Acta virologica 53: 197 – 202, 2009 doi:10:4149/av_2009_03_197

Genomic sequencing and characterization of a Chinese isolate of Bovine viral diarrhea virus 2

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Received February 2, 2009; accepted June 25, 2009

Summary. – The complete genomic sequencing and characterization of Bovine viral diarrhea virus (BVDV) isolate XJ-04 originated from cattle in China was described. The genomic RNA of the isolate was 12,284 nt long and contained short 5'- untranslated region (UTR), 3'-non-coding regions (NCR), and one open reading frame (ORF) encoding a large polyprotein of 3,895 amino acids with 20 potential N-glycosylation sites. The identity of the isolate XJ-04 with reference strains NADL (BVDV-1) and 890 (BVDV-2) in autoprotease (N^{pro}) gene and structural genes (C, E^{rns}, E1, E2) was analyzed. The percentage of nt and aa identity in analyzed genes indicated that the isolate XJ-04 was closer to the reference strain 890 (BVDV-2) than to NADL (BVDV-1). Phylogenetic analysis revealed that the isolate belonged to BVDV-2a subtype. Furthermore, comparison analysis indicated that the isolate XJ-04 did not contain any genomic insertions that can be directly related to the cytopathic phenotype.

Keywords: BVDV-2; cytopathic effect; genomic sequencing; phylogenetic analysis;

Bovine viral diarrhea viruses 1 and 2 are important pathogens of cattle and cause severe economic losses primarily due to the decreased fertility, abortion, diarrhea, respiratory symptoms, and subclinical or persistent infection (Hamers *et al*., 1998; Houe 1999, 2003). BVDV together with Border disease virus (BDV), Classical swine fever virus (CSFV) and tentative Pestivirus of giraffe constitute the genus *Pestivirus* of the family *Flaviviridae* (Fauquet *et al*., 2005).

BVDV consists of a single-stranded positive-sense RNA genome of approximately 12.3 kb in length with a single large ORF flanked by 5'-UTR and 3'-NCR (Deng and Brock, 1992). The ORF encodes a polyprotein of about 3,990 aa that is cleaved into mature viral protein by cellular protease or autoprotease. The order of viral proteins in the genome is as follows: N^{pro}-C-E^{rns}-E1-E2-p7-NS2/3- NS4A-NS4B-NS5A-NS5B (Rümenapf *et al*., 1993; Tautz *et al*., 1997; Collet *et al*., 1988).

Based on the nucleotide sequence of 5'-UTR, the two genotypes BVDV-1 and BVDV-2 are recognized (Ridpath *et al*., 1994; Tajima *et al*., 2001; Vilcek *et al*., 2001; Couvreur *et al*., 2002). BVDV-1 can be divided into at least 11 genetic groups (Vilcek *et al*., 2001; Jackova *et al*., 2008). BVDV-2 isolates were classified into two subtypes termed as BVDV-2a and BVDV-2b (Flores *et al*., 2002). Initially, BVDV-2 viruses were identified in severe outbreaks of acute bovine viral diarrhea disease in North America in late 1980s (Perdrizet *et al*., 1987; Corapi *et al*., 1989). Now, BVDV-2 infection is widely distributed in Asia, namely in Japan (Nagai *et al*., 2001), Korea (Kim *et al*., 2006), and India (Mishra *et al*., 2008). Recently, a BVDV-2 strain isolated from cattle in China has been reported (Zhu *et al*., 2009).

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Abbreviations: BDV = Border disease virus; BVDV-1, 2 = Bovine viral diarrhea virus 1 and 2; ORF = open reading frame; NCR = non-coding region; UTR = untranslated region; NS = non-structural protein; MDBK = Madin-Darby bovine kidney cells; cpBVDV = BVDV of cytopathic type; CSFV = Classical swine fever virus

Table 1. Primers used for the amplification of isolate XJ-04 genomic fragments

Fragment	Length[a] (bp)	Location[a] (nt)	Upstream primer	Downstream primer
A	282	108-389	5'-CAT GCC CAT AGT AGG AC-3′	5'-CCA TGT GCC ATG TAC AG-3'
B	773	142-914	5'-TAGCGGTAGCAGTGA GTCCAT TG-3'	5'-TAGCACCCTTACTCCCTTCATCG-3'
C	1089	690-1778	5'-CGCTGGAACTATGTAGGGAGGC-3'	5'-CCCTTGCTCCCTCAATGGTGT-3'
D	872	1511-2382	5'-CCGCTTARGG AGTGTGCTGTG-3'	5'-GGAAAGCTGTGGTGGTGGCA-3'
E	1017	2061-3087	5'-TGGTGGTTCTGTCTGACTTTGC-3'	5'-CAGTCCCTCTGAGTTGACGAAGT-3'
F	1015	2466-3580	5'-TCCCWGAATGCAARGAGGGMTTC-3'	5'-ACCCATRGCYATCTGCTCAG-3'
G	634	3481-4124	5'-GTTCATAATCATAGCAGTGGTCGCGTT-3'	5'-GTGGCAAGGTGGCAGGTTCTCT-3'
H	1576	3823-5398	5'-GGACCCATGCTTTCTTCTC-3'	5'-TACCGAGCTGATCCCTCCT-3'
I	996	4955-5950	5'-AGAAATGAAGCCGTCCACAG-3'	5'-TAGTTTGGTGGGCTTGGAAT-3'
J	1282	5689-6970	5'-CGACTCAGGGTGTCCCGAAGGT-3'	5'-TCTCCTATTGTGACTGCCATTCTC-3'
K	1010	6726-7735	5'-CCAATGCCATAGAGTCAG GTG Tv-3'	5'-CAAGCCTTCCTTTGTAGACTGG-3'
L	917	7580-8496	5'-AGGACTGACGGCAAGGACTC-3'	5'-TTTCCTATGGTCTGGACTGCTTC-3'
M	929	8243-9171	5'-TTGCTAGGTACGGGCATAAGT-3'	5'-TCTGGTCGGTCACCCAAATA-3'
N	1006	8928-9933	5'-GACCTCCAAACTTCAGAGTAACAA-3'	5'-ATTGCTGTTGTGGTTTTCCTCT-3'
O	1098	9716-10813	5'-AGAGCAAGAACGGCCAAGAATAT-3'	5'-GGCTGCTCCCTTACGGTTTATC-3'
P	1044	10582-11625	5'-TGCTGGTATTAGGCTGGAGAAAC-3'	5'-TCCTCAAATCTGTATGCCACTT-3'
Q	943	11506-12448	5'-AACAGAAAGAGGACTGGGATTGA-3'	5'-TGCTTAGTGCATTGGAGGTAGTG-3'
5'-3'-end	about 410		5'-AAGACTGAGGCTTATGCTGATGAC3'	5'-CAATGAACTCACTGCTACCGCTA-3'

[a]Primer locations and length were listed according to the strain 890 (Acc. No. U18059).

In November 2004, the tissue samples including the spleen, lymph node, liver, and kidney were collected from a 3-months old calf in small farm in Xinjiang province. The calf showed clinical symptoms of pestivirus infection such as severe diarrhea and conjunctival hemorrhage. A viral isolate XJ-04 was isolated from the collected samples in our laboratory and preliminarily identified as BVDV-2. The isolate was able to produce a cytopathic effect in the culture of Madin-Darby bovine kidney (MDBK) cells (unpublished data). Here, we report the complete genomic sequence of the isolate XJ-04 and compare it with the genomic sequences of other BVDV strains.

Seventeen pairs of primers named alphabetically from A to Q were designed, based on the information of 6 full-length genomic sequences of BVDV-2 available from GenBank (Acc. Nos. U18059, AF502399, AY149215, AF0 02227, AF145967, and AY149216). The primers were used to amplify 17 DNA fragments that overlapped each other for assembling the complete genome sequence of XJ-04 except the 5'- and 3'-ends.

To determine the 5'- and 3'-ends with the method of RNA ligation and PCR, a pair of primers was designed based on the known partial sequence near the 5'- and 3'-ends (Table 1).

Viral RNA was extracted from the supernatant of virus stocks using Trizol LS Reagent (Invitrogen) following the manufacturer's instructions. Isolated RNA was dissolved in RNase-free water and applied as template for the synthesis of the first strand cDNA. The RT step was carried out using the PrimeScriptTM 1st strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. 17 overlapping cDNA fragments were further amplified by PCR using Taq DNA polymerase (New England Biolabs) and appropriate primers (Table 1). PCR products were purified using the TIANgel Midi Purification Kit (Tiangen) and ligated into pMD-19 simple T vector (TaKaRa) according to the manufacturer's specifications and then transformed into *Escherichia coli* DH5α by a standard procedure. To obtain a precise sequence for each amplified region, 3 recombinant clones derived from 3 independent PCRs were selected for sequencing by commercial service (Shanghai Sangon Biological Engineering Technology & Services).

For precise determination of the 5'- and 3'-end sequences, the RNA ligation method was utilized prior to the first cDNA synthesis as described previously (Brock *et al*., 1992; Becher *et al*., 1998) with some modification. For this analysis, genomic RNA was prepared using the QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions. RNA (2 μg) was self-ligated via its 5'- and 3'-end using 120 U of T4 RNA ligase (New England Biolabs) in a 60 μl reaction mixture containing 50 mmol/l Tris-HCl (pH 7.8), 10 mmol/l $MgCl_2$, 10 mmol/l dithiothreitol, 1 mmol/l ATP, and 120 U RNasin (Promega) for 5 hrs at 37°C. After phenol-chloroform extraction and ethanol precipitation, the pellet was resuspended in 10 μl of RNase-free water. 3 μl of the solution were used as the template for amplification of the

Table 2. Potential N-linked glycosylation sites in structural proteins E^{ms}, E1, and E2 of reference strains SD-1, 890, and isolate XJ-04

Structural glycoprotein	Amino acid position SD-1 (BVDV-1)	Amino acid position 890 (BVDV-2)	Amino acid position XJ-04 (BVDV-2)
E^{ms}	272	272	N
	281	281	281
	296	296	296
	335	335	335
	365	365	365
	370	370	370
	413	413	413
	487	487	487
E1	N_1	N_1	516
	597	597	597
E2	809	807	807
	878	876	876
	922	920	920
	N_2	N_2	951
	990	988	988

N = not found at the position aa 272; N_1 = not found at the position aa 516; N_2 = not found at the position aa 951.

junction region of the 5'-3' self-ligated genomic RNA. Then six 5'-3'ligation clones were subjected to sequencing.

In total, 18 overlapping fragments covering the complete genome of XJ-04 were available ranging from 282 to 1,576 bp in size. All the nucleotide sequences were manually proof-read with the help of biosoftware DNASTAR (Dnastar) and assembled into consecutive sequence. Thus, the complete genome 12,284 nt long was generated containing a large ORF of 11,694 nt flanked with 5'-UTR of 385 nt and 3'-NCR of 205 nt. The ORF encoded a single polyprotein of 3,897 aa with the relative molecular mass M_r = 438.3 K. The genomic organization of isolate XJ-04 is consistent with that of other BVDV viruses (Collet *et al*., 1988; Deng and Brock 1992; Rümenapf *et al.*, 1993; Ridpath and Bolin 1995; Xu *et al*., 2006; Nováckova *et al.*, 2008). Based on the putative processing sites, the structure of the entire XJ-04 genome from 5'-UTR to 3'-NCR including nucleotide positions was as follows: 5'-UTR, 1–385; N^{pro}, 386–886; C, 887–1195; E^{rns}, 1196–1876; E1, 1877–2461, E2, 2462–3577; p7, 3578–3787; NS2/3: 3788–7195; NS4A, 7196–7387; NS4B, 7388–8428; NS5A, 8429–9919; NS5B; 9920–12076 ; 3'-NCR, 12077–12284. The complete genomic sequence of XJ-04 was deposited in GenBank (Acc. No. FJ527854).

The ORF encoded a single polyprotein of 3,897 aa with M_r of 438.3 K. Amino acid sequence comparison analysis showed that the point mutations are mainly distributed in the structural protein E^{rns}, E1, and E2 (data not shown). The polyprotein contained 20 potential N-glycosylation sites (Asn-X-Ser or Asn-X-Thr). Among those 14 glycosylation sites were located in the structural proteins E^{rns}, E1, and E2. Compared with strains 890 (BVDV-2) and SD-1 (BVDV-1), the isolate XJ-04 contained similar predicted glycosylation sites except variations at three positions, i.e. deletion sites at aa 272 in E^{rns}, aa 516 in E1 and one additional site at aa 951 in E2, respectively (Table 2).

For typing of the isolate XJ-04, 243 bp (genome position of 129–371 nt in the strain 890) of the sequences within 5'-UTR regions and the complete genomic sequences were used to perform a phylogenetic analysis. The sequences were processed with Bioedit software and used to generate the phylogenetic tree by biosofteware MEGA 4.1 using the neighbor-joining method (Hall, 1999; Kumar *et al*., 2008). Bootstrap probabilities were calculated with 1000 replicates. The phylogram constructed with complete genome sequences revealed that the isolate XJ-04 belonged to BVDV-2 (Fig. 1a). Furthermore, a tree constructed with the regions of 5'-UTR grouped the isolate into the branch of BVDV-2a (Fig. 1b). The region coding for viral autoprotease N^{pro} gene is also usually used for virus typing. Comparative analysis of the N^{pro} gene showed that nt and aa identity values were also closer to BVDV-2 reference strain 890 (87.8% and 98.4 %, respectively) than to BVDV-1 reference strain NADL (67.1% and 94.8%, respectively). Additionally, this classification was supported by the nt identity analysis of the complete genome. The genomic similarity to the strain 890 was 90.1%, but to the strain NADL was only 68.0%.

For further characterization of XJ-04, the nt and aa identity analysis in the structural proteins C, E^{rns}, E1 and E2 between XJ-04 and BVDV reference strains was performed. The identity shared by XJ-04 with strains 890 and

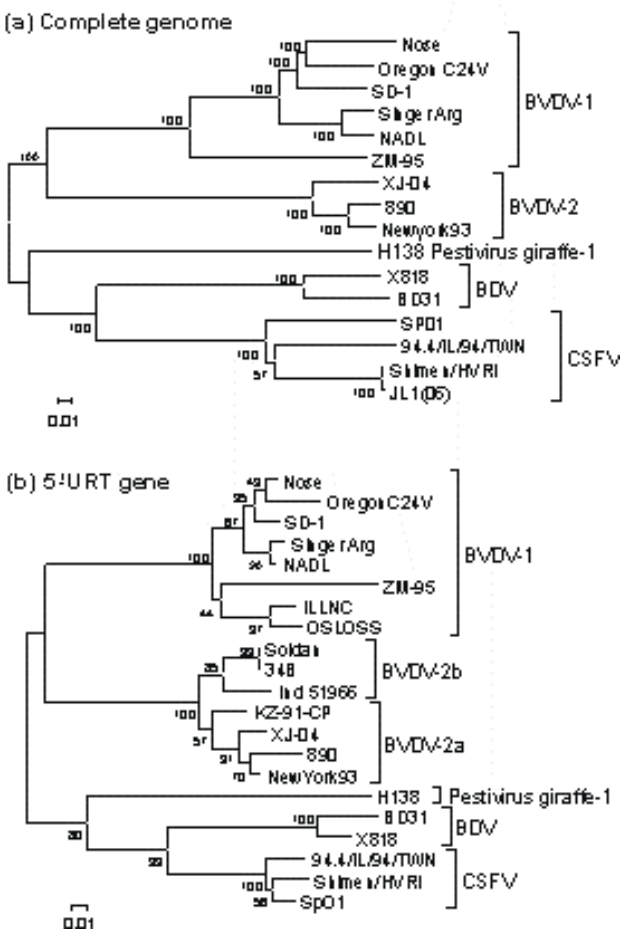

Fig. 1

Phylogenetic trees of various pestivirus strains based on the complete genomic sequence (a) and 5'-UTR (b)

The scale indicates the number of substitutions per site. Sequences of individual strains are available in GenBank.

NADL varied from 90.8% to 92.7% and from 65.1% to 80.0% at nt level, respectively. However, at aa level XJ-04 shared high similarity to both 890 and NADL in the range of 97.3%–99.3% and 88.7%–96.9%, respectively (Table 3). The discrepancy of low identity at nt level and high identity at aa level suggested that many synonymous codons were employed. Table 2 also showed that E2 gene in XJ-04 coding for the most immunogenic protein was poorly homologous to NADL, what was in accord with the fact that low serological cross-reactivity was observed between these two viruses (unpublished data).

BVDV of cytopathic (cpBVDV) and non-cytopathic type are distinguished by their effect on the cell culture (McClurkin *et al*., 1985; Baker, 1987). Cytopathic type of BVDV is correlated with the presence of insertions of cellular sequences, duplication of viral sequences with or without insertions, deletions, and point mutations in the genomes (Baroth *et al*., 2000; Becher *et al*., 2002; Quadros *et al*., 2006). Reported insertions in cpBVDV are distributed mainly at three positions including aa 1161, aa 1545, and aa 1589 at the area of NS2/3 genes (Ridpath *et al*., 2006). Also, insertions outside of the NS2 /3 genes in BVDV strain CP8 were reported (Müller *et al*., 2003). However, alignment of the entire genomic sequence for XJ-04 and other BVDV isolates available in GenBank showed that no insertion existed within or outside of NS2/3 gene in the isolate XJ-04 (data not shown). Similarly, cpBVDV-1 Oregon C24V does not contain any insertions and a relation between point mutations in the NS2 region and the cytopathic type of this virus has been confirmed (Kümmerer *et al*., 1998). Definitely, a reason for the cytopathic phenotype of isolate XJ-04 needs further study. To our knowledge a BVDV-2 strain with cytopathic phenotype and without insertions has not been reported yet.

In this report, we described the first complete genome sequence of BVDV-2 isolate XJ-04 originated from China. The phylogenetic analysis revealed that XJ-04 was located in the branch of BVDV-2 and can be further subtyped as BVDV-2a. This classification was supported by similarity of XJ-04 to BVDV-2 reference strain 890 at both nt and aa level in the structural genes and N^{pro} gene. In spite of the fact that isolate XJ-04 displayed a cytophatic phenotype, we found no insertion in its genome. The presented data supplement the general knowledge about the BVDV genomics and provide further information about the pestivirus genetic diversity in China.

Table 3. Nucleotide and amino acid sequence identity (%) of isolate XJ-04 in comparison with reference strains 890 and NADL

Reference strain	Analyzed region[a]			
	C	E^{ms}	E1	E2
890 (BVDV-2)	90.9	90.8	91.1	92.7
	99.3	*97.3*	*98.2*	*97.7*
NADL (BVDV-1)	68.3	80.0	72.0	65.1
	96.9	*95.1*	*95.6*	*88.7*

[a]Roman script and italic script represent the nucleotide and amino acid sequence identities, respectively.

Acknowledgement. The authors are grateful to Dr. Leonard Bello, University of Pennsylvania for providing MDBK cells. This work was supported by the grant No. BE2008363 from the Science and Technology Department of Jiangsu province, grant No. 08KJA230002 from the Jiangsu High Education Basic Research, and grant No. 30771603 from the Chinese National Science Foundation.

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