

## Genetic Typing of Bovine Viral Diarrhea Viruses (BVDV) Circulating in Korea

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To characterize the genetic diversity of bovine viral diarrhea viruses (BVDV) circulating in Korea, 11 BVDV isolates were obtained from 467 field samples collected during 2005~2006 in Korea. All of the BVDV isolates were identified as non-cytopathic (non-cp) BVDV biotypes. The 5' noncoding region (NCR) genes of the isolates were sequenced and analyzed. In total, ten BVDV isolates were typed as BVDV-1 by comparing the genomic sequences to the 5' NCR. One isolate (05R169) showed 98.6% nucleotide sequence identity with the BVDV-2 reference strain and was therefore typed as BVDV-2. Our results indicate that BVDV-1 is the main genotype circulating in the cattle population of Korea.

**Key Words:** BVDV, Genotype, Isolation, Cattle

### INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a pestivirus belonging to the family *Flaviviridae*. BVDV acts as the causative agent of bovine viral diarrhea-mucosal disease (BVD-MD) in cattle and is a common bovine pathogen that causes significant economic losses worldwide (4,5). Other examples of pestiviruses with veterinary importance include classical swine fever virus and border disease virus in sheep. BVDV infection can lead to various clinical symptoms including diarrhea, fever, respiratory disease, and abortion or early embryonic death of fetuses in pregnant cattle. BVDV has a genome size of 12.5 kb, and on the basis of 5' noncoding region (NCR) gene analysis, appears

to be segregated into two genotypes: BVDV-1 and BVDV-2 (10,11). Based on recent comparisons of sequences derived from three genetic regions (i.e., 5' NCR, a coding region for the autoprotease N<sup>pro</sup>, and a coding region for the structural protein E2), the BVDV-1 genotype has been subdivided into at least 11 BVDV-1 genetic subgroups (9,10,15). In addition to BVDV-1, BVDV-2 has been identified in many countries including Korea (1,2,6~8,12,16) and is associated with more severe clinical symptoms such as hemorrhagic diarrhea and abortion (8,11). During the first trimester of gestation, viral infection in dams may produce persistently infected (PI) calves due to immunotolerance effects. PI animals are the main source of viral transmission because they continuously shed large amounts of virus into the environment and are permanent carriers of BVDV. In addition, the presence of PI animals leads to an increased risk of mucosal disease (3). In this study, we investigated which genotypes of Korean BVDV isolates were circulating in cattle populations during 2005~2006.

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## MATERIALS AND METHODS

### 1. Virus and cells

BVDV, NADL (National Animal Disease Laboratory) strain of reference virus and field isolates were propagated in Madin-Darby Bovine Kidney (MDBK) cells cultured in  $\alpha$ -minimum essential medium (MEM: Gibco BRL, Grand Island, NY, USA) with antibiotics (penicillin, 100 IU/ml, streptomycin, 100  $\mu$ g/ml), 5% fetal bovine serum (FBS: Gibco BRL) free of BVDV and antibodies against BVDV. The virus infected cell cultures were maintained in  $\alpha$ -MEM supplemented with 5% FBS.

### 2. Virus isolation

To isolate BVDV, samples were obtained from organ suspensions of 46 aborted fetuses and homogenized tissues of 37 cattle that displayed clinical signs of BVD. Tissue samples were membrane-filtered and later used in the BVDV isolation procedure. All samples were obtained from tissues that were submitted to the Pathology Division, National Veterinary Research and Quarantine Service (NVRQS), Korea, between January 2005 and June 2006. To detect PI calves with BVDV, a total of 384 plasma samples were collected from the calves which were between 3 days and 6 months old in 4 provinces (Table 1). The samples were initially examined for the detection of BVDV genome by using RT-PCR; PCR-positive samples were further used to isolate BVDV. Specifically, MDBK monolayers in 24-well microplates were washed twice with PBS and then inoculated with 100  $\mu$ l of the membrane-filtered samples. Following adsorption, the inoculated samples were replaced with 1 ml MEM containing 3% FBS. These microplates were incubated for 7 days and then screened for cytopathic effects and examined using an immunofluorescence antibody (IFA) test. All isolates were cloned three times using the limit dilution method, propagated on MDBK cells, and examined using an IFA test.

### 3. RNA extraction and RT-PCR

RNA was isolated from 250  $\mu$ l of BVDV-infected cell

culture supernatants using an RNA isolation kit (Bioneer, Deajeon, Korea) according to the manufacturer's instructions. Conventional RT-PCR using the pan-pestivirus primer pair V324/V326 for the 5' NCR of the pestivirus was used for genetic detection (14). PCR was carried out in a reaction mixture containing 10  $\mu$ l of denatured RNA, 10  $\mu$ l of 5 $\times$  buffer (12.5 mM MgCl<sub>2</sub>), 2  $\mu$ l of enzyme mix (reverse transcriptase and *Taq* polymerase), 1  $\mu$ l of each primer (50 pmol) and 26  $\mu$ l of distilled water (Qiagen, Hilden, Germany) for a 50  $\mu$ l final volume. The cycling profile was run as follows: cDNA synthesis at 42 $^{\circ}$ C for 30 min; followed by 35 cycles with denaturation at 94 $^{\circ}$ C for 30s, annealing at 50 $^{\circ}$ C for 30s and extension at 72 $^{\circ}$ C for 30s; and a final extension at 72 $^{\circ}$ C for 10 min. The PCR products were visualized using electrophoresis on 1.5% agarose gel containing ethidium bromide. Purified PCR products using the gel extraction kit (Qiagen) were ligated with pGEM-T easy vector (Promega, Madison, WI, USA). Plasmid DNA was isolated from amplified *Escherichia coli*, and recombinant plasmids were identified using *EcoRI* restriction enzyme digestion (Promega).

### 4. Sequencing and phylogenetic analysis

Sequencing reactions were performed using recombinant plasmids and the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Wellesley, MA, USA). Phylogenetic analysis was performed on the 5' NCR nucleotide sequence data from 11 Korean isolates and 30 reference BVDV strains. Sequence data for the reference strains were obtained from GenBank (National Center for Biotechnology Information, NCBI). Phylogenetic trees and sequence pair distances of the nucleotides were obtained using the DNASTAR software program (DNASTar, Madison, WI, USA). Homology analysis was performed using DNASIS software (Hitachi software, Tokyo, Japan).

## RESULTS

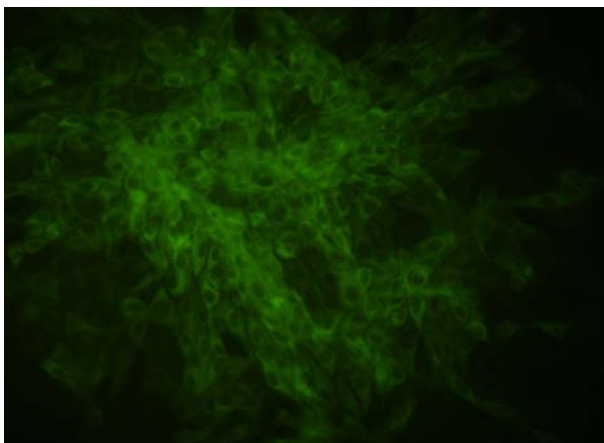
### 1. Virus isolation

In total, 11 BVDVs were isolated from 7 provinces (Table 1). Six BVDV isolates (designated 05R169, 05R203,

**Table 1.** Bovine viral diarrhea viruses isolated from cattle in Korea and GenBank accession numbers of their 5'NCR nucleotide sequences

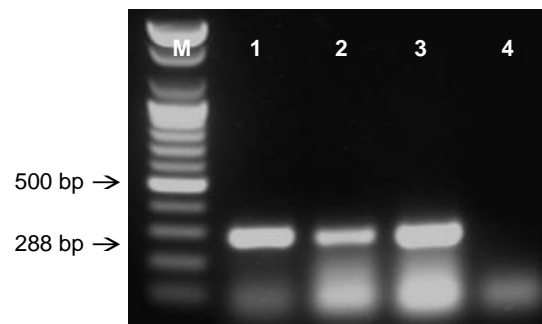
| Virus isolate | Source of sample  | Isolated province | Breed | Biotype | Genotype | Accession number |
|---------------|-------------------|-------------------|-------|---------|----------|------------------|
| 05D26         | Tissue homogenate | Chungbuk          | K*    | NCP†    | 1b       | DQ973172         |
| 05D73         | Tissue homogenate | Gyeonggi          | H     | NCP     | 1b       | DQ973173         |
| 05Q122        | Aborted fetus     | Chungnam          | H     | NCP     | 1b       | DQ973175         |
| 05D137        | Aborted fetus     | Jeonbuk           | K     | NCP     | 1a       | DQ973174         |
| 05Q174        | Tissue homogenate | Gangwon           | H     | NCP     | 1b       | DQ973176         |
| 05R169        | Blood plasma      | Chungnam          | H     | NCP     | 2        | DQ973177         |
| 05R203        | Blood plasma      | Gyeongnam         | K     | NCP     | 1b       | DQ973178         |
| 05R204        | Blood plasma      | Gyeongbuk         | H     | NCP     | 1a       | DQ973179         |
| 06R447        | Blood plasma      | Gangwon           | K     | NCP     | 1a       | DQ973180         |
| 06Z71         | Blood plasma      | Chungnam          | K     | NCP     | 1c       | DQ973181         |
| 06Z127        | Blood plasma      | Chungnam          | K     | NCP     | 1c       | DQ973182         |

K: Korean native cattle, H: Holstein dairy cattle. †NCP: non-cytopathic.



**Figure 1.** Immunofluorescence of 05R169 isolate by IFA test using monoclonal antibody against BVDV in MDBK cell.

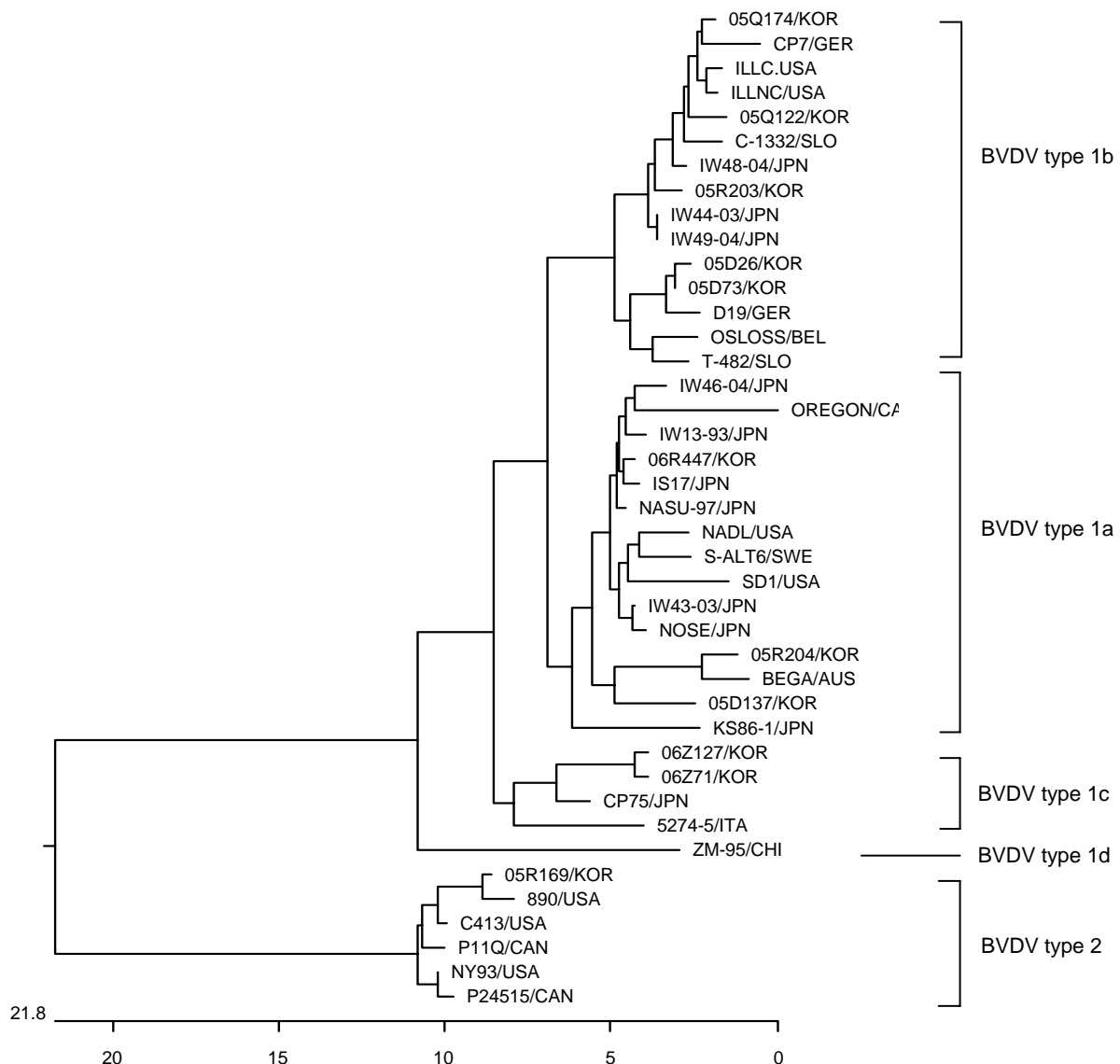
05R204, 06R447, 06Z71, and 06Z127) were obtained from the 384 plasma samples, three isolates were obtained from tissue samples of cattle demonstrating clinical symptoms (05D26, 05D73, 05Q174), and two isolates were obtained from aborted fetuses (05Q122, 05D137; Table 1). All the isolates were identified as non-cp BVDV biotypes by no appearance of cytopathic effect and IFA test positive using BVDV specific monoclonal antibody (IDEXX, Westbrook, Maine, USA). The BVDV-specific fluorescences were noticed in the cytoplasm of the virus infected cells (Fig. 1). Six BVDV isolates were isolated from native Korean cattle (Table 1).



**Figure 2.** RT-PCR products of BVDV isolates were detected on 1.5% agarose gel. The expected size was 288 bp. M; 100 bp DNA ladder, Lane 1; 05D26, Lane 2; 05D73, Lane 3; NADL strain, Lane 4; negative sample.

## 2. Identification and genotyping of the isolates by RT-PCR and sequence analysis

The RT-PCR using specific primers of BVDV could amplify 5' NCR gene of the isolates. The PCR products of 5'NCR were detected with 288 base pairs on 1.5% agarose gel (Fig. 2). The nucleotides of these 11 BVDV isolates were sequenced, and all data were deposited in GenBank under accession numbers DQ973172-DQ973182. Phylogenetic analysis classified these viruses into two different genotypes (BVDV-1 and BVDV-2) as described previously (Fig. 3). We found that isolates 05D137, 05R204, and 06R447 were clustered with subgenotype 1a; isolates 05D26, 05D73, 05Q122, 05Q174, and 05R203 were clustered with



**Figure 3.** Phylogenetic tree of the 5' non-coding regions (NCR) of BVDV strains and isolates. The tree was generated from comparative alignment of sequences from 245 bp of the 5'NCR of the BVDV genome. The multiple sequences were constructed by the neighbor joining method. Genotypes were given on the right of tree. Abbreviations: Korea (KOR), Australia (AUS), Germany (GER), Canada (CAN), Slovenia (SLO), Belgium (BEL), Japan (JPN), China (CHI), Italy (ITA), Sweden (SWE), United State of America (USA).

subgenotype 1b; and isolates 06Z71 and 06Z127 were segregated into subgenotype 1c. In contrast, isolate 05R169 obtained from a sample of Chungnam Province was clustered with the genotype 2 virus (Fig. 3).

## DISCUSSION

BVD occurs worldwide in most cattle-producing countries, and the prevalence of BVDV antibody-positive herds

is on average approximately 55% (4). Vilcek *et al.* (13) reported that virus typing was the same whether sequences from the 5'NCR or N<sup>pro</sup> coding regions were used. In this study, we examined the genetic diversity of Korean BVDV isolates by identifying the 5'NCR nucleotide sequences of 11 field isolates. Sequence analysis showed that ten of the BVDV isolates were classified as the BVDV-1 genotype and only one was classified as the BVDV-2 genotype. These results indicate that the type 1 BVDV genotype is

the predominant virus circulating in the cattle population of Korea. In addition, the phylogenetic tree analysis indicated that isolates 05D137, 05R204, and 06R447 belong to the BVDV-1a subtype, whereas isolate 05R169 clustered with the BVDV-2 genotype. Moreover, isolate 05R169 is very closely related to strain 890, which is the North American type 2a reference strain. In fact, the genetic similarity of the 5'NCR nucleotide sequence between isolate 05R169 and strain 890 was 98.6%.

Ridpath *et al.* (14) reported that BVDV-1 is generally used in vaccine production, and BVDV-2 is isolated predominantly from fetal bovine sera. This BVDV-2 isolate is particularly common in PI calves delivered from dams vaccinated against BVDV and cattle that have died from hemorrhagic syndrome (an acute form of BVDV). Since little information is currently available regarding PI calves and BVD-MD cases in Korea, further studies are required to investigate the pathological and clinical characteristics of BVDV-2 isolates.

In addition, local vaccines against BVDV are produced from BVDV-1 strains only in Korea. For the prevention of cattle from 2 genotypes of BVDV infection, it is necessary to develop effective BVDV vaccine using both BVDV-1 and BVDV-2 strains.

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