3. Phylogenetic tree of the 5’ untranslated region sequences of the Japanese bovine viral diarrhea virus strains compared with those available in GenBank. The maximum likelihood feature in the MEGA6 software was used to establish the phylogenetic tree (1,000 bootstrap replicates). The number at each branch point indicates ≥50% bootstrap values in the bootstrap interior branch test. The current Japanese strains identified are indicated using black circles.
Fig. 4. Phylogenetic tree of (A) genome sequences and (B) full-length E2 gene sequences of the Japanese bovine viral diarrhea virus strains compared with those available in GenBank. The maximum likelihood feature in the MEGA6 software was used to establish the phylogenetic tree (1,000 bootstrap replicates). The number at each branch point indicates ≥50% bootstrap values in the bootstrap interior branch test. The current Japanese strains identified are indicated using black circles.

sequences when comparing BVDV 1b to others. Further research is needed to perform cross-neutralization tests of the current BVDV isolates obtained in this study. Although there are limitations as mentioned above to this study, our findings reveal the presence of 1a, 2a, and 1b subgenotypes in Tokachi Province, suggesting that the combination of live-attenuated vaccines with 1a/2a strains and inactivated vaccine containing 1b strain should be recommended for bovine vaccination program in this region.

CONFLICT OF INTERESTS. The authors declare that they have no conflict of interest.

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