Front. Agric. China 2007, 1(3): 344–351
DOI 10.1007/s11703-007-0058-z

RESEARCH ARTICLE

ZHAO Yuelan, QIN Jianhua, GUO Hongbin, ZUO Yuzhu, ZHANG Baoning, ZHANG Lei

Cloning and sequence analysis of genetic variation on *NS*2-3 of bovine viral diarrhea virus (HB-DCZ) strain in Hebei Province, China

Abstract The objective of this research is to analyze the genetic characterization of a bovine viral diarrhea virus (BVDV) strain (HB-DCZ strain) isolated from China and describe its relationship with other BVDV strains. Special primers (forward: 5′- gagatctcgggaggtac -3′, reverse: 5′-cctctcggcatgatcccgaaa -3′) are used to amplify partial *NS*2-3 sequence of HB-DCZ strain by reverse transcription polymerase chain reaction (RT-PCR). The product of PCR is cloned into pMD18-T vector, and then transfected into JM109. The recombinant plasmids are extracted and identified by *Eco*R I and *Hin*d III enzyme digestion. The *NS* 2–3 nucleotide fragment of the isolated virus is sequenced, and then amino acid sequence is deduced. Nucleotide sequence and deduced amino acid sequence of the strain are analyzed and compared with other BVDV strains from Genebank with the aid of DNAstar software. The results show that the obtained fragment of HB-DCZ strain contains 665 bp nucleotides, which indicate that there is no insertion in the isolated virus genome. The homologies of nucleotide sequences show that HB-DCZ strain has 99.1%, 97.4%, 92.3%, 77%, 76.9%, 76.4% and 76.2%, sequence similarity with 184, ZM195, Osloss, Oregon C_{24}V, Singer, NADL, and S D-I, respectively. According to the nucleotide sequences of the obtained fragments, the 208 corresponding amino acids are deduced. The homologies of amino acid sequences show that HB-DCZ strain had 100%, 93.3%, 91.3% and 83.2% sequence similarity with VEDEVAC, Osloss, ILLC and Oregon C_{24}V, respectively. In conclusion, HB-DCZ strain has no exogenous sequence insertion, no gene recombination, no gene rearrangement and no gene deficiency. HB-DCZ strain is closely related to BVDV Osloss strain, and belongs to subtype Ib.

Keywords bovine viral diarrhea virus (BVDV), HB-DCZ virus strain, cloning, sequencing, *NS*2-3 gene

1 Introduction

Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus* of the family Flaviviridae, which also includes classical swine fever virus and Border disease virus (Collett, 1992; Wengler et al., 1995; Pringle, 1999). The BVDV strains exist as two biotypes, cytopathic (CP) and noncytopathic (NCP), based on their lytic activity in in vitro cultures (McClurkin et al., 1985; Meyers and Thiel, 1996). By means of genetic analysis, it was possible to segregate BVDV into genotypes and subgenotypes (Donis, 1995). There are two genotypes, types 1 and 2, based on specific genetic differences (Pellerin et al., 1994, Ridpath et al., 1994). Type 1 strain can be further divided into types 1a and 1b (Pellerin et al., 1994; van Rijin et al., 1997; Ridpath and Bolin, 1998). However, more extensive analyses indicated that there are at least 11 genogroups of BVDV-1 (Baule et al., 1997; Vilcek et al., 2001). In cattle populations, BVDV-1 strains are predominant in most parts of the world, whereas BVDV-2 only appears in North America (Pellerin et al., 1994; Ridpath et al., 1994), Europe (Wolfmeyer et al., 1997; Letellier et al., 1999) and Japan (Nagai et al., 1998).

Bovine viral diarrhea virus is widespread in cattle and is associated with significant economic losses to the livestock industry worldwide (Baker, 1987; Houe, 1999). Infection with BVDV may cause various symptoms such as depression, inappetance, oral erosions and ulcerations, diarrhea, respiratory disorders, and immunosuppression (Baker, 1995; Houe, 1995; Potgieter, 1995; Taniyama et al., 1995; Grooms et al., 1996). When pregnant cows are infected with BVDV, reproductive failure, such as abortion, teratogenic effects and stillbirths, as a result of transplacental infection will occur

Translated from *Chinese Agricultural Science Bulletin*, 2007, 23(3): 1–6 [译自: 中国农业通报]

ZHAO Yuelan (✉), GUO Hongbin, ZHANG Lei
College of Chinese Traditional Veterinary Science, Agricultural University of Hebei, Dingzhou 073000, China
E-mail: yuelanzhao@126.com

QIN Jianhua (✉), ZUO Yuzhu, ZHANG Baoning
College of Animal Science and Technology, Agricultural University of Hebei, Baoding 071001, China
E-mail: qjhqqq@126.com

according to the gestation age (Moennig and Liess, 1995). Importantly, virus infection during the first trimester of gestation may produce persistently infected (PI) calves that are immunotolerant to the infective virus. The PI calves continue to play a role as a very significant reservoir shedding large quantities of the virus in its lifetime and make mucosal diseases occur when contracting antigen similar cytopathic BVDV as a fatal form in contrast with single CP or NCP BVDV infection (Malmquist, 1968; Roeder and Drew, 1984; Nettleton and Entrican, 1995; Thiel et al., 1996), by which fatal watery-bloody diarrhea and ulcerations of the intestinal tract is characterized.

Studies estimated that the prevalence of PI animals was $1\% \pm 2\%$ and that of antibody-positive adult cattle was $60\% \pm 85\%$ in most countries of the world (Liess, 1990; Houe, 1999). In China, positive rates of antibodies against BVDV of serum samples from cattle were 46.15%–54.9% in most investigated provinces (Wang et al., 1993; Gao et al., 1999). However, up to now, few papers have been published on the genetic characterization of BVDV strain in China. Recently, BVD mainly occurred in dairy farms in Hebei Province of China, and a BVDV strain (HB-DCZ strain), was isolated from dejection and blood samples of diarrhea and aborted bovine on these farms (Zhao et al., 2006). Therefore, this study was designed to analyze the genetic characteristics of HB-DCZ strain and describe its relationship with other BVDV strains.

2 Materials and methods

2.1 Virus strain

Bovine viral diarrhea virus standard strain OregonC_{24}V, as a reference strain, was obtained from China Institute of Veterinary Drug Control. BVDV (HB-DCZ strain), which may cause cytopathic effects (CPE) in Madin–Darby bovine kidney (MDBK) cells, was isolated from cases of the mucosal disease in diarrhea and aborted cattle in dairy farms in Hebei Province of China (Zhao et al., 2006).

2.2 MDBK cells

Madin–Darby bovine kidney (MDBK) cells were obtained from the China Institute of Veterinary Drug Control. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and nonessential amino acids. The cultured MDBK cells used for virus propagation and the serum used for cell growth were tested and found free from pestivirus contamination.

2.3 Design and synthesis of primers

A pair of genus-specific primers was designed according to the published *NS*2-3 genetic sequences (OrengonC_{24}V) by Primer 5.0 software, synthesized by TaKaRa (Dalian) Biotechnology Co., Ltd. (China). Expected amplified fragment was located on nucleotide sequence alignment 4583–5247 regions of BVDV OregonC_{24}V strain. The forward primer and reverse primer were located on both sides of exogenous insertion sequence domain of BVDV reference Osloss and NADL strain, respectively. Expected amplified fragment was 893 bp (Osloss strain, with insertion sequence), 935 bp (NADL, with insertion sequence) and 665 bp (with no insertion sequence and NCP strain). Primer sequences are listed below:

forward primer p1: 5′- gagatctcgggaggtac -3′,
reverse primer p2: 5′- cctctcggcatgatcccgaaa -3′.

2.4 Multiplication of viruses

OregonC_{24}V and isolated HB-DCZ virus were inoculated to MDBK monolayer cells. After adsorbing for 1–2 h, maintain liquid (DMEM) was added to the cell flask. The viruses were further propagated in MDBK cell lines for 72 h, then harvested and stored at −70°C after freezing and thawing three times.

2.5 Extraction of RNA of BVDV genome

Total RNA viruses was isolated from infected cells using TRNzol according to the protocol indicated in the TRNzol Reagent kit (TIANGEN BIOTECH (BEIJING) CO., LTD.). The RNA pellet was rinsed with 70% ethanol and resuspended in 50 μL diethylpyrocarbonate (DEPC)-treated water, and stored at −70°C. All procedures of RNA manipulation were performed using disposable pipettes, tubes and DEPC-treated water.

2.6 Synthesis of the first-strand cDNA

The cDNA synthesis was carried out according to the protocol of the supplier with M-Mulv (Promega). Briefly, in 1.5 mL Eppendorf tube, 5 μL viral RNAs mixed with 1 μL primer2 and DEPC-ddH_2O were incubated at 70°C for 5 min to melt secondary structure within the template and cooled immediately on ice for 2 min to prevent secondary structure from reforming, then mixed gently by flicking the tube, followed by adding 10 μL Reverse Transcriptase buffer, 1.25 μL 10 mmol deoxynucleoside triphosphate (dNTP), 0.5 μL RNAase inhibitor (40 U/μL), 1 μL M-MLV RT (200 U/μL), DEPC-ddH_2O up to 25 μL. The tubes were then incubated at 42°C for 60 min, 95°C for 5 min, and chilled on ice.

2.7 PCR amplification of *NS*2-3 genomic fragment

With the first-strand DNA as a template, the cDNA fragment was amplified by Taq DNA polymerase adopting 25 μL reaction system. The optimal reaction mixture contained 5 μL template, 2.5 μL $10 \times$ PCR buffer (Mg^{2+} plus, 2 μL dNTP Mixture (2.5 mmol of each), 1 μL of each primer (p1/p2 (20 μmol) and 0.25 μL Taq DNA polymerase (5 U/μL), DEPC-ddH_2O up to 25 μL. The amplifications were

performed in a program that involved 30 cycles as follows: 3 min at 94°C for predenaturation, 30 sec at 94°C for denaturation, 45 sec at 54°C for annealing, 45 sec at 72°C for elongation, and 8 min at 72°C for the last step of prolonged elongation. PCR products were visualized under UV light on ethidium bromide-stained 1% agarose gel after electrophoresis. The references of OregonC_{24}V and MDBK culture were used as controls.

2.8 Cloning and identification of amplified target fragment

Polymerase chain reaction (PCR) products are usually presented by individual electrophoretic bands. The amplified target fragments with the expected size were excised from the gel and purified using the PCR Fragment Agarose Gel DNA Purification Kit (TaKaRa (Dalian) Biotechnology Co., Ltd.) according to manufacturer's instructions. Purification fragments were cloned into the pMD18-T Vector, and then transfected into JM109, cultivated at 37°C for filtration of blue and white clone. Original independent white bacterial colonies were selected and then cultured. The resulting recombinant plasmids were extracted by Plasmid Small-scale Extraction Kit (centrifugation columniation type, TIANGEN BIOTECH (BEIJING) CO., LTD.) and identified by *Eco*R I/*Hin*d III enzyme digestion and PCR amplification.

2.9 Sequencing and analysis of *NS*2-3 genomic fragment

The fragment of *NS*2-3 gene of the isolated virus HB-DCZ strain was sequenced in both directions using the p1 and p2 primers on an ABI DNA sequencer (Pekin Elmer). It was performed by TaKaRa (Dalian) Biotechnology Co., Ltd. Nucleotide sequence and deduced amino acid sequence of the strain were analyzed and compared with other BVDV strains from Genebank with the aid of DNAstar software.

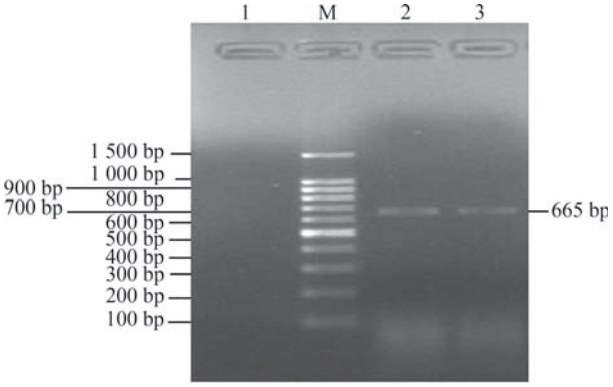
3 Results

3.1 Result of reverse transcription polymerase chain reaction (RT-PCR) of *NS*2-3 genomic fragment of BVDV (HB-DCZ) strain

A band of specific BVDV *NS*2-3 genomic fragment was visualized in OregonC24V and HB-DCZ strains by 1.0% agarose gel electrophoresis. The obtained specific band approximated 665 bp (Fig. 1), which was consistent with the expected. Therefore, the genome of the cpBVDV isolates examined did not contain insertions within the *NS*2-3 in the region comprised between primers 1 and 2, as was ascertained by agar gel electrophoresis of PCR products.

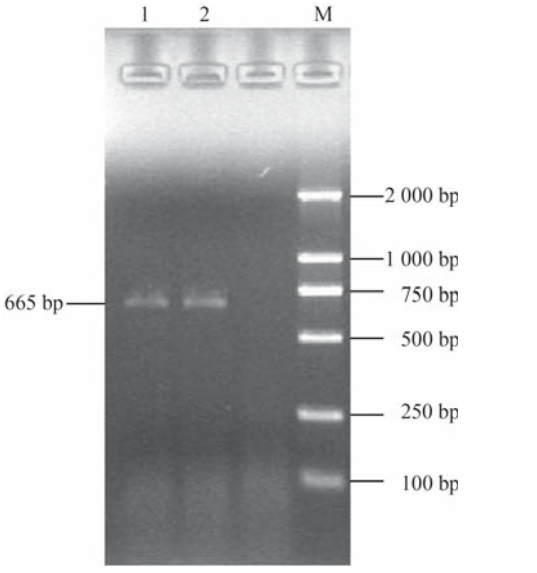
3.2 Result of cloning and identification of amplified target fragment

Polymerase chain reaction amplification resulted in a specific band with the recombinant plasmids as a template and with primers p1/ p2. The target fragment of isolated HB-DCZ strain approximated 665 bp (Fig. 2), which was consistent with anticipation. *Eco*R I and *Hin*d III digestion of the recombinant plasmids resulted in two bands with sizes of 702 bp and 2635 bp (Fig. 3) that were consistent with the theoretical values.



Note: 1 stands for MDBK culture, 2 for isolated HB-DCZ strain, 3 for BVDV standard strain OregonC_{24}V, and M for 100 bp ladder marker.

Fig. 1 Result of RT-PCR

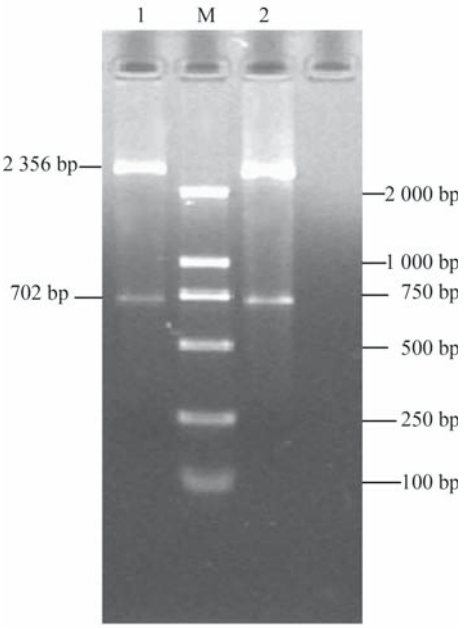


Note: 1 stands for PCR product of recombinant plasmid DNA, 2 for PCR product of genome DNA, and M for DL2000 DNA marker.

Fig. 2 PCR identification of recombinant plasmid

3.3 Results of sequence and analysis of cloned target fragment

The sequence result showed that the cloned *NS*2-3 fragment of HB-DCZ strain was 665 bp. It was consistent with the expected value in length. The sequence *NS*2-3 fragment of HB-DCZ strain differed from other BVDV strains. The HB-DCZ virus strain had no exogenous sequence insertion, gene recombination, gene rearrangement or gene deficiency.



Note: 1 and 2 stand for results of recombinant plasmid enzyme digestion, and M for DL2000 DNA marker.

Fig. 3 Recombinant plasmid digested by *Hin*d III/ *Eco*R I

However, some nucleotide sequences were replaced. It was consistent with internal report (Fig. 4). The result of the homologies of nucleotide sequences showed that HB-DCZ strain had 100%, 93.8%, 90.1% and 79.8% sequence similarity with VEDEVAC (AJ544866), Osloss (M96687), ILLC (U86599) and Oregon C_{24}V (AF09165), respectively.

3.3.1 Deduced amino acid sequence

Coding protein of *NS*2-3 genetic domain of HB-DCZ strain was composed of 208 amino acids, containing 38 alkaline amino acids (HKR), 24 acidic amino acids (DE), 77 water repulsion amino acids (AVILFW), and 34 polarity amino acids (STCYNQ). The result of the homologies of amino acid sequences showed that HB-DCZ strain had 100%, 93.3%, 91.3% and 83.2% sequence similarity with VEDEVAC (AJ544866), Osloss (M96687), ILLC (U86599) and Oregon C_{24}V (AF09165), respectively (Fig. 5).

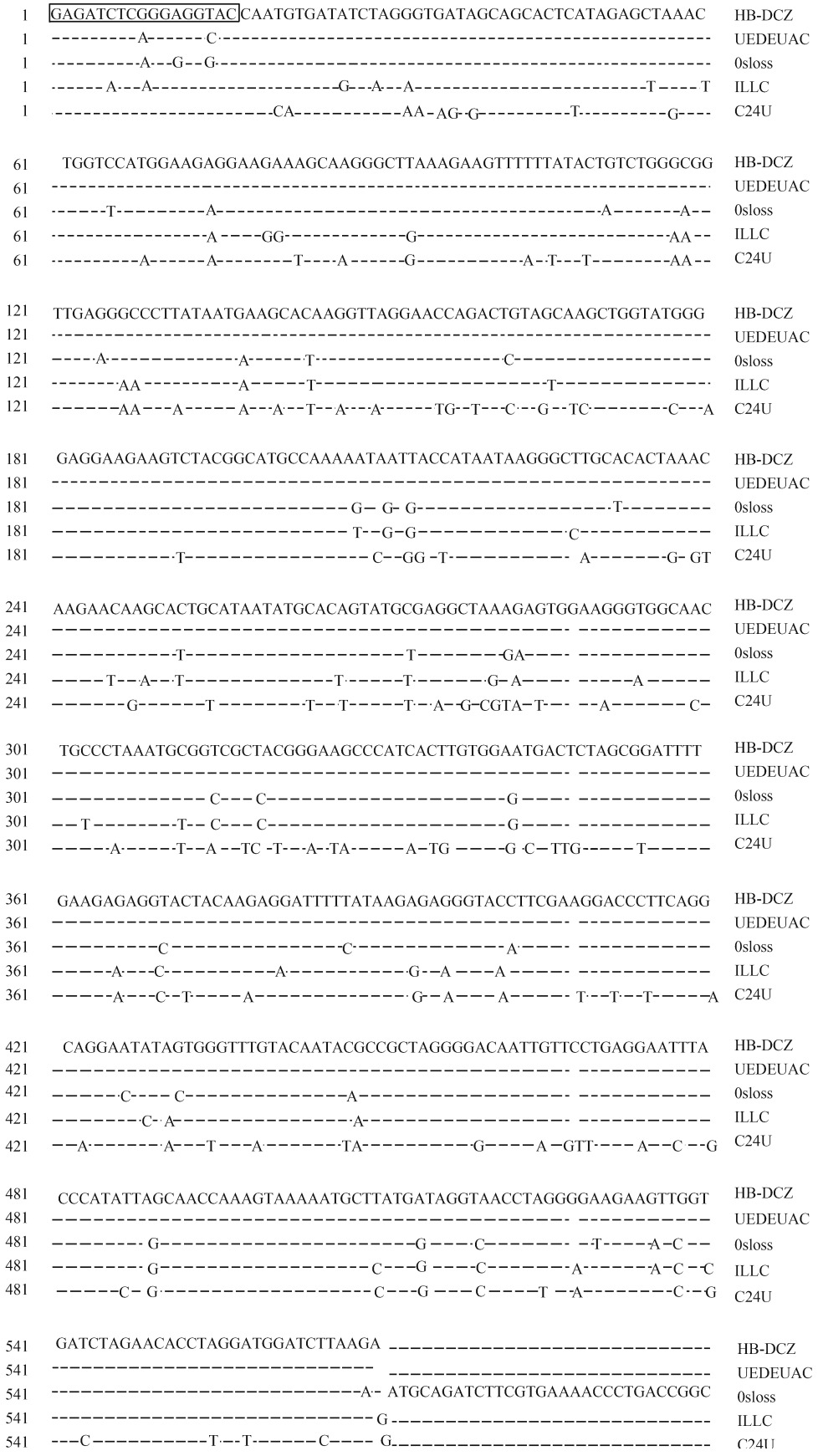
3.3.2 Phylogenetic tree analysis of BVDV strains

The homologies of nucleotide sequence of *NS*2-3 genetic domain of HB-DCZ strain with other BVDV strains were 100% (VEDEVAC), 99.1% (184), 97.4% (ZM195), 95.4% (H), 92.3% (Osloss), 89.5% (ILLC), 83.1% (Draper), 77.5% (3142), 77% (OregonC_{24}V), 76.9% (Singer), 76.4% (NADL), 76.2% (SD-I), 75.9% (3887), 75.6% (D) and 74.2% (YAK), respectively. The HB-DCZ was closely related to BVDV strains EDEVAC, Osloss, ILLC, 184, H and ZM195 (Fig. 6).

4 Discussion

The special primers were designed according to the *NS*2-3 genetic sequences, located on nucleotide sequence alignment 4583–5247 regions of BVDV OregonC_{24}V strain. This region contained exogenous insertion, rearrangements, including duplication in majority of BVDV. The length of amplified fragment of this region revealed that isolates contained RNA insertions and gene duplication was not in the *NS*2-3 gene. The theoretical length of 665 bp of the expected amplified fragment showed that it had no exogenous sequence insertion, gene recombination or gene rearrangement. The genome of the cpBVDV Osloss strain contained an integration of a cellular sequence—an ubiquitin-coding sequence (Wang et al., 1996). The cellular homologue inserted into the NADL genome, termed *cIns*, has been recently identified. The expected amplified fragment was 893 bp (Osloss strain, with insertion sequence), 935 bp (NADL, with insertion sequence), respectively. In the present study, our results showed that the length of amplified fragment of this region of the HB-DCZ isolates was 665 bp. The result indicated that the HB-DCZ isolates did not contain detectable insertions or gene duplication within the region examined *NS*2-3 gene (Qi et al., 1992; Ridpath and Neill, 2000). In the present study, the homologies of nucleotide sequences showed that HB-DCZ strain had 100%, 99.1%, 97.4%, 95.4%, 92.3%, 89.5%, 83.1%, 77.5%, 77%, 76.9%, 76.4%, 76.2%, 75.9%, 75.6% and 74.2% sequence similarity with VEDEVAC, 184, ZM195, H, Osloss, ILLC, Draper, 3,142, Oregon C_{24}V, Singer, NADL, S D-I, 3,887, D and YAK, respectively. The homologies of amino acid sequences showed that HB-DCZ strain had 100%, 93.3%, 91.3% and 83.2% sequence similarity with VEDEVAC, Osloss, ILLC and Oregon C_{24}V, respectively. Therefore, all the results of our study proved that HB-DCZ strain had no exogenous sequence insertion, no gene recombination, no gene rearrangement, and no gene deficiency. HB-DCZ strain was closely related to BVDV EDEVAC strain, and belonged to subtype Ib (Neill and Ridpath, 2001).

Two widely used cpBVDV strains (Osloss and NADL) were the first to reveal that cpBVDVs originate from their *ncp* counterparts by diverse genetic mechanisms, usually as a result of RNA recombination. Insertions of cellular sequences were accompanied or not by duplication of viral sequences, and duplications and rearrangements of viral sequences were identified in the genomes of cpBVDVs (Meyers et al., 1991; Tautz, et al., 1994; Vilcek et al., 2000). Efficient proteolytic release of nonstructural protein 3 (*NS*3) from the viral polyprotein was considered to be crucial for the cytopathogenicity of pestiviruses. Recently, a novel cytopathogenic (cp) bovine viral diarrhea virus strain (BVDV CP8) with a complex insertion composed of viral and cell-derived sequences, including two fragments of the cellular J-domain protein Jiv (J-domain protein interacting with viral protein) located in the N-terminal region of the polyprotein was described. BVDV CP8 expressed a Jiv fusion protein of 513 amino acids in addition


1 GAGATCTCGGGAGGTACCAATGTGATATCTAGGGTGATAGCAGCACTCATAGAGCTAAAC HB-DCZ
UEDEUAC
0sloss
ILLC
C24U
61 TGGTCCATGGAAGAGGAAGAAAGCAAGGGCTTAAAGAAGTTTTTTATACTGTCTGGGCGG HB-DCZ
UEDEUAC
0sloss
ILLC
C24U
121 TTGAGGGCCCTTATAATGAAGCACAAGGTTAGGAACCAGACTGTAGCAAGCTGGTATGGG HB-DCZ
UEDEUAC
0sloss
ILLC
C24U
181 GAGGAAGAAGTCTACGGCATGCCAAAAATAATTACCATAATAAGGGCTTGCACACTAAAC HB-DCZ
UEDEUAC
0sloss
ILLC
C24U
241 AAGAACAAGCACTGCATAATATGCACAGTATGCGAGGCTAAAGAGTGGAAGGGTGGCAAC HB-DCZ
UEDEUAC
0sloss
ILLC
C24U
301 TGCCCTAAATGCGGTCGCTACGGGAAGCCCATCACTTGTGGAATGACTCTAGCGGATTTT HB-DCZ
UEDEUAC
0sloss
ILLC
C24U
361 GAAGAGAGGTACTACAAGAGGATTTTTATAAGAGAGGGTACCTTCGAAGGACCCTTCAGG HB-DCZ
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C24U
421 CAGGAATATAGTGGGTTTGTACAATACGCCGCTAGGGGACAATTGTTCCTGAGGAATTTA HB-DCZ
UEDEUAC
0sloss
ILLC
C24U
481 CCCATATTAGCAACCAAAGTAAAAATGCTTATGATAGGTAACCTAGGGGAAGAAGTTGGT HB-DCZ
UEDEUAC
0sloss
ILLC
C24U
541 GATCTAGAACACCTAGGATGGATCTTAAGA HB-DCZ
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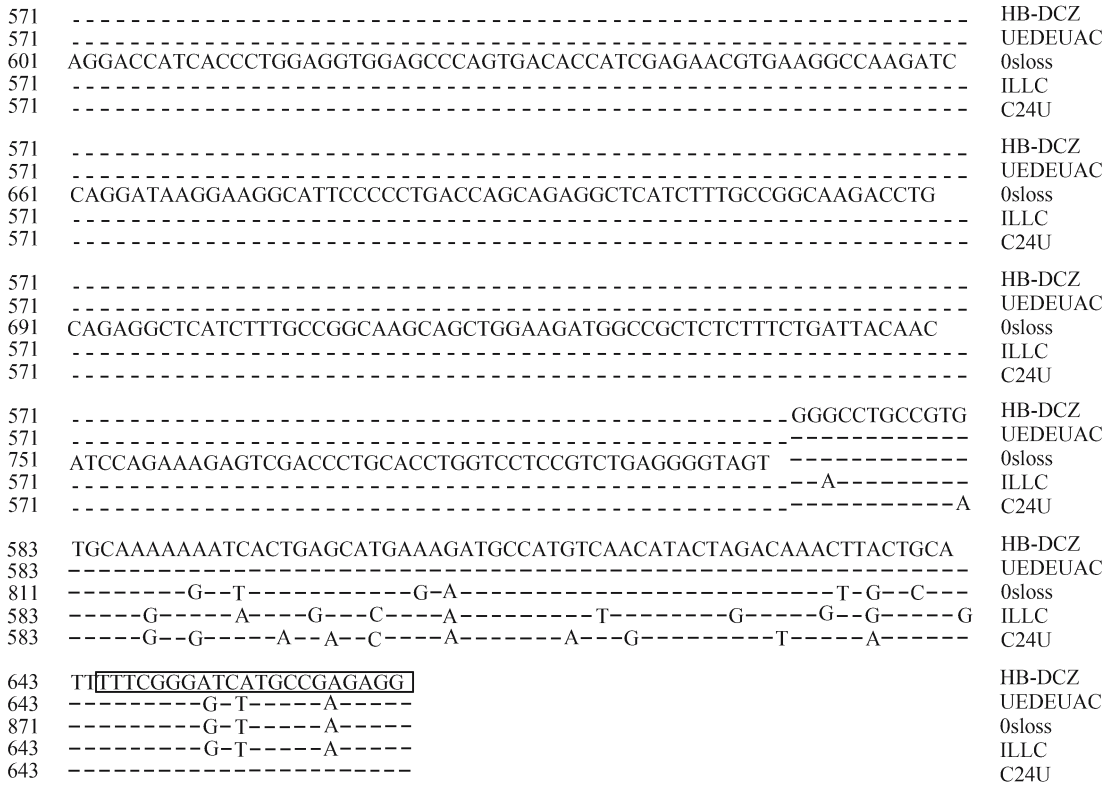

Fig. 4 Comparison of nucleotide sequences of *NS*2-3 gene of HB-DCZ strain with other BVDV strains

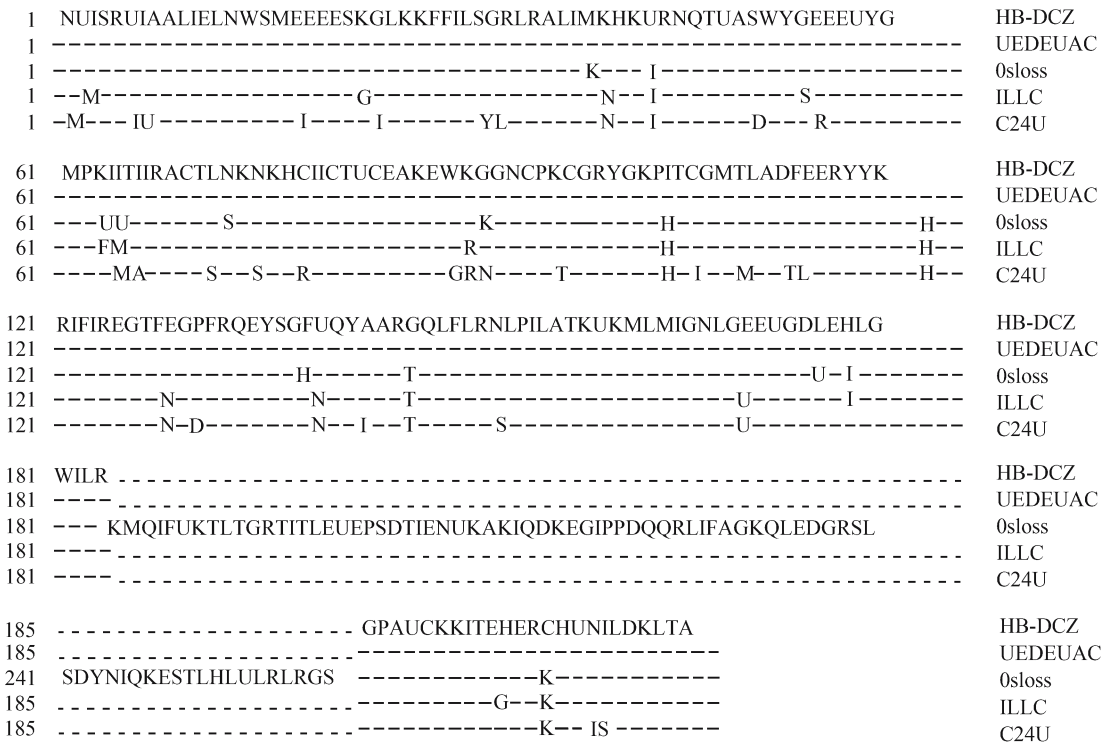

Fig. 5 Comparison of amino acid sequences of *NS*2-3 gene of HB-DCZ with other BVDV strains

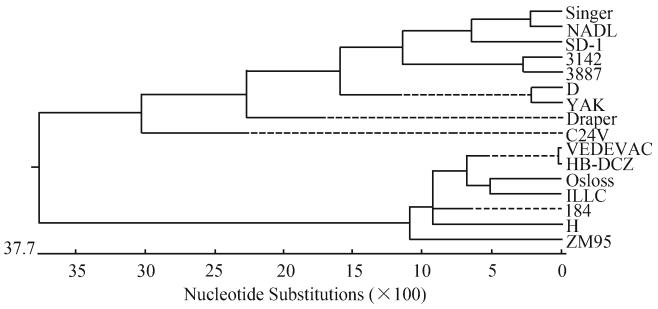

Fig. 6 Phylogenetic tree analysis of various BVDV strains

to a complete set of viral proteins. This protein had the capacity to induce *NS*2-3 cleavage in trans. Accordingly, CP8 was a representative of a novel type of cp pestivirus with a cp-specific mutation located outside of the *NS*2-3 gene (Muller et al., 2003). BVDV CP8 contained cellular insertions and viral sequence duplications in the N-terminal region of the polyprotein and not in the *NS*2-3 boundary as most isolates characterized thus far. Thus, it is tempting to speculate that the number of mechanisms of generation of *NS*3 will increase as more cpBVDV isolates are characterized. In genomes containing a duplicated *NS*3 gene, the expression of *NS*3 polypeptide is believed to occur by direct translation of the duplicated gene (Vilcek et al., 2000; Muller et al., 2003). The study on genome of cpBVDV Oregon, a cp strain whose genome did not contain *NS*2-3 insertions or *NS*3 duplication, revealed a novel mechanism of *NS*3 generation. In the genome of BVDV Oregon and other cp isolates including the Singer strain, the information necessary for *NS*2-3 processing resides within the *NS*2 gene and probably involves a set of point mutations that somehow affect the cleavability of *NS*2-3. These results demonstrated that, in addition to RNA recombination, a different genetic mechanism, point mutations within the *NS*2 gene, might be responsible for the production of cpBVDV. The study of a large number of cpBVDV isolates indicated that this mechanism seemed to be more frequent than previously expected (Kummerer et al., 1998; Kummerer et al., 2000). Here, the cpBVDV HB-DCZ strain had no *NS*2-3 exogenous sequence insertion, gene recombination, gene rearrangement or gene deficiency. The results presented that there may be additional mechanisms of transform of biologytype of the viral genomes (cp/ncpBVDV)

Acknowledgements This research was supported by the National Science and Technology Tackle Key Problem Project Foundation of China (No. 2006 BAD04A10) and Science and Technology Research Project Foundation of Hebei Bureau of Animal Science (No. 2002-4).

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