Acta virologica **53**: 131 – 134, 2009 doi:10:4149/av_2009_02_131

# Identification of a Bovine viral diarrhea virus 2 isolated from cattle in China

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*Received February 4, 2009; accepted April 23, 2009*

**Summary. –** The identification and characterization of bovine viral diarrhea virus 2 (BVDV-2) strain SD-06 isolated from cattle in China is reported. We performed sequence analysis of 5'-untranslated region (5'-UTR) and E2 sequences and the identity at the nucleotide and amino acid level indicated that the isolate was closely related to BVDV-2. The BVDV-2 strain New York'93 showed the highest sequence homology with the isolate SD-06. The phylogenetic analysis revealed that the isolate SD-06 belonged to BVDV-2a subtype. Furthermore, immunofluorescence assay with the monoclonal antibody specific for BVDV-2 glycoprotein E2 confirmed this identification. Thus, the strain SD-06 was the first isolate of BVDV-2 identified in China.

**Keywords:** Bovine viral diarrhea virus 2; cattle; E2; phylogenetic analysis; 5'-UTR

BVDV is the infectious agent of bovine viral diarrhea disease occurring in many countries and causing big economical losses to cattle farms (Moerman *et al*., 1993; Houe, 1999, 2003; Ridpath *et al*., 2006). Based on the nucleotide sequence of 5'-UTR, BVDV is divided into two genotypes BVDV-1 and BVDV-2 (Wolfmeyer *et al*., 1997; Heinz *et al*., 2004). BVDV-1 strains can be further divided into at least 11 genetic subgroups (Vilcek *et al*., 2001). BVDV-2 is antigenically and genetically distinct from BVDV-1 and is classified into two subtypes BVDV-2a and BVDV-2b (Evermann and Ridpath, 2002; Giangaspero *et al*., 2008). Initially, BVDV-2 isolates were identified in severe outbreaks of acute bovine viral diarrhea disease in North America in the late 1980s (Perdrizet *et al*., 1987; Corapi *et al*., 1989; Carman *et al*., 1998), and are known as highly pathogenic (Pellerin *et al*., 1994; Ridpath *et al*., 1994). Later, BVDV-2 viruses were identified in South America (Flores *et al*., 2000, 2002; Jones *et al*., 2001), Germany (Wolfmeyer *et al*., 1997; Beer *et al*., 2002), Belgium (Letellier *et al*., 2005), Italy (Pratelli *et al*., 2001), Austria (Vilcek *et al*., 2003) and other countries. Furthermore, BVDV-2 was also found in the countries flanking China such as Japan (Nagai *et al*., 2001), Korea (Kim *et al*., 2006) and India (Mishra *et al*., 2008). Our study reports about the first identification of BVDV-2 strain SD-06 isolated from the cattle in China.

In December 2006, seven tissue samples including trachea, lung, spleen, liver, kidney, intestine and lymph nodes from a dead calf and 13 blood samples were collected from the cattle (3–5 months old) in a small farm in Shandong province. The collected samples were examined for the presence of BVDV-1, 2. Clinical conditions of diseased animals as severe diarrhea, cough, and conjunctival hemorrhage indicated bovine viral diarrhea disease. At necropsy, the bronchopneumonia, catarrhal pneumonia and catarrhal-hemorrhagic enterocolitis were observed.

Presence of BVDV-1, 2 was initially investigated from the samples by RT-PCR with the primers described previously for amplification of the 5'-UTR (Ridpath and Bolin, 1998). RNA was isolated with TRIZOL® LS Reagent (Invitrogen) according to the manufacturer's instructions. Viral

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**Abbreviations:** BVD = Border disease virus; BVDV-1, BVDV-2 = Bovine viral diarrhea virus 1 and 2; BVDV-2a, BVDV-2b = Bovine viral diarrhea virus, subtype 2a and 2b; CSFV = Classical swine fever virus; MDBK = Madin-Darby bovine kidney; MAb(s) = monoclonal antibody(ies); PBST = PBS containing 0.05% Tween 20; 5'-UTR = 5'-untranslated region

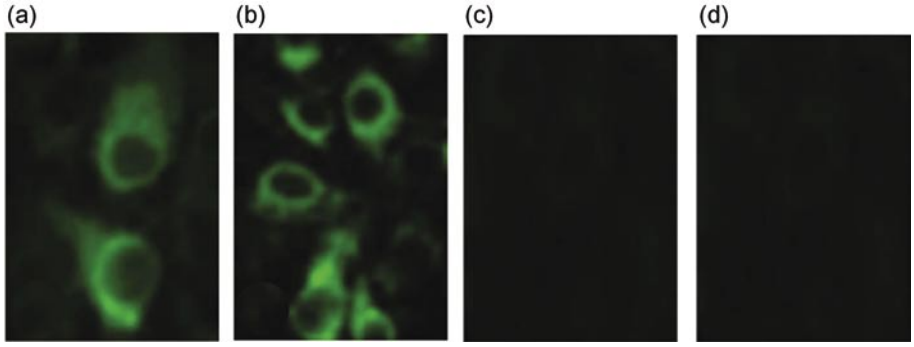


**Fig. 1**

**Immunofluorescence assay of MDBK cells infected with BVDV-2 strains 890 and SD-06**

Panels a, c: strain SD-06-infected MDBK cells stained with MAbs Bz-53 and D89, respectively. Panels b, d: strain 890-infected MDBK cells stained with MAbs Bz-53 and D89, respectively.

RNA (1 μg) was used as a template for synthesis of the first strand cDNA with 6-mer commercial random primers using PrimeScriptTM 1st strand cDNA Synthesis Kit (TaKaRa) following the manufacturer's instructions. The amplified product was cloned into pMD-19 simple T vector (TaKaRa) and sequenced commercially by Shanghai Sangon Biological Engineering Technology & Services. The sequence was submitted to GenBank (Acc. No. FJ795044). Comparison with other 5'-UTR sequences revealed that it was highly homologous to BVDV-2 reference strain New York'93 sharing 97.1% identity at the nucleotide level (Acc. No. AF502399).

The blood samples positive for BVDV were subjected to the virus isolation. Leukocytes isolated from 2 ml of blood were inoculated into 25 $cm^2$ flasks of sub-confluent Madin-Darby bovine kidney (MDBK) cells. One isolate of BVDV was obtained from the tested samples and signed as SD-06. The isolated virus showed cytopathic effect during serial passages of infected MDBK cells.

Further characterization and identification of viral antigens was carried out by immunofluorescence assay. After 5 passages of SD-06, the infected cells in 96-plate were fixed and permeabilized with methanol (supplemented with 0.3% $H_2O_2$). After washing with PBS containing 0.05% Tween 20 (PBST), the cells were blocked with PBST containing 2.5% BSA. Then, the cells were reacted with monoclonal antibodies (MAbs) Bz-53 or D89, which specifically recognized glycoprotein E2 of BVDV-2 and BVDV-1, respectively (Wang *et al*., 2005), for 1 hr in a moist chamber at 37°C. After incubation, the cells were gently washed 3 times with PBST and FITC-conjugated rabbit anti-mouse secondary antibody (Sigma) was added to each well and incubated for 40 mins at 37°C. Finally, the cells were washed and examined under a fluorescence microscope (IMT2 Olympus). BVDV-2 strain 890, a reference strain of subtype 2a, was used as a positive control. Both strains SD-06 and 890 showed strong reactivity with MAb Bz-53 and no reactivity with MAb D89 (Fig. 1). Thus, the strain SD-06 was confirmed as BVDV-2, since it could be recognized specifically by MAb Bz-53 directed against BVDV-2 E2 glycoprotein.

Phylogenetic tree was constructed by the comparison of 243 bp sequence within 5'-UTR (genome position 129–371 bp) of the strain 890, SD-06, and other pestiviruses available in the GenBank. The sequences were trimmed and aligned with the Bioedit software (Hall, 1999). The truncated sequences were used to generate the phylogenetic tree by biosoftware MEGA 4.1 (Kumar *et al*., 2008) using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap probabilities were calculated with 1000 replicates. The phylogram revealed that the isolate SD-06 belonged to BVDV-2a (Fig. 2). Interestingly, the Chinese isolate SD-06 showed closer relationship to the North American strain NewYork'93, rather than to the geographically neighboring Indian strain Ind 51966 (Acc. No. EU371402).

In order to amplify the envelope glycoprotein E2 gene, we designed degenerate primers. The locations of the primers in the strain 890 genome and the sequences were as follows: forward primer EF (nt 2463–2485), 5'-TCCCWGAAT GCAARGAGGGMTTC-3'; reverse primer ER (3577–3558 nt) 5'-ACCCATRGCYATCTGCTCAG-3'. The amplified fragment of 1116 bp was cloned and sequenced similarly as 5'-UTR sequence described above. The sequence was submitted to the GenBank (Acc. No. FJ795045). Comparison analysis using 1116 bp sequence of the strain SD-06 revealed that the highest nucleotide sequence identity 92.3% and amino acid identity 97.7% was found with the BVDV-2 strain 890. In comparison with the isolate York'93, the sequence identity was 91.5%

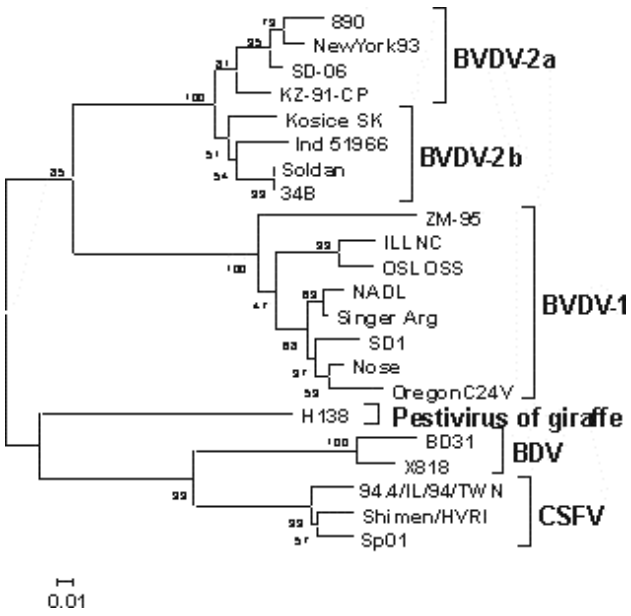


**Fig. 2**

**Phylogenetic tree of pestivirus isolates based on the sequence of 243 bp within 5'-UTR**

Acc. Nos. of BVDV-1 isolates: Nose (AB019670), NADL (M31182), Singer_Arg (DQ088995), Oregon C24V (AF091605), ZM-95 (AF526381), OSLOSS (M96687), ILLNC (U86600), SD1 (M96751); BVDV-2a isolates: KZ-91-CP (AB003619), New York'93 (AF502399), 890 (U18059), SD-06 (FJ795044); BVDV-2b isolates: Soldan (U94914), 34B (AF244952), Ind 51966 (EU371402), Kosice_SK (EU360934); BDV isolates: BD31 (U70263), X818 (AF037405); CSFV isolates: Shimen/HVRI (AY775178), Sp01 (FJ265020), 94.4/IL/94/TWN (AY646427); Pestivirus of giraffe H138 (AF144617).

at the nucleotide level and 97.5% at the amino acid level. Furthermore, amino acid sequence comparison with the strain 890 showed that the glycoprotein E2 of the strain SD-06 contained four potential N-glycosylation sites (Asn-X-Ser or Asn-X-Thr), which were located at the same sites as in the strain 890 (data not shown).

Taken together, the obtained results showed that the virus SD-06 isolated from the cattle in China was BVDV-2. To our knowledge, a BVDV-2 isolate originating from China has not been described yet and our report is the first one describing the isolation and identification of such isolate. Also, the search for a source of BVDV-2 infection in the cattle farm and a thorough characterization of SD-06 isolate remain the subjects of further investigation.

**Acknowledgements.** This work was supported by the Science and Technology Department of Jiangsu province (grant No. BE2008363). The authors are grateful to Dr. LJ. Bello, University of Pennsylvania, for providing the MDBK cells, BVDV-2 reference strain 890 and the MAbs D89 and Bz-53.

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