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Full Length Research Paper

Identification and characterization of 16 recent Chinese isolates of bovine viral diarrhea virus genotype 2 (BVDV-2)

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Eighty calf blood samples were collected from different areas of China and were examined. Sixteen non cytopathic isolates of bovine viral diarrhea virus genotype 2 (BVDV-2) were isolated by specific BVDV-2-directed RT-PCR and sandwich enzyme-linked immunosorbent assay (ELISA). The results were verified by the indirect immunofluorescence assay and the ultra-thin sections. Genes of 5'-UTR, E2 and N^{pro} from all the 16 field isolates were sequenced. The sequence identities at the nucleotide and amino acid levels were higher than 99.0%. The phylogenetic analysis revealed that the BVDV-2 USA strain, AzSpin, had the highest sequence homology with each of the Chinese isolates.

Key words: Bovine viral diarrhea virus genotype 2 (BVDV-2), isolation and characterization, phylogenetic analysis.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an enveloped positive-stranded RNA virus in the genus *Pestivirus* of the family *Flaviviridae* (Collett et al., 1988; Meyers et al., 1989; Ridpath and Bolin, 1995; Becher et al., 1997). It's the causative agent of bovine viral diarrhea, characterized by high fever, diarrhea and cough (Moerman et al., 1993; Waleley et al., 2004; Ridpath et al., 2006). Following analysis of the 5'-UTR of the viral genome, BVDV isolates are classified into two genotypes: BVDV-1 and BVDV-2 (Flores et al., 2000). BVDV-1 is the predominant species found in cattle and widely spread throughout the world. In contrast, BVDV-2 was first described in the early 1990s in the US and Canada (Paton et al., 1995; Pellerin et al., 1994; Arias et al., 2003) and following also, detected in Japan, Brazil and several European countries including UK, Germany, Belgium, France, and Italy (Becher et al., 1995; Sakoda et al., 1999; Couvreur et al., 2002; Flores et al., 2002). In 2004, three BVDV-2 isolates from cattle were first reported in China (Zhu et al., 2009a).

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In this study, we collected and examined 80 blood samples of calves in several dairy farms from different provinces in order to consider the situation for BVDV-2 infection in China and to further investigate whether there are any differences among the BVDV-2 isolates, geographically, after isolation and identification.

MATERIALS AND METHODS

Samples, antigen testing and viral isolates

Eighty blood samples of calves (3 to 5 months old), which had the clinical symptoms, such as diarrhea, cough and dyspnea, were randomly collected in dairy farms from different provinces in China. The blood (serum) samples were initially screened for BVDV (BVDV-1 or BVDV-2) antigen presence by using monoclonal antibody-based sandwich ELISA method established in our laboratory (unpublished data). All positive samples were subjected to the virus isolation by inoculating them on the sub confluent monolayers of Madin Darby bovine kidney (MDBK) cells with Dulbecco's Modified Eagle Media (DMEM) containing 1% BVDV-free horse serum (HS).

After three cycles of freezing and thawing, cultures, including cells and culture media, were harvested to prepare RNA with TRIZOL® LS Reagent (Invitrogen) according to the manufacturer's instructions. Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) with the specific BVDV-2-directed primers described previously for amplification of the 5'-UTR of the BVDV genome was

Table 1. Primers used to generate sequence information.

Name	Sequence	Length
5' UTR primers		
FBVDVI-II	5'-CATGCCCATAGTAGGAC-3'	
BBVDVI-II	5'-CCATGTGCCATGTACAG-3'	283bp
N^{pro} primers		
AP2	5'-TAGCGGTAGCAGTGAGTCCATTG-3'	
AP1	5'-TAGCACCCTTACTCCCTTCATCG-3'	774bp
E2 primers		
SBVE2-1	5'-GGGCTATTGTGGCTGATGC-3'	
SBVE2-2	5'-TCTTCAGTATTCACTCCAGCACC-3'	1175bp

used as a further confirmation method (Ren et al., 2008). To further characterization of viral antigen of the BVDV isolates, immunofluorescence assay was carried out, described previously (Zhu et al., 2009b).

PCR, DNA cloning and sequencing analysis

The viral RNA 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 5'UTR, N^{pro} and E2 genes from the genomes of BVDV-2 isolates were PCR-amplified and PCR products were purified using the TIANgel Midi Purification Kit (Tiangen) and ligated into pGEMT vector according to the manufacturer's specifications, then transformed into *Escherichia coli* DH5α by routine procedure. After screening for the positive recombinants, the recombinant clones were sent for sequencing by commercial service (Shanghai Sangon Biological Engineering Technology and Services). The sequence of PCR primers and the length of the genome amplified are shown in Table 1.

The retrieved sequences were trimmed and aligned with the Bioedit software (Kim et al., 2006). The phylogenetic tree and sequence pair distances of the nucleotide were obtained using the neighbor-joining method by MEGA4.1 (Saitou et al., 1987; Juliá et al., 2009).

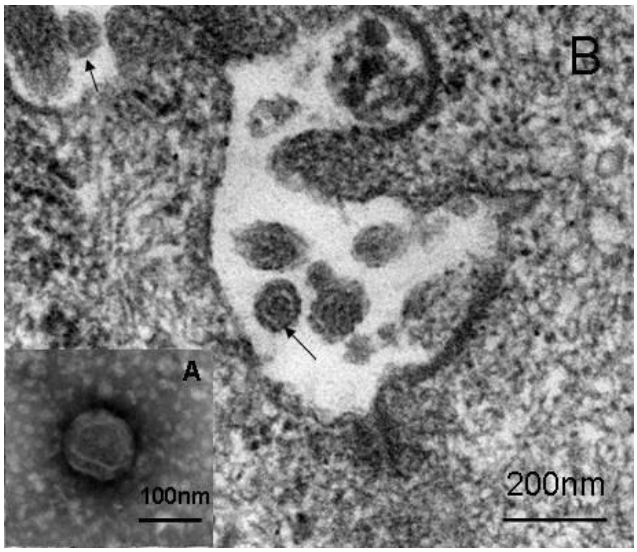

Figure 1. Morphology of the BVDV-2 isolates by negative staining and ultra-thin sections from the infected MDBK cells under the TEM. A: the virion for negative staining; B: the virion in the endocytoplasmic reticulum of infected MDBK cell.

RESULTS AND DISCUSSION

Altogether, 16 positive samples were detected and confirmed by combined methods of both sandwich ELISA and the specific BVDV-2-directed RT-PCR. The 16 MDBK cells-infected BVDV isolates were isolated from the positive blood (serum) samples respectively, which reacted specifically with the mouse-originated polyclonal antibody against recombination E2 glycoprotein from BVDV-2 and monoclonal antibody (MAbBz-53) specific to BVDV-2 with fluoresce lights within the cytoplasms in the indirect immunofluorescence assay, and no reactivity with MAb D89 specific to BVDV-1. None of them could cause characteristic cytopathic effect (CPE) for serial passages in BVDV-infected MDBK cells for up to 10 generations. By preparing the ultra-thin sections from the isolates-infected MDBK cells (Wang et al., 2000), there were virus-like particles, roughly about the size of 60 nm in diameter located in endoplasmic reticulums observed (Figure 1B) and the morphology of negative staining of phosphotungstic acid for the purified virions were round, enveloped, with size of 60 nm in diameter under the transmission electronic microscope (TEM) (Figure 1A).

Based on the sequence data of the 5'-UTR, N^{pro}, and E2 genes from the 16 isolates, the comparison analysis results showed that their nucleotide sequence identity were higher than 99.0%, they were very closely related to BVDV-2. Very probably, the sixteen isolates may originate from the same BVDV-2 strain. The 5'-UTR and N^{pro} genes have proved to be well-suited for the study of genetic relationships within the genus *Pestivirus* (Becher et al., 1995). As shown in phylogenetic tree of 5'-UTR (Figure.2),

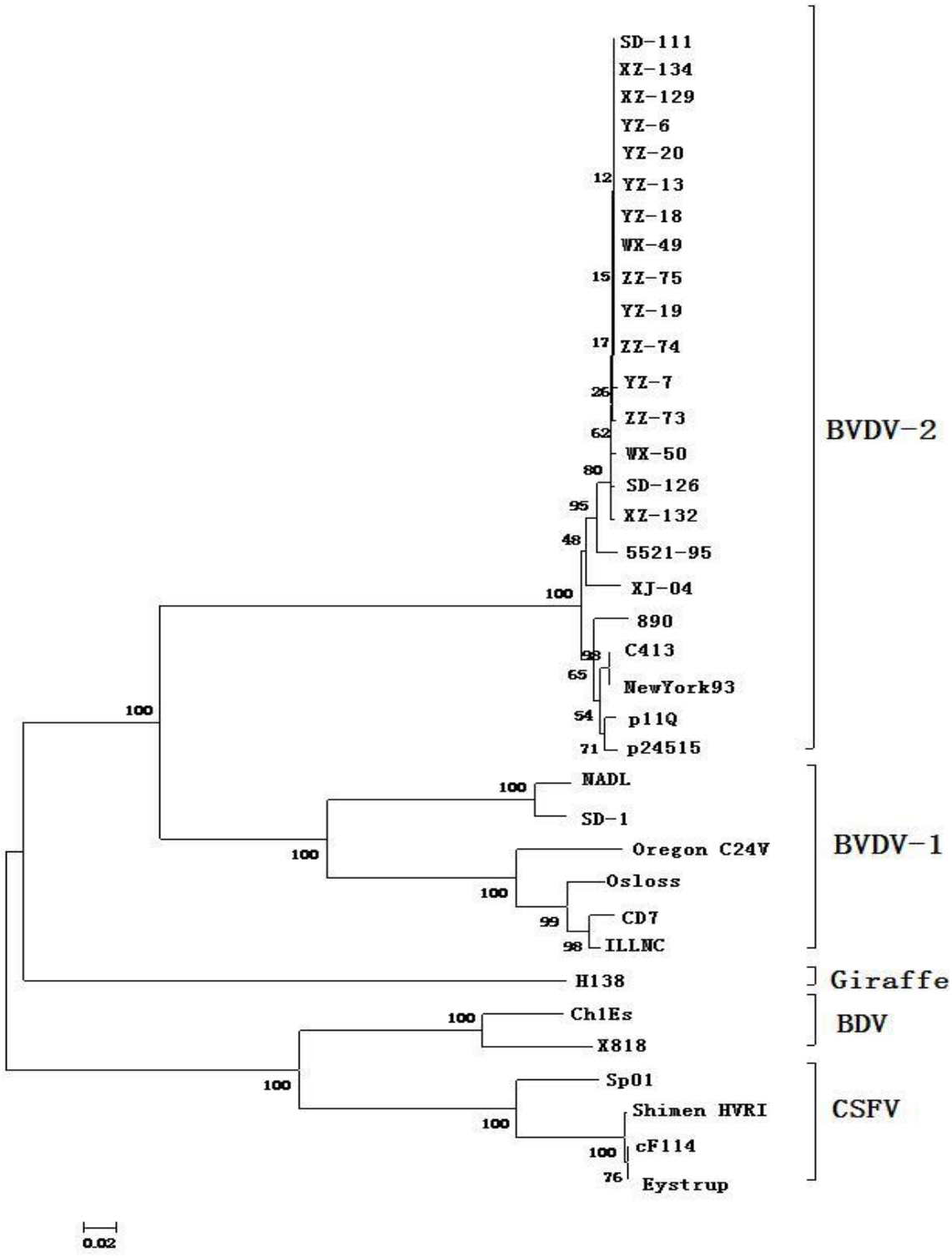

Figure 2. Genetic typing of 5' -UTR nucleotide sequences. SD-111, XZ-134, XZ-129, YZ-6, YZ-20, YZ-13, YZ-18, WX-49, ZZ-75, YZ-19, ZZ-74, YZ-7, ZZ-73, WX-50, SD-126, XZ-132 were the strains isolated in this survey.

the results confirmed that all the sixteen isolates belonged to BVDV-2. We also conducted the phylogenetic tree of N^{pro} which showed that the highest similarity was observed with BVDV2-AzSpin strain from USA (data not shown). In addition, comparative analysis of E2 gene revealed that the highest nucleotide sequence

identity 94.7 to 95.1% and amino acid identity 92.5 to 93.3% was found with the BVDV-2 strain AzSpin. In comparison with the Chinese strain XJ-04 which is the first BVDV-2 strain identified in China (Zhu et al., 2009a), the nucleotide and amino acid identity were 91.9 to 92.2%, 90.9 to 91.4%, respectively. Interestingly, the 16 BVDV-2 isolates showed much closer relationship to the USA strain rather than the Chinese strain. Amino acid sequence comparison between the strain AzSpin and XJ-04 revealed that the glycoprotein E2 of the sixteen isolates contained five potential N-glycosylation sites (Asn-X-Ser or Asn-X-Thr) located at the same sites. However, there is a little difference in one of the five potential N-glycosylation sites between 16 BVDV-2 isolates, AzSpin and XJ-04 strains, which is Asn-Thr-Ser in 16 BVDV-2 isolates and Asn-Ala-Ser in AzSpin and XJ-04 strains.

BVDV of both genotypes may occur in cytopathogenic (CP) and non-cytopathogenic (NCP) forms, classified according to whether or not it produces visible change in cell cultures (Xue at al., 2010). Generally, the existence of insertions within the region coding for the NS2/3 is a sign of cp-BVDV (Baroth et al., 2000; Quadros et al., 2006; Becher et al., 2002). In order to confirm whether there were insertions in the 16 isolates or not, primers (Space-P2: GTTCATAATCATAGCAGTGGTCGCC; Space-P1: GTGGCAAGGTGGCAGGTTCTCT) were designed according to the NS2/3 genes in GenBank. The PCR results showed there were no insertions within the NS2/3 genes, while a 228 nucleotide insertion appeared in 890 (data not shown). This is in agreement with the report by Ridpath et al. (2006).

From this study, we confirmed that BVDV-2 infection is widespread in China and all the BVDV-2 strains isolated, clustered within the same genotype. Currently, there are not enough data on epidemiology and distribution of BVDV-2 in Asia (Kadir et al., 2008). Hence, it will be valuable to continue to characterize more isolates from different regions of China as well as from neighbouring countries to clarify epidemiological patterns in that area, which will also help to understand global epidemiology of BVDV. The genetic data presented in this work enrich the general knowledge about the prevalence of BVDV-2 isolates circulating in China. All this attempts will be useful for the development of molecular diagnostic assays for BVDV infection in order to control and prevent this disease.

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REFERENCES

Arias P, Orlich M, Prieto M, Cedillo Rosales S, Thiel HJ, Alvarez M, Becher P (2003). Genetic heterogeneity of bovine of bovine viral diarrhoea viruses from Spain. J. Vet. Microbiol., 96: 327-336.

Baroth M, Orlich M, Thiel HJ, Becher P (2000). Insertion of cellular NEDD8 coding sequences in a pestivirus. J. Virol., 278: 456-466.

Becher P, König M, Paton DJ, Thiel HJ (1995). Further characterization of border disease virus isolates: evidence for the presence of more than three species within the genus *Pestivirus*. J. Virol., 209: 200-206.

Becher P, Orlich M, Shannon AD, Horner G, König M, Thiel HJ (1997). Phylogenetic analysis of pestiviruses from domestic and wild ruminants. J. Gen. Virol., 78: 1357-1366.

Becher P, Thiel HJ, Collins M, Brownlie J, Orlich M (2002). Cellular sequences in pestivirus genomes encoding gamma aminobutyric acid (A) receptor-associated protein and golgi-associated ATPase enhancer of 16 Kilodaltons. J. Virol., 76: 13069-13076.

Collett MS, Larson R, Gold C, Strick D, Anderson DK, Pruchio AF (1988). Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. J. Virol., 165: 191-199.

Couvreur B, Letellier C, Collard A, Quenon P, Dehan P, Hamers C, Pastoret PP, Kerkhofs P (2002). Genetic and antigenic variability in bovine viral diarrhea virus strain SD-1. J. Virol., 191: 867-879.

Meyers G, RuÈmenapf T, Thiel HJ (1989). Molecular cloning and nucleotide sequence of the genome of hog cholera virus. J. Virol., 171: 555-567.

Moerman A, Straver PJ, de Jong MC, Quak J, Baanvinger T, van Oirschot JT (1993). A long term epidemiological study of bovine viral diarrhoea infections in a large herd of dairy cattle. J. Vet. Rec., 132: 622-625.

Flores EF, Gil LHVG, Bottn SA, Weiblen R, Ridpath JF, Kreutz LC, Pilati C, Driemeyer D, Moojen V, Wendelstein AC (2000). Clinical, pathological and antigenic aspects of bovine viral diarrhea virus (BVDV) type 2 isolates identified in Brazil. J. Vet. Microbiol., 77: 175-183.

Flores EF, Ridpath J, Weiblen R, Vogel FSF, Gil LHVG (2002). Phylogenetic analysis of Brazilian bovine viral diarrhea virus type 2 (BVDV-2) isolates: Evidence for a subgenotype within BVDV-2. J. Virus Res., 87: 51-60.

Juliá S, Craig MI, Jiménez LS, Pinto GB, Weber EL (2009). First report of BVDV circulation in sheep in Argentina. J. Prev. Vet. Med., 90: 274-277.

Kim IJ, Hyun BH, Shin JH, Lee KK, Cho KO, Kang MI (2006). Identification of Bovine Viral Diarrhea Virus type 2n Korean native goat (*Capra hircus*). J. Virus Res., 121: 103-106.

Paton DJ, Lowings JP, Sands JL, Smith JE, Ibata G, Edwards S (1995). A proposed division of the pestivirus genus using monoclonal antibodies, supported by cross-neutralisation assays and genetic sequencing. J. Vet. Res., 26: 82-109.

Pellerin C, van den Hurk J, Lecomte J, Tussen P (1994). Identification of a new group of bovine diarrhea virus strains associated with severe outbreaks and high mortalities. J. Virol., 203: 260-268.

Quadros VL, Mayer SV, Vogel FS, Weiblen R, Brum MC, Arenhart S, Flores EF (2006). A search for RNA insertionsand NS3 gene duplication in the genome of cytopathic isolates of bovine viral diarrhea virus. J. Braz. J. Med. Biol. Res., 39: 935-944.

Ren M, Jiao HH, Jiang Y, Liu ZH, Wang CB, Zhu GQ (2008). Establishment of RT-PCR assay for detection of bovine viral diarrhea virus genotype 2. J. Chin. J. Prev. Vet. Med., 9: 665-668.

Ridpath JF, Bolin SR (1995). The genomic sequence of a virulent bovine viral diarrhea virus (BVDV) from the type 2 genotype: Detection of a large genomic insertion in a noncytopathic BVDV. J. Virol., 212: 39-46.

Ridpath JF, Neill JD, Vilcek S, Dubovi EJ, Carman S (2006). Multiple outbreaks of severe acute BVDV in North America occurring between 1993 and 1995 linked to the same BVDV 2 strain. J. Vet. Microbiol., 114: 196-204.

Sakoda Y, Ozawa S, Damrongwatanapokin S, Sato M, Ishikawa K, Fukushho A (1999). Genetic heterogeneity of porcine and ruminant pestiviruses mainly isolated in Japan. J. Vet. Microbiol., 65: 75-86.

Saitou N, Nei M (1987). The neighbour-joining method: a new method

for reconstructing phylogenetic trees. J. Mol. Biol. E., 4: 406-409.
Wakely PR, Turner JLE, Ibata G, King SP, Sandvik T, Howard P, Drew TW (2004). Characterisation of a type 2 bovine viral diarrhoea virus isolated from cattle in the UK. J. Vet. Microbiol., 102: 19-24.
Wang Z, Min G, Li M, Teng J, Ding M (2000). Study on the morphological processing of classical swine fever virus in cultured cells. Wei Sheng Wu Xue Bao., 40: 237-242.
Xue E, Zhu YM, Zhu LC, Ren XG, Feng JK, Shi HF, Gao YR (2010). Genotyping of bovine viral diarrhea viruses from cattle in China between 2005 and 2008. J. Vet. Microbiol., 143: 379-383.
Zhu LQ, Ren M, Lin YQ, Ding XY, Zhang GP, Zhao X, Zhu GQ (2009). Identification of a Bovine viral diarrhea virus 2 isolated from cattle in China. J. Acta Virol., 53: 131-134a.
Zhu LQ, Lin YQ, Ding XY, Ren M, Tao J, Wang JY, Zhang GP, Zhu GQ (2009). Genomic sequencing and characterization of a Chinese isolate of Bovine viral diarrhea virus 2. Acta Virol., 53: 197-202b.