Comparative analysis on the 5′-untranslated region of bovine viral diarrhea virus isolated in Korea

J.S. Park [a], H.J. Moon [a], B.C. Lee [b], W.S. Hwang [b], H.S. Yoo [c], D.Y. Kim [d], B.K. Park [a,*]

[a] *Department of Veterinary Medicine Virology Lab, The Xenotransplantation Research Center, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Republic of Korea*
[b] *Department of Veterinary Medicine Theriogenology Lab, The Xenotransplantation Research Center, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Republic of Korea*
[c] *Department of Veterinary Medicine Infectious Disease Lab, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Republic of Korea*
[d] *Department of Veterinary Medicine Pathology Lab, The Xenotransplantation Research Center, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Republic of Korea*

Abstract

Samples of 249 bovine abortuses, one intestine, and four diarrheal stools from 254 cows were collected, and bovine viral diarrhea virus (BVDV) was detected by reverse transcription-polymerase chain reaction (RT-PCR). Subsequently, virus isolation was preformed with PCR-positive samples, and then PCR product of 5′-untranslated region (UTR) of BVDV isolate was sequenced and analyzed. Among the samples collected, 20 (from 17 bovine abortuses, one intestine, and two diarrheal stools) were positive for BVDV RT-PCR; four BVDVs (from two bovine abortuses, one intestine, and one diarrheal stool) were isolated. When the four isolates were biotyped in cell culture, one BVDV isolate from a bovine abortus was cytopathic and the others were non-cytopathic. In addition, three isolates were genotyped as BVDV-1 and one isolate from a diarrheal stool as BVDV-2. In phylogenetic analysis, it suggested that the BVDV-2 isolate in Korea is closer to the North American strains than Asian strains. This is the first report on the identification and isolation of BVDV-2 in Korea.
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1. Introduction

Bovine viral diarrhea virus (BVDV) is widely distributed in cattle-producing areas and countries (Houe, 1999). Clinically severe disease outbreaks associated with acute, uncomplicated BVDV infection have been reported in the US, Britain, Canada, and Germany (Carman et al., 1998). BVDV infection causes various clinical symptoms such as diarrhea, respiratory disorders, and immunosuppression (Thiel et al., 1996). Meanwhile, infection with a highly virulent virus, BVDV-2 could result in prolonged fever, leukopenia, severe thrombocytopenia, and diarrhea (Bolin and

Ridpath, 1992; Corapi et al., 1989). In particular, transplacental infection of BVDV in pregnant cows results in reproductive failure such as abortion, mummification or congenital defects according to the gestation stage (Nagai et al., 2001). During the first trimester of gestation the virus infection may produce persistently infected (PI) calves, which are immunotolerant to the infected virus. Thereafter, PI calves maintain significantly a large reservoir, shedding large quantities of the virus in their lifetime, in addition to being a risk group for the mucosal disease (Nettleton and Entrican, 1995; Thiel et al., 1996).

Together with classical swine fever virus (CSFV) and border disease virus (BDV), BVDV belongs to the genus *Pestivirus* of the family *Flaviviridae*. The virus genome consists of a positive-sense, single-stranded RNA (approximately 12.5 kb in length), which has one large open

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reading frame (ORF) coding about 4,000 amino acids (Collett et al., 1988; De Moerlooze et al., 1993; Deng and Brock, 1992) and its polyprotein is proteolytically processed during virus replication to generate viral structural and non-structural proteins (Meyers and Thiel, 1996). Further, the genome contains untranslated regions (UTRs) at both ends (5′ and 3′ UTRs). Pestivirus 5′-UTR includes several short ORFs of unknown function and has been predicted to form a highly structured RNA element that may serve as an internal ribosome entry site to initiate cap-independent translation of ORFs (Brown et al., 1992). The 5′-UTR is particularly attractive for the purpose of viral differentiation because it is highly conserved among members within each pestivirus species (Boye et al., 1991; Berry et al., 1992). On the basis of the 5′-UTRs analysis, BVDV was segregated into two genotypes, BVDV-1 and BVDV-2. Sequence homology within each group was over 93%, while between groups 1 and 2 dropped to 74% (Ridpath et al., 1994). Moreover, BVDV-1 was subdivided into BVDV-1a including reference strain NADL, and BVDV-1b including reference strain Osloss (Pellerin et al., 1994; van Rijn et al., 1997). In general, BVDV was also subdivided into cytopathic BVDV (cpBVDV) and non-cytopathic BVDV (ncpBVDV) biotypes based on cell culture results.

The objectives of this study were to establish reverse transcription-polymerase chain reaction (RT-PCR) for BVDV detection with field samples, develop differential RT-PCR for genotyping BVDV isolates, analyze 5′-UTR sequence of the isolates and finally to establish phylogenetic relationships between the isolates.

2. Materials and methods

2.1. Preparation of samples

Two hundred and forty-nine aborted fetuses, one calf intestine, and four adult cow diarrheal stools were collected from 254 cattle on 228 farms. With each aborted fetus, a piece of tissue from lung, thymus, liver, spleen, kidney, and brain were taken routinely for BVDV detection. Mixed tissues and a calf intestine were chopped, homogenized, and made into 10% suspensions (v/v) using phosphate-buffered saline (0.1 M PBS, pH 7.2). The suspensions were centrifuged for 30 min at 3,000*g*. However, diarrheal stools were made into 50% suspensions (v/v) using PBS. The preparations were further diluted into 1:10 using PBS, vortexed, and clarified by centrifugation for 10 min at 4,800*g* to eliminate large debris. Then, the clarified supernatants were kept at −70 °C until use. For the primary virus passage, the supernatants were filtrated through 0.8- and 0.2-μm pore-sized syringe filters (Nucleopore, CA, USA).

2.2. Virus and cell

BVDV, NY-1 strain as a reference virus was used and proliferated onto Madin-Darby bovine kidney (MDBK) cell in minimal essential medium (MEM, Gibco BRL, Uxbridge, MA, USA) supplemented with 5% ultra-low IgG fetal bovine serum (Gibco BRL), 200 mM Hepes buffer, 300 mM L-glutamine, 0.2% (w/v) sodium bicarbonate, and antibiotics (penicillin G sodium 10,000 unit/ml, streptomycin sulfate 10,000 μl/ml). RT-PCR positive-samples for BVDV detection were also cultured onto MDBK cells in MEM described above.

2.3. Extraction of nucleic acid

RNA extraction was performed using TRIzol (Gibco BRL), a commercially available mixture of guanidine isothiocyanate and phenol. Extraction and purification of total RNA were performed according to the manufacturer's recommendations. Briefly, 500 μl of TRIzol reagent was mixed with 200 μl of 10% tissue homogenate or 500 μl of the clarified supernatant of diarrheal stool. Subsequently, 100 μl of chloroform was added, and the suspension was centrifuged for 10 min at 12,000*g*. The RNA-containing aqueous phase was precipitated with isopropanol of the same volume, maintained at −70 °C for 2 h, and centrifuged for 10 min at 12,000*g*. The RNA pellet was washed with 1 ml of 75% ethanol, centrifuged for 10 min at 12,000*g*, dried, and followed by resuspension in 30 μl of diethyl pyrocarbonate (DEPC)-treated deionized water.

2.4. Design of primers

Primer pairs were designed on the 5′-UTR to detect both BVDV-1 and BVDV-2 in clinical samples. BVDV primers prepared for RT-PCR were based on the nucleotide sequences of National Center for Biotechnology Information (NCBI) Genbank database accession numbers M31182 (BVDV-1, NADL strain) and U18059 (BVDV-2, 890 strain). The common sense primer BVDV F1 starts from a nucleotide position 105 at the 5′-UTR end, and the common antisense primer BVDV CR1 starts from a nucleotide position 320. All primers were commercially synthesized, and the locations and sequences of the primers are summarized in Table 1.

2.5. Reverse transcription-polymerase chain reaction

RT was carried out using random hexamers (Takara, Japan). A mixture of 1 μl of random hexamer(100 pmol) and 30 μl of RNA solution was denatured by heating at 95 °C and immediately placed on ice. Subsequently, 10 μl of 5× first strand buffer (50 mM Tris–HCl, 75 mM

Table 1
Primers used in reverse transcription-PCR to amplify specific fragments from bovine viral diarrhea virus

Primer	Sequence (5′ → 3′)	Nucleotide position
BVDV F1	GCC ATG CCC TTA GTA GGA CT	105–124
BVDV CR1	GCW RCA CCC TAT CAG GCT GT	320–339
BVDV R2	AGA TCG GTC CTG GTT TGA TA	631–650

W: A or T; R: A or G.

KCl, 3 mM MgCl$_2$), 5 µl of 10 mM DTT, 2 µl of 0.3 mM each of dNTP, and 100 units of M-MLV reverse transcriptase were added to make a final volume of 50 µl. The mixture was incubated at 37 °C for 60 min, and the reaction was stopped by heating at 95 °C for 2–3 min. cDNA was amplified immediately or stored at −20 °C.

Amplification was carried out using GeneAmp PCR systems 2400 model (Perkin–Elmer, Applied Biosystems, Inc., Foster City, CA, USA). For the detection of BVDV in clinical samples, 3 µl of cDNA was added to make a reaction volume of 25 µl containing the final concentrations of 10× PCR buffer (10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100), 2.5 mM of MgCl$_2$, 0.3 mM each of dNTP, 20 pmol each of BVDV F1 and BVDV CR1 primers, and 1.25 unit of *Taq* DNA polymerase. Thermal cycles consisted of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 45 s. Upon the completion of cycling, samples were kept at 72 °C for 5 min prior to cooling. Amplification was performed for 30 cycles and then PCR products were analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide. For the detection of BVDV-2 from the isolates, BVDV F1 and BVDV R2 primers were used. BVDV R2 starts from a nucleotide position 631 at N^{pro} gene (Table 1). Amplification conditions were the same as described above, except that the annealing temperature was modified to 58 °C.

2.6. RT-PCR sensitivity and specificity test

In order to determine the sensitivity of RT-PCR for BVDV detection in clinical samples, BVDV, NY-1 strain ($10^{5.0}$ TCID$_{50}$/ml) was serially diluted in MEM and the diluted virus was detected by RT-PCR. Two hundreds microliter of the diluted virus was added to 100 µl of homogenized tissue solution that was negative for BVDV. RNA of the preparation was extracted and assayed as described above. BVDV F1 and BVDV CR1 primers were used for the sensitivity test. To determine the specificity of RT-PCR, BVDV, NY-1 strain, Aka-bane virus, parainfluenza-3 (PI-3) virus, and infectious bovine rhinotracheitis (IBR) virus were amplified by BVDV F1 and BVDV CR1 primers.

2.7. Isolation of BVDV in cell culture

Among the samples of 249 aborted fetuses, one calf intestine and four diarrheal stools from adult cows, twenty BVDV-positive samples determined by RT-PCR were used for virus isolation. MDBK cell monolayer was prepared in a six-well cell culture cluster. The prepared cell monolayer was washed with PBS 3 times, and the filtered supernatants were inoculated. After 1 h adsorption at 37 °C under 5% CO$_2$, the inoculated cell monolayer was washed with PBS 3 times and incubated in MEM supplemented with 5% ultra-low IgG fetal bovine serum at 37 °C under 5% CO$_2$. After incubation for 5 days, blind passage was continued until passage 3. However, forth and fifth passages were performed in a 25-cm^2 cell culture flask. Viral RNA was extracted from the inoculated cells, and RT-PCR was carried out using the same procedure described previously.

2.8. Sequencing of 5′-UTR

Genetic difference of BVDV was analyzed by carrying out sequence analysis at the nucleotide positions 105–339 in 5′-UTR of BVDV. The viral genomic sequences were derived by direct sequencing of PCR amplification products as described previously (Ridpath et al., 1994). All sequencing reactions were performed in duplicate, and all sequences were confirmed by sequencing both strands. The sequencing data were aligned in ClustalW 1.8 program from BCM (Baylor College of Medicine) Launcher web site (http://search-launcher.bcm.tmc.edu), and homology analysis was performed using BLAST (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST/). For the sequence analysis, BVDV-1 isolates were aligned with BVDV, NY-1 strain (Accession No.: AF039178) as a reference strain, and BVDV-2 isolate was aligned with BVDV-2, 890 strain (Accession No.: U18059).

2.9. Phylogenetic analysis

Phylogenetic analysis was performed on 5′ UTR nucleotide sequence data from 16 BVDV strains which included four Korean isolates, nine reference BVDV-2 strains and three reference BVDV-1 strains. The DNA sequences were analyzed with clustal X. Phylogenetic trees were constructed with the MEGA 2.1 software (Kumar et al., 2001) using Neighbor-Joining method and 1,000 bootstrap replications. The cut-off point of bootstrap replication was determined with above 60%. The sequence data of reference strains were retrieved from GenBank.

3. Results

3.1. Sensitivity and specificity for BVDV detection

Through RT-PCR BVDV was detected at a concentration of 2×10^1 $TCID_{50}$/ml. The specificity test revealed that Akabane, PI-3, and IBR viruses were not amplified by the BVDV specific primer set. The specific product size of BVDV was 235 bp. The sensitivity and specificity of RT-PCR are shown in Fig. 1.

3.2. Detection of BVDV from clinical samples

In the detection of BVDV from clinical samples using BVDV F1 and BVDV CR1 primer set, 17 of 249 the abortus, the calf intestine, and two of the four diarrheal stool samples were positive for RT-PCR.

3.3. Isolation of BVDV in cell culture

Twenty BVDV-positive samples by RT-PCR were inoculated into MDBK cell monolayers, from which four BVDVs were isolated. Among them, three BVDVs (Bo333 isolate, Bo373 isolate, Bo388 isolate) were ncpBVDV and the remaining one (Bo41 isolate) was cpBVDV (Table 2).

3.4. Genotyping BVDV isolates by RT-PCR

After BVDV isolation was confirmed by routine RT-PCR, BVDV F1 and BVDV R2 primer set was used for the detection of BVDV-2 among the four isolates. BVDV, Bo388 isolate was determined to be BVDV-2

and the other three isolates were BVDV-1 (Table 2 and Fig. 2). Subsequently, 5'-UTR region of PCR product of BVDV isolate was sequenced. The alignment analysis was performed at the nucleotide positions 105–339 in 5'-UTR of BVDV, NY-1, strain and four BVDV isolates. Sequence identity between BVDV-1 or BVDV-2 isolate was 92–97%, including the Korean isolates, and that identity between BVDV-1 and BVDV-2 isolate was 69–78%. However, the homology between BVDV-2 isolate and BVDV-2 reference strains ranged from 93% to 97%, while that of BVDV-2, Bo388 isolate and BVDV-1, NY-1 strain was 76%. Table 3 summarizes the results.

3.5. Phylogenetic analysis

Phylogenetic analysis of partial 5'-UTR gene of BVDV-1 and BVDV-2 strains could be grouped into two distinct clusters (Fig. 3). In BVDV-1, the clustering of Korean isolates was statistically supported by 91% of bootstrap replicates, Korean BVDV-1 isolates made a domestic own cluster and were distinct from reference BVDV-1 strain, NY-1. Korean BVDV-2 isolate was grouped into different cluster with 890 strain, used as reference strain in this study with support of 93% of bootstrap replicates.

4. Discussion

Acute postnatal infection with BVDV causes various clinical pictures ranging from inapparent infections to wide range of symptoms. In particular, the major economic damage caused by BVDV in susceptible animals
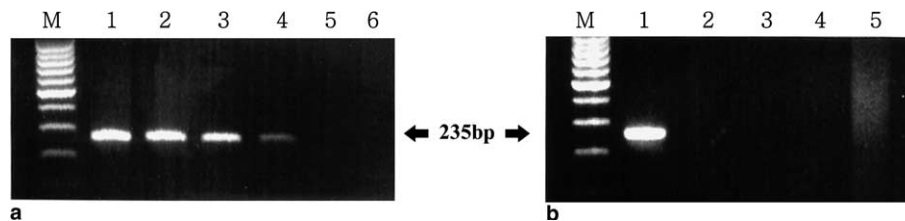

Fig. 1. Sensitivity of reverse transcription-PCR using a specific primer set, BVDV F1 and BVDV CR1, for the detection of bovine viral diarrhea virus (a). Specificity of reverse transcription-PCR using a specific primer set, BVDV F1 and BVDV CR1, for the detection of bovine viral diarrhea virus (b). (a) Lane M: 100 bp DNA ladder. Lanes 1–5: reactions in 10-fold dilutions of BVDV, NY-1 strain (10^5–10^1 $TCID_{50}$/sample). Lane 6: negative control. (b) Lane M: 100 bp DNA ladder. Lane 1: BVDV, NY-1 strain. Lane 2: negative control. Lane 3: Akabane virus, OBE-1 strain. Lane 4: PI-3 virus, NADL strain. Lane 5: IBR virus, PQ7 strain.

Table 2
Biotypes and genotypes of bovine viral diarrhea virus isolates

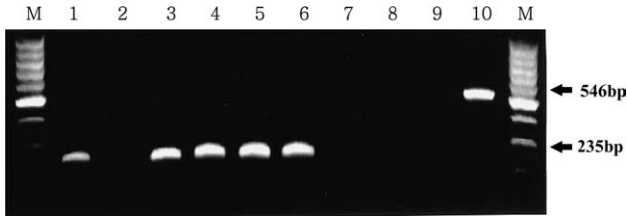| Isolate	Sample origin	Biotype	Genotype
BVDV, Bo41 isolate	Aborted fetus	Cytopathic	1
BVDV, Bo333 isolate	Aborted fetus	Non-cytopathic	1
BVDV, Bo373 isolate	Intestine	Non-cytopathic	1
BVDV, Bo388 isolate	Diarrheal stool	Non-cytopathic	2

Fig. 2. Differential reverse transcription-PCR for genotyping bovine viral diarrhea virus isolates. Lanes 3–6 were amplified with primer set, BVDV F1 and BVDV CR1, whereas lanes 7–10 were amplified with primer set, BVDV F1 and BVDV R2. Lane M: 100 bp DNA ladder. Lane 1: BVDV, NY-1 strain. Lane 2: negative control. Lanes 3 and 7: BVDV, Bo41 isolate. Lanes 4 and 8: BVDV, Bo333 isolate. Lanes 5 and 9: BVDV, Bo373 isolate. Lanes 6 and 10: BVDV, Bo388 isolate.

is primarily the result of intrauterine infection (Doherty, 1986). Pathological studies of the virus predominantly focused on the establishment of persistent infections and the development of mucosal disease. Originally

identified in outbreaks of severe disease, BVDV-2 was initially thought to be a new and invariably virulent virus. Subsequent studies demonstrated that the virus of genotype 2 has been circulating among the North American cattle population for at least 20 years (Carman et al., 1998). Genotype 2 has been identified in Europe (Wolfmeyer et al., 1997), South America (Canal et al., 1998) and North America (Evermann and Ridpath, 2002), indicating that it is likely to be identified in other countries as well as the epidemiological investigation proceeds (Canal et al., 1998; Evermann and Ridpath, 2002).

Characterization of BVDV genotype has revealed distinction in the 5′ non-coding region of pestivirus RNA (Harasawa, 1994; Harasawa and Sasaki, 1995). In this study, primers for BVDV detection were designed on 5′-UTR to determine two BVDV genotypes. The sensitivity of the established RT-PCR was reasonable to detect BVDV from clinical samples. In addition, the

Table 3
Homology of nucleotide sequence in the 5′-untranslated region of bovine viral diarrhea virus

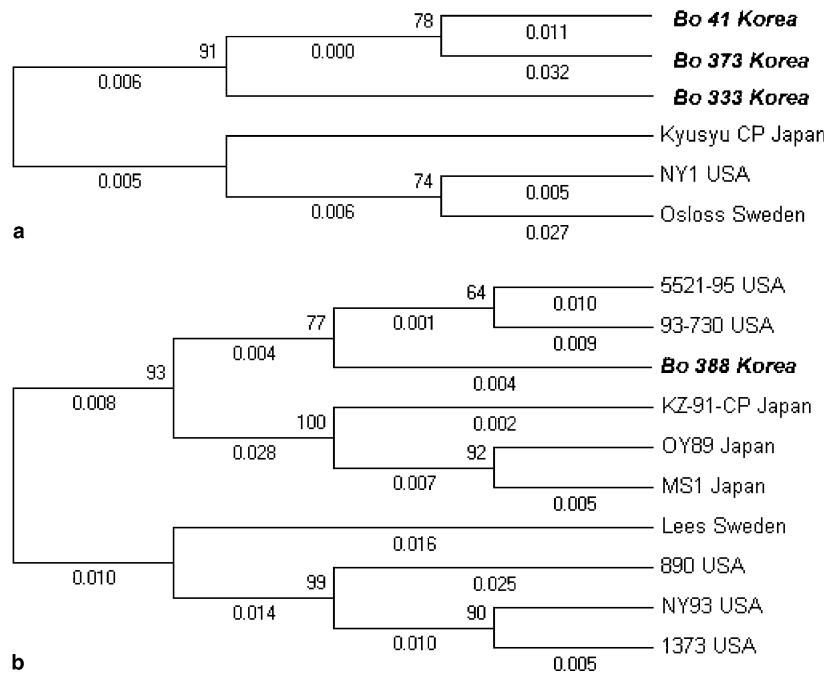| BVDV isolate	BVDV-1				BVDV-2	
	Bo41	Bo333	Bo373	NY-1	Bo388	890 strain
Bo41	100	96	92	95	72	78
Bo333		100	95	97	73	77
Bo373			100	93	69	74
NY-1				100	76	73
Bo388					100	93
890 strain						100

Fig. 3. Phylogenetic trees of BVDV-1 (a) and BVDV-2 (b). Tree constructed with Neighbor-Joining method using MEGA 2.1 software. The figures under the line were generated with "*p-distance*" model, and above the line were bootstrap value with 1,000 replications.

specificity of PCR was distinct from other bovine viruses. Because CSFV does not infect cattle and BDV is not present in Korea, these viruses were excluded in the test. Most of the collected samples were aborted fetuses derived from bovine reproductive studies, and the ratio of abortion related to BVDV infection was approximately 6.8%. This result was similar to that of a recent report in Korea (Lee et al., 1999), in which a major cause of 73 abortions or stillbirths determined by pathological findings was neosporosis (21%). The report suggested that BVDV only or co-infection of other viruses and bacteria was 8%. For genotyping the isolates, BVDV-2 specific primer set was designed on the N^{pro} region, with which was appropriate for differential RT-PCR. This study clarified that two genotypes, BVDV-1 and BVDV-2, are present in the Korean cattle population. It was also reported that a Korean isolate of BVDV-2 was a non-cytopathogenic virus. Sequencing efforts were restricted to the four viruses that were able to grow in culture. But, it would have been better to include all 20 positives in this analysis. This would also have helped in determining the relative abundance of type 1 versus type 2 viruses, although this study showed for the first time the existence of type 2 viruses in Korea. It would not be able to discriminate as to whether or not animals are infected with the two types of virus simultaneously. Through direct fluorescent assay, the possibility to be a bovine parvovirus, bovine coronavirus, bovine rotavirus, and bovine adenovirus was excluded.

In an alignment analysis performed to support the differential RT-PCR results among BVDV isolates, homologies between BVDV, NY-1 strain and BVDV-1 isolates and 93% homology between BVDV-2, 890 strain and BVDV, Bo388 were similar to those of previous reports. Further homology analysis through the BLAST search revealed that BVDV, Bo388 strain had 97% and 95% homologies with BVDV, 5521-95 strain (Accession No.: AF039174) and BVDV, KZ-91-CP isolate (Accession No.: AB003619) that were known to be a BVDV-2. These results indicate that BVDV, Bo388 strain is more similar to BVDV, 5521-95 strain than BVDV, 890 strain. Thus, it was suggested that BVDV, Bo388 isolate may be a variant of BVDV-2. This is the first report on the isolation and identification of BVDV-2 in Korea.

In phylogenetic analysis, other published strains were lower than 60%, the tree was constructed if higher than 60%. It was difficult to compare with other strains from various geographical origins, because the first isolation of Korean BVDV-1 was reported in 1987, becoming endemic in the Korean bovine herds (Seok, 1987). BVDV-1 had been existed in Korea for more than 20 years, it may be suggested that Korean isolates (Bo 41, Bo 333, Bo 373) could make a domestic own cluster. In BVDV-2, the first Korean isolate was grouped into a different cluster with reference BVDV-2 strains, 890 and NewYork '93 strains. The Korean isolate was closer

American strains than Japanese strains. This was supported by 77% of bootstrap replicates.

Previous reports showed that BVDV-2 could also be isolated from healthy or aborted fetuses, and cattle with respiratory disease, neurologic symptoms, and hemorrhagic syndrome (Letellier et al., 1999; Flores et al., 2000). Because healthy fetuses were not sampled in our study, a further study remains to be screened with normal samples.

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